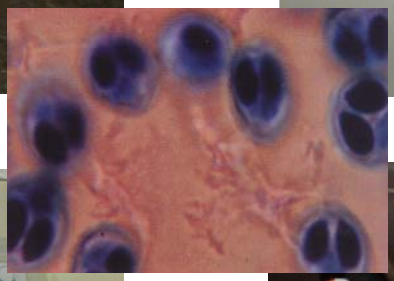
National Wild Fish Health Survey

Laboratory Procedures Manual



U.S. Fish & Wildlife Service

Division of Fish Hatcheries
Washington, D.C.
3.1 Edition - March 2006

This publication was originally produced in 2000 for the U.S. Fish and Wildlife Service, to describe the protocols and procedures utilized in the National Wild Fish Health Survey (NWFHS). The Third edition was completed in June 2005 to include new PCR methodologies for bacterial testing, to revise *Renibacterium salmoninarum* PCR procedures, to add formal reference sections, make minor text edits, and to reformat the entire manual. Edition 3.1, finished in March 2006, was completed to make minor edits in addresses, *Renibacterium salmoninarum* PCR extraction, bacteria sampling, and acknowledgements.

The NWFHS Laboratory Procedures Manual is intended for use by USFWS Fish Health Centers, but may also be helpful to fishery professionals, the technically oriented fishery industry, and students of biological or fishery sciences.

This Manual is accessible via the internet at http://wildfishsurvey.fws.gov/. CD-ROM copies may be requested from the U.S. Fish and Wildlife Service at the address on the title page. All recipients are requested to provide feedback on their use of this Manual and offer suggestions for improvements. The material may be updated annually if significant changes are made in the detection or corroborative testing methods, or in the overall Survey procedures.

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PREFACE

There are many published sources for laboratory procedures used in the diagnosis of finfish diseases. The National Wild Fish Health Survey (NWFHS) – Laboratory Procedure Manual is not intended to be comprehensive in its treatment of this large subject area. Many of the major fish diseases that occur within the United States and throughout the world have not been documented in wild fish populations. Consequently, the NWFHS (Survey) has targeted major fish pathogens that are considered a risk to both natural and artificially produced fish populations. The NWFHS Laboratory Procedure Manual (Manual) contains protocols for these major pathogens, and also provides a general scheme of approach to allow detection of new or exotic agents through the disciplines of virology, bacteriology, parasitology and histology. Pathogens of Regional Importance (PRI) are included for informational purposes; however specific protocols have not been included, in most cases, for these pathogens.

The Survey includes methodologies that provide the highest sensitivity for detection of target fish pathogens in subclinical wild fish populations. For this reason, Polymerase Chain Reaction[1] (PCR) technology is included in the Survey as an additional tool to corroborate detection of specific diseases (Whirling Disease, Bacterial Kidney Disease, Ceratomyxosis, and viral agents such as IHNV and VHSV). In this application of PCR as a "corroborative test method", it is not the intent of the Survey to imply that PCR is to replace "gold standards" for detecting or confirming specific fish pathogen. Rather, PCR is being utilized as a secondary detection tool to corroborate the presence of a pathogen that has been detected by standard methods. The intent of the Survey is to test the PCR technique along side standard detection methods such as Pepsin-Trypsin Digest, ELISA, and Virology. This PCR data will add to our knowledge of both fish diseases and the performance of molecular tools as detection methodologies. Traditional confirmation tests such as histology (for Whirling Disease and ceratomyxosis), and serum neutralization for viral agents are also included for the Survey.

The foremost purpose of this Manual is to provide a working document of very detailed information for the U.S. Fish and Wildlife Service - Fish Health Center staff and clients regarding the daily procedures in which we conduct the Wild Fish Health Survey. Where appropriate, the methods herein follow those described in Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens, 2004, 6th edition. American Fisheries Society, Fish Health Section, Bethesda, Maryland, commonly referred to as the AFS-FHS Blue Book (2004). Additional protocols are included for the Enzyme Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) techniques. In several instances protocols cited from other investigators in the published literature have been referenced as well. As with most laboratory manuals, this document will be updated as new and improved procedures become available.

NOTE: Mention of brand names or trademarks, or any specific equipment in the text of this Manual is not an endorsement of any particular product by the U.S. government or the Fish and Wildlife Service. The products mentioned serve only as descriptive models for the reader, any comparable product can be used at the discretion of the reader.

[1]The PCR Process is covered by Patents owned by Hoffman-LaRoche, Inc.

EDITOR

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ACKNOWLEDGMENTS

This Manual would not be possible without the generous contribution by Theodore R. Meyers of the Alaska Department of Fish and Game. The first edition of the Manual was based in large part on the Alaska Department of Fish and Game, Fish Pathology Section Laboratory Manual (Meyers, 1997). We have edited, updated and adapted their manual to reflect the needs of the Wild Fish Health Survey. The USFWS recognizes and thanks the efforts of Dr. Meyers, Jill Follett, Joan Thomas, Marie Fried, Sally Short, and Tamara Burton for their research and compilation of much of the information in the first edition.

Several other key individuals contributed to the basis of the information and protocols contained in this Manual. Of special note are Dr. James Winton of the Western Fisheries Research Center (USGS-BRD), Seattle, Washington for his technical review of this manuscript; Ron Pascho (USGS-BRD) for his development of the ELISA for detection of Bacterial Kidney Disease; Dr. Karl Andree and Dr. Ron P. Hedrick of the University of California (Davis) for the methodology for detecting Whirling Disease by Polymerase Chain Reaction (PCR); and many other dedicated researchers and technicians who continue to contribute to our knowledge of fish diseases.

A special acknowledgment is in order for Mary Ellen Mueller and William Knapp of the Division of Hatcheries for their support of the National Wild Fish Health Survey during its inception in 1996-1997.

And finally, recognition and appreciation to the fish health biologists from the nine fish health centers across the country. These individuals have worked diligently over the past several years to make the Survey successful by developing partnerships, collecting thousands of fish samples, performing the laboratory testing, and managing the information in the NWFHS Database. In addition to the work required for the Survey, each Fish Health Center contributed to the Third Edition by updating the written protocols that comprise this Manual.

CHAPTER 1

Overview of the National Wild Fish Health Survey

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I. Background

The Fish and Wildlife Service requested and received a $1 million annual increase in appropriations for fish disease work. Six hundred thousand dollars was used to initiate a *National Wild Fish Health Survey* (Survey) under the leadership of Service Regional Fish Health Centers (Centers), and in cooperation with stakeholders such as states, Tribes, and the aquaculture industry. This project incorporates standardized diagnostic and data management methods to ensure national comparability, identifies target pathogens, fish species, and habitats for survey, and is developing a systematic and interagency approach to fish health management of important watersheds.

Because initial funds were limited, every effort was made to collaborate with those collecting fish for other purposes with the aim of maximizing efforts in pathogen and parasite analysis rather than sample collection. In addition, a National Wild Fish Health Survey Database (Database) has been established to receive data from the Survey. The Database is accessible electronically via the Internet at www.wildfishsurvey.fws.gov

In November, 1996 a group composed of fish health biologists from each of the Service's nine Regions, state fishery managers from Oregon and Alaska, researchers in fish disease from the University of California-Davis, the Leetown Science Center and Western Fisheries Research Center (USGS), and a representative from the private aquaculture industry met in Denver, CO to develop an implementation plan for the Survey. The initial document (1997 Protocols & Procedures) provided a framework and procedures for implementation of the Survey as developed by this group. Given this was the first endeavor of its kind, this group recognizes that this plan would change as new information arose. In the first year of implementation, as the document was widely distributed, we received many comments and suggestions for revisions. The *NWFHS Laboratory Procedures* Manual (Manual) was further developed in 1998-1999 by contributions from fish health biologists across the country to provide a comprehensive Manual that includes optimum detection methods and standardized protocols for all aspects of the Survey. This Manual is meant to be dynamic and adaptive to best meet the needs and intent of the project. We expect to incorporate comments and suggestions received through yearly revisions of the document.

II. Acknowledgements

Many individuals have contributed to the Survey throughout its development and implementation. William E. Knapp and Mary Ellen Mueller, of the Division of National Fish Hatcheries, were catalysts for the conceptual development and funding initiatives that brought this Survey to light. Many other researchers made significant contributions to the procedures and protocols developed over the first and second years. We would like to especially thank Dr. Jim Winton of the Western Fisheries Research Center (USGS) in Seattle, and Dr. Ron Hedrick and Dr. Karl Andree of University of California, Davis. These researchers offered immeasurable help in transferring technical capabilities to Fish Health Centers through hands-on training and workshops.

In developing this Manual, the Service would like to recognize and thank Dr. Theodore Meyers who graciously offered the Alaska Fish Pathology Section Laboratory Manual as a template for our protocols, as well as support through technical and editorial review of this Manual.

Many fish health biologists from the nine regional Fish Health Centers developed and contributed individual chapters for the sampling methods and laboratory assays described in the initial version of the Manual (Version 1.0 – June 2001). The following individuals made significant contributions to the sections listed:

Patricia Barbash Lamar Fish Health Unit	*Renibacterium salmoninarum* PCR Non-Lethal Methodology
Ray Brunson Olympia Fish Health Center	Virology
Norm P. Heil Warm Springs Fish Health Center	Sample Collection and Submission
Becky Lasee LaCrosse Fish Health Center	Sample Receipt and Laboratory Tracking Parasitology & Internal Review
Terrence Ott LaCrosse Fish Health Center	Cell Culture
Kenneth Peters Bozeman Fish Health Center	Ceratomyxa shasta PCR
Kimberly True California-Nevada Fish Health Center	Editor (2001, 2004) ELISA, Parasitology, Bacteriology Manual Revision Committee Chair

Manual revisions will occur annually, or as needed to address the changing body of scientific knowledge and to incorporate new methodologies for emerging pathogens. The procedures for annual revisions of the Manual are described in Chapter 15, Procedure for Revisions to the National Wild Fish Health Survey Laboratory Procedures Manual.

Appendix 15.A and 15.B in Chapter 15 lists current Manual Revision Committee Members and Associate Editors for prior editions.

III. Purpose

The purpose of the National Wild Fish Health Survey is to determine the distribution of specific pathogens in wild fish populations.

IV. Justification

A. Knowledge of the distribution of pathogens in wild fish will contribute to:

1. Protect threatened or endangered species
2. Provide more options for better fish management
3. Provide a cohesive national perspective for better fish health management
4. Develop standardized fish health and fish transport regulations that are scientifically defensible

Why a National Wild Fish Health Survey?

- The discovery that whirling disease was decimating wild trout in the intermountain west focused the Nation's attention on the fact that very little is known about diseases among wild fish.
- The most important weapon needed to control or prevent fish diseases is knowledge. Currently, there is very little information about the relationship between presence of the pathogen in wild fish and its likelihood of producing disease in either wild or hatchery reared fish.
- Valuable stocks of fish are at risk because of our lack of knowledge about the distribution of pathogens and parasites in wild fish.
- A standardized approach is necessary to allow for comparisons from state to state or watershed to watershed to help identify why a pathogen or parasite in one area has negative impacts on certain fish stocks while not in others.
- Scientific information is needed to provide a biological basis for management decisions regarding stocking and fish transport activities.

V. Partnerships

The success of the Survey depends on establishing productive partnerships. Within the Fish and Wildlife Service, fish health biologists involved in the Survey work closely with other fishery biologists in the Fisheries, Ecological Services, and International Affairs programs. This ensures cohesiveness between the Survey and related aquatic activities, such as those involving environmental contaminants, endangered species, refuges and aquaculture. It also adds a valuable fish health dimension to those activities.

Partnerships will continue to be formed with other organizations active in fish health, fishery biology and fishery management. Included are other Federal agencies, State and Tribal agencies, conservation and professional organizations, universities and foreign nations. Care has been taken to identify and include partners early in designing and planning the Survey. Priority has been placed on adopting an overall approach that is broadly inclusive and one that is flexible in attracting and accommodating a variety of different kinds of partners.

Partnerships are based on common interests, responsibilities and activities. Some partners, like Federal, Tribal, and State fishery managers have been involved in several ways. Some provide fish taken from areas identified as high priority sampling areas. Others have provided funds to expand the Survey to areas that otherwise might not be targeted. Still others may not have participated in the Survey itself, but may have benefited from the data generated, or may have voiced their support for the Survey at critical times when national priorities and budgets were being established.

As more and more people and organizations become aware of the Survey and benefit from it, interests in forming partnerships will grow. Initially, the Service has focused on reaching four primary constituencies:

- Other Federal agencies with fishery management responsibilities either on their lands or through cooperative management arrangements;
- States and Tribes;
- Conservation and professional organizations; and
- Universities and other research institutions.

During the first year, FY 1997, attention was focused on planning and designing a scientifically sound survey that could provide additional fishery management capabilities in both the public and private sector. Survey design was coordinated carefully with representatives of each of the four primary constituencies to ensure its utility and attractiveness. As the Survey became operational and sampling began toward the later half of FY 1997, the Service broadened its efforts to increase understanding and awareness of the Survey and establish partnerships. Awareness of the Survey will be expanded by presentations made at professional and industry meetings, articles in professional and trade journals, and by communications and interactions among professionals engaged in private and public fishery management.

The Survey will always benefit from new partnerships and, in turn, will be shaped and directed by those partnerships. The Service will be challenged to maintain a flexible outlook in order to be responsive to diverse group of partners and at the same time guide the Survey in the direction intended by Congress.

VI. Fish of Primary Interest

The initial focus of the Survey has been on the following fish: trout, salmon, paddlefish, perch, sturgeon, suckers, sunfish, herring, catfish, bass, carp and minnows. Numerous species have

been added to the Survey since it's inception to address partner interest and regional concern regarding fish diseases.

VII. Target Pathogens

Each fish is evaluated for target pathogens and parasites that are known to infect that particular species. In addition, the standard methods used in the Survey will detect the major salmonid fish pathogens should they exist in other species. Refer to Appendix 1.A – Glossary of Terms for terms and pathogen abbreviations. The following list includes bacterial, viral, and parasitic pathogens of interest, and their abbreviation.

A. Viruses

1. Infectious Hematopoietic Necrosis Virus (IHNV)
2. Infectious Pancreatic Necrosis Virus (IPNV)
3. Viral Hemorrhagic Septicemia Virus (VHSV)
4. *Oncorhynchus Masou* Virus (OMV)
5. Largemouth Bass Virus (LMBV)

B. Bacterial pathogens

1. *Aeromonas salmonicida* (AS), Furunculosis
2. *Edwardsiella ictaluri* (ESC), Enteric Septicemia
3. *Renibacterium salmoninarum* (RS), Bacterial Kidney Disease
4. *Yersinia ruckeri* (YR), Enteric Redmouth

C. Parasites

1. *Myxobolus cerebralis* (WD), Whirling Disease

VIII. Pathogens of Regional Importance (PRI)

In addition to the pathogens and parasites listed above, the Service's Fish Health Centers have identified several Pathogens of Regional Importance (PRI) for which additional diagnostic procedures may be conducted as part of the Survey. These parasites and pathogens are included in laboratory protocols when either fish health professionals or fishery managers identify them as a potential risk to fish health in a watershed or ecosystem. PRI include the following:

A. Viruses

1. Infectious Salmon Anemia Virus (ISAV)
2. White Sturgeon Iridovirus (WSIV)
3. White Sturgeon Herpesvirus (WSHV2)
4. Spring Viremia of Carp Virus (SVCV)

B. Bacteria

1. *Flavobacterium columnare*
2. *Flavobacterium psychrophilum*
3. *Citrobacter freundii*
4. *Edwardsiella tarda*

C. Parasites

1. *Ceratomyxa shasta* (salmonid ceratomyxosis)
2. *Bothriocephalus acheilognathi* (Asian tapeworm)

The following table (Table 1.1) lists primary fish pathogens that are targeted by the Survey and may be associated with specific fish families. Pathogens of Regional Importance are denoted with (PRI).

IX. Table 1.1 - Fish Family and Target Pathogens*

Family	Bacterial Pathogens	Viral Pathogens	Parasites
Acipenseridae (Sturgeon)	*A. salmonicida* *E. ictaluri* *Y. ruckeri*	IPNV WSIV (PRI) WSHV2 (PRI)	
Catostomidae (Suckers)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV	
Centrarchidae (Sunfishes)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV LMBV	
Clupeidae (Herring)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV VHSV	
Cyprinidae (Minnows/Carp)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV SVCV (PRI)	*B. acheilognathi* (PRI)
Ictaluridae (Catfish)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV	
Percichthyidae (Temperate Basses)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV	
Percidae (Perch)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IPNV	
Polyodontidae (Paddlefish)	*A. salmonicida* *E. ictaluri* *Y. ruckeri*	IPNV WSHV-2	
Salmonidae (Trout/Salmon)	*A. salmonicida* *E. ictaluri* *R. salmoninarum* *Y. ruckeri*	IHNV ISAV (PRI) IPNV OMV VHSV	*M. cerebralis* *C. shasta* (PRI)

*** Targeted pathogens may not be found in all families.**

X. Priority Selection Criteria for Determining Areas of Focus

Available resources are not sufficient to accommodate all requests to sample specific waters.

A. Ranking criteria applied to determine which areas to sample:

1. Presence of pure wild (unmanipulated) stock of fish
2. Geographical area or species of concern to our State and Tribal partners
3. Geographical area that has never been sampled for fish diseases
4. Species of special management concern (high concern e.g., threatened or endangered species)
5. Study area of special management concern (high concern e.g., recovery project or suspected disease)
6. Sampling site is Federally managed
7. Historical data available from site (population, biodiversity, water quality, etc.)
8. Area is a broodstock or egg collection site
9. Other relevant data is being collected that enhances survey context (examples: contaminants, population estimates, year classes, species abundance/diversity or community structure, environmental parameters such as D.O., temperature, habitat type, pH, hardness, flow rate, etc.)
10. Partnerships will significantly leverage funds.

XI. Fish Collection Protocols

The Survey uses existing collection activities by cooperators to the fullest extent possible. Methods include either active or passive types of collection as described by Murphy and Willis, (1996). All collection methods described have advantages and disadvantages that must be recognized.

Study objectives, environmental characteristics, animal behavior, and size are additional factors that influence sampling methods. For the purpose of the Survey, collection methods that accurately reflect the relative abundance of animals sampled and allow the investigator to obtain live specimens are preferred.

For these reasons, investigators should focus on active collection methods that include, but are not limited to, electrofishing, seines, trawls, and dredges that generally define a more accurate

sampling effort and are more likely to provide live or fresh samples. Passive collection methods include, but are not limited to, gill nets, hoop nets, fyke nets, scoop traps, and rotary screw traps. Care must be used in passive collections to ensure fresh samples suitable for fish health analysis. However, since the study parameters are national in scope and include diverse aquatic habitats and cooperators, any collection method that gives a close approximation of the population for each habitat and provides opportunities for valid tissue collection should be considered.

Fish collection methods must be identified by each investigator and included in the database to enable valid comparison of health data collected.

XII. Specimen Receiving and Custody Procedures

A. General

Good sample documentation ensures proper identification and storage of samples, and proper tracking of the samples as they move through the diagnostic procedures in the fish health laboratory.

B. Procedures

All submissions should comply with the following:

1. Each submission will be documented on a NWFHS SUBMISSION FORM and specimens will be collected in accordance with AFS-FHS Blue Book (2004). For tests specified that are currently not found in Blue Book, the collection and processing of samples will follow those outlined in this Manual.

2. Each Submission Form will be reviewed to verify that it contains all appropriate information to process the accession. For most routine submissions this information includes:

    a. Submitter (fish biologist, fish health specialist, other)

    b. Date of collection

    c. Location of collection (GIS coordinates and common name)

    d. Capture procedure

    e. Site description

    f. Remarks

    g. Number of samples submitted

    h. Sample I.D. Numbers (i.e. 1-15 = samples numbered 1 through 15)

i. Genus and species and/or common name of fish samples (age, size and sex if known)

j. Specimen type (tissue)

k. Media type (if submission is by culture or preserved histology sample)

l. Number of samples for this group of fish

m. Number of fish per sample (pooled samples)

3. The section labeled for *Lab Use Only* will be completed as laboratories receive the samples, therefore initiating the chain of custody tracking for each submission. Information entered will include:

   a. Date received

   b. Coordinating Inspector/Pathologist

   c. Case Number (if applicable)

   d. Condition of samples

   e. Remarks

4. Receiving laboratory personnel will check and verify by signature all samples received. Further information on sample tracking and details of chain of custody procedures can be found in Chapter 3 -Sample Receipt and Laboratory Tracking.

XII. Diagnostic Protocols

A. Viruses

Procedures used for virology and cell culture techniques in this Manual largely follow those outlined in the AFS-FHS Blue Book (2004). Quality Assurance and Control measures for Virology are specifically addressed in cell culture propagation (Chapter 10) and in viral testing (Chapter 11). Specifically, cell lines are standardized among all Centers. Cell lines are tested annually for mycoplasma infections, and every three years for viral sensitivity.

B. Cell Culture Lines

Species sampled and applicable cell lines for preliminary viral testing

1. Salmonids and Herring: EPC and CHSE-214

2. Sturgeon: WSSK1 and CHSE-214

3. Ictalurids: BB and CHSE-214

4. Centrarchids: FHM and CHSE-214

5. Cyprinids: EPC and CHSE-214

All Fish Health Centers use reference cell lines from American Type Culture Collection (ATCC) that have been tested for viral sensitivity and mycoplasma infections. Cell lines are tested annually for mycoplasma infections, and every three years for viral sensitivity with the assistance of the Western Fisheries Research Center, USGS in Seattle, Washington. Each Center maintains these reference cell lines in liquid nitrogen, or ultra-low cryopreservation. Every three years, the existing cell lines are re-tested for viral sensitivity and the optimum cell lines are re-distributed to each Fish Health Center for virology testing performed under the Survey.

XIV. Corroborative Methods

For target pathogens and Pathogens of Regional Interest (PRI), the following corroborative methods will be performed.

A. Virus

Identification by specific antibody tests: serum neutralization, immunoblot, or FAT. Polymerase Chain Reaction (PCR) will only be used for viruses with appropriately defined specific known and labeled nucleotide sequences, i.e. North American VHSV, IHNV, IPNV, OMV, ISAV, LMBV, SVCV, and WSHV. The finding of SVCV requires immediate notification to the USDA, Animal and Plant Health Inspection Service (APHIS). Suspect SVCV isolates should be submitted to APHIS laboratories for confirmation testing and repository (See Appendix 1.B for APHIS procedures regarding reportable diseases).

B. Bacteria

The procedures described here largely follow those outlined AFS-FHS Blue Book with the following additional methodologies:

1. *Renibacterium salmoninarum* (Bacterial Kidney Disease)

    a. Sample: Kidney

    b. Preliminary methods: ELISA

    c. Corroborative testing: Nested PCR with specific primers

2. *Aeromonas salmonicida* (Furunculosis)

    a. Sample: Kidney and/or spleen

b. Preliminary methods: Isolation on BHIA and biochemical assays suggested for identification of *Aeromonas salmonicida*.

c. Corroborative testing: Specific antibody tests (DFAT, agglutination)

3. *Yersinia ruckeri: type I and type II* (Enteric Redmouth)

   a. Sample: Kidney, spleen

   b. Preliminary methods: Isolation on BHIA and biochemical assays suggested for identification of *Yersinia ruckeri*.

   c. Corroborative testing: Specific antibody tests (DFAT, agglutination)

4. *Edwardsiella ictaluri* (Enteric Septicemia)

   a. Preliminary methods: Isolation on BHIA and biochemical assays suggested for identification of *Edwardsiella ictaluri.*

   b. Corroborative testing: Specific antibody tests (DFAT, agglutination)

5. *Citrobacter freundii*
   a. Sample: Kidney, Spleen

   b. Preliminary methods: Isolation on BHIA and identification of colony and cell morphology consistent with *Citrobacter freundii*.

   c. Corroborative diagnosis: Specific antibody tests (agglutination test, DFAT)

6. *Edwardsiella tarda*

   a. Sample: Kidney, Spleen

   b. Preliminary methods: Isolation on BHIA and identification of colony and cell morphology consistent with *Edwardsiella tarda*

   c. Corroborative diagnosis: Specific antibody tests (agglutination test, DFAT)

7. *Flavobacterium columnare* (Columnaris Disease)

   a. Sample: Kidney, Spleen

   b. Preliminary methods: Isolation on TYES and identification of colony and cell morphology consistent with *Flavobacterium columnare*.

c. Corroborative diagnosis: Specific antibody tests (agglutination test, DFAT), or PCR.

8. *Flavobacterium psychrophilum* (Coldwater Disease)

   a. Sample: Kidney, Spleen

   b. Preliminary methods: Isolation on TYES and identification of colony and cellular morphology consistent with *Flavobacterium psychrophilum*

   c. Corroborative testing: Specific antibody tests (DFAT, agglutination test), or PCR.

B. Parasites

1. *Myxobolus cerebralis* (Whirling Disease)

   a. Sample: Half head or cartilage plug for preliminary detection method, and remaining half head for archiving for corroborative testing by PCR or histology.

   b. Preliminary methods: Pepsin-Trypsin Digest (PTD) method with observation of typical spores

   c. Corroborative diagnosis: PCR of halved head or cartilage plug, or presumptive spores detected by digest method, with nested primers. Histological sections showing spores in cranial cartilage can also be used for corroboration.

2. *Bothriocephalus acheilognathi* (Asian Tapeworm)

   a. Sample: Intestine

   b. Preliminary methods: Microscopic examination of morphology, intestinal squash for visualization.

   c. Corroborative diagnosis: Morphological criteria for Asian tapeworm.

3. *Ceratomyxa Shasta*

   a. Sample: Posterior intestine

   b. Preliminary methods: Wet mount observation of multicellular myxosporean trophozoites.

   c. Corroborative diagnosis: PCR with specific primers or histological sections.

XV. Quality Assurance and Quality Control

Many aspects of Quality Assurance/ Quality Control are addressed in the specific sections of this Manual for laboratory techniques such as ELISA, PCR, Tissue Culture, and Virology. For a full review of the USFWS Quality Assurance / Quality Control program please refer to the Quality Assurance/Quality Control Procedures Manual, Volume 5, of the USFWS Handbook.

XVI. Cost Accounting

The cost estimate for a 1-5 fish pool for bacterial, viral, and parasitic determinations is approximately $35.00 per sample. This does not include histology. A complete pathogen screening including histology is approximately $54.00 per fish.

A. Allowable cost categories for planning and budgeting include:

1. Capital Equipment Costs - *outlay for capitol equipment such as PCR and ELISA*;

2. Diagnostic tests (75% of annual funding each year) - *Supplies and labor for all laboratory work*;

3. Technical Assistance - *Labor for providing advise and support to cooperators, WFS conferences, meetings, and stakeholder outreach*;

4. Field Sampling Costs - *Labor and supplies outside laboratory costs;*

5. Data verification/data entry costs - *Data checking and input into the Database*;

6. Travel expenses - *Airfare, common carrier, and items on travel voucher (hotel, per diem, etc.) - not to include labor*

Fish Health Centers provide annual reports to the Regional Office and Director for use in budget development and execution. Expenditures are reported in each of the five categories above.

XVII. Data Verification and Information Transfer Protocols

A. Lab and field data generated and/or documented by the Fish Health Centers is "verified" prior to entry into the National Wild Fish Health Survey Database (Database).

B. Verifier authorization will be given to the Director for each Fish Health Center. Additionally, the Director can also delegate immediate support staff to verify information on their behalf.

C. Authorized verifiers must be familiar with the requirements of the Database and ensure that the quality of the data meets standards for:

1. Field Sampling
2. Chain of Custody
3. Diagnostic Tests
4. Database Fields
5. Information Transfer

D. Formal training has been provided to all Fish Health Centers as well as continuing education via Internet communication and periodic updates of the Database data-entry program.

E. Authorized verifiers will sign with their Verifier ID # (or name).

F. Field samples identified/confirmed to be positive for a certain pathogen (determined to be important for species/pathogen management) will be identified by the Fish Health Center. Prior notification of these results will be given to the provider, and responsible resource management agency for the sample.

G. Data entry is limited to target pathogens and PRIs. The addition of new pathogens to the Database requires approval of all Center Directors as described in Chapter 15 – Procedure for Revision. Additional fish species are allowed to be entered in the Database as these species are encountered and tested in the Survey.

H. Washington Office Liaison for Fish Health Centers and the Regional Fisheries ARD will also be notified of the finding. Test result data regarding such a sample will not be entered into the Database for up to fifteen (15) days from the date of the verification. The specific resource management agency that might be affected by the finding may approve an earlier data entry than 15 days.

I. The Database will be accessible to all Internet users in a read-only format.

XVIII. National Wild Fish Health Survey Database

A. Concept

The Database is Internet - World Wide Web accessible with a graphical interface, and includes spatial (geographical) data. The system has proper security measures for authorized personnel to upload data and edit as necessary as well as protect the integrity of the data. All edit and query capabilities are in real time for those with authorization. Edit and upload capabilities for each authorized user is limited to their particular area or Region of jurisdiction. For public viewing on the Internet, data is in read-only format with download capabilities in ASCII text and GIS to allow for use by other databases.

B. Data

Data fields consist of location, date, species of fish sampled, size range, collection method, numbers of samples, diagnostic tests performed, pathogens found, environmental parameters (when available during sample collection), and testing facility. The Database has the ability to accept and integrate additional fields as necessary.

C. Queries

Data can be queried for certain specific parameters and by any logical field parameter that authorized user access allows. Public query variables can include geographical area, species of fish, pathogen, and date. Queries include summary information by field.

D. Display

Basic display of the Database includes a Home Page screen with explanation and links to view the data, download the data and run specific or user defined queries. Geographical displays show points or areas of data collections and related information.

E. Reports

The Database is able to provide reports in text format and GIS format as desired by users of the data. Database administrators provide reports of access logs, on-line use, and system status.

Who Benefits from a National Wild Fish Health Survey?

We all benefit from a National Wild Fish Health Survey. Most especially, those of us who want to preserve and protect our valuable wild and native stocks, those of us who enjoy recreational fishing opportunities, and fishery managers that need sound biological information to better manage our Nation's resources. Specific benefits include:

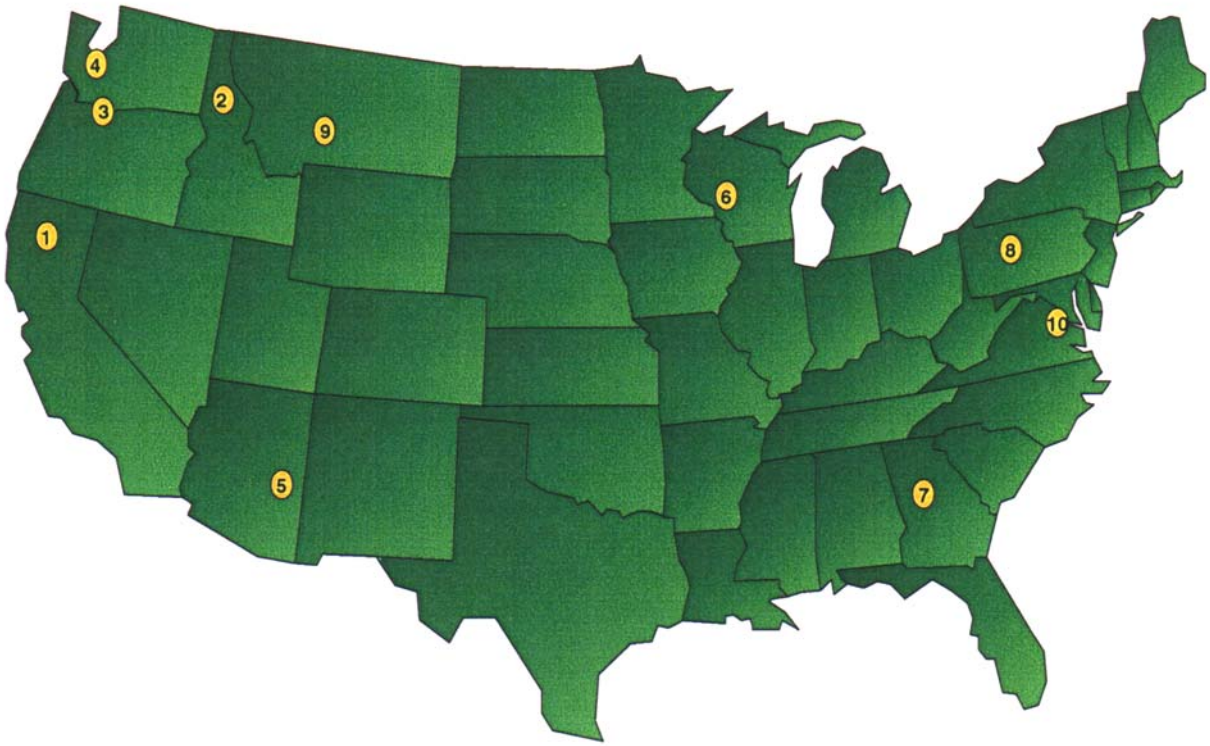
- Fishery Managers (State, Federal and Tribal) - Fishery managers are faced with the dual mission of protecting wild stocks of fish and providing fish for recreational opportunities. Knowledge gained in the Survey and accessed through a National Fish Health Data Base will provide mangers critical information for meeting their challenge. Knowledge of distribution of parasites and disease will allow managers to make sound decisions about control and containment strategies for areas or species that may already harbor a pathogen as well as identify areas or species where pathogens or parasites have not been detected and which may warrant special protection.

- Anglers – Over 50 million Americans engage in angling. Anglers will benefit from information gained from the Survey because managers will have additional tools and information available to better manage for recreational fishing opportunities, both for wild stocks and for those managed through enhancement with cultured fish.

- American economy – Throughout our country, income gained from tourist and trade associated with angling is an important part of the economy. Recreational fishing provides over 1.3 million jobs nationwide and generates over 70 billion dollars in total economic output. Information provided through the Survey will provide additional measures to safeguard these precious resources ensuring the continued economic benefits from angling.

- Aquaculture industry – Information about the distribution of pathogens in wild stocks will help the aquaculture industry by strengthening the biological basis for laws and regulations that govern the transport and sale of aquaculture products. Information from the Survey may show that in some places, regulations limiting or prohibiting movement of hatchery fish for commercial purposes can be relaxed for certain pathogens without jeopardizing wild stocks.

XIX. U.S. Fish and Wildlife Service Fish Health Centers



1	**California-Nevada Fish Health Center**	**6**	**LaCrosse Fish Health Center**
2	**Idaho Fish Health Center**	**7**	**Warm Springs Fish Health Center**
3	**Lower Columbia Fish Health Center**	**8**	**Lamar Fish Health Center**
4	**Olympia Fish Health Center**	**9**	**Bozeman Fish Health Center**
5	**Pinetop Fish Health Center**	**10**	**Washington Office, U.S. Fish and Wildlife Service**

See Chapter 2 – Sample Collection and Submission, Appendix 2.A for a full listing of Fish Health Center contacts and shipping addresses.

References

Murphy, B. R., and D. W. Willis, editors. 1996. Fisheries Techniques, 2nd edition. American Fisheries Society, Bethesda, Maryland.

Appendix 1.A – Glossary of Terms and Abbreviations

Aeromonas salmonicida – bacterial agent responsible for causing furunculosis.

Agglutination – a reaction in which particles (e.g. bacteria) suspended in a liquid collect into clumps in a response to the addition of a serologic antibody.

BB – cell culture of brown bullhead cells.

BHIA – brain-heart infusion agar.

BKD – Bacterial Kidney Disease, caused by the bacteria *Renibacterium salmoninarum*

Bothriocephalus acheilognathi – Asian tapeworm parasite.

Cell culture & virus isolation – specimens are inoculated onto appropriate cell culture lines, the cultures are incubated, virus-specific cytopathic effect (CPE) is observed, and viruses are identified.

Centers – collectively**,** the nine U.S. Fish and Wildlife Service Fish Health Centers located throughout the country.

Ceratomyxa shasta – parasite responsible for causing ceratomyxosis disease in salmonids.

CFR – Code of Federal Regulations

Chain of Custody – documentation of sample identification, tracking, and disposition during laboratory testing.

CHSE-214 – cell culture of Chinook salmon embryo cells.

***Citrobacter freundii* –** an opportunistic bacterium causing general septicemia.

CNS – central nervous system

Database – National Wild Fish Health Survey Database.

d-H_2O – distilled water

DO – dissolved oxygen, the amount of elemental oxygen, O_2, in solution under existing atmospheric pressure and temperature.

Edwardsiella ictaluri – bacterial agent responsible for causing enteric septicemia of catfish.

Edwardsiella tarda – bacterial agent responsible for causing enteric septicemia of catfish.

ELISA – enzyme-linked immunosorbent assay.

EPC – cell culture of *Epithelioma papillosum cyprini* cells.

Exophthalmia – Abnormal protrusion of the eyeball from the orbit.

FAT – fluorescent antibody test (and DFAT, direct fluorescent antibody test), where a type specific label is applied to a sample and examined with a fluorescent light microscope.

FHM – cell culture of fat head minnow cells.

Flavobacterium columnare – bacterial agent responsible for columnaris disease.

Flavobacterium psychrophilum – bacterial agent responsible for coldwater disease.

GIS – Geographical Information System, location coordinates based on longitude and latitude.

Hardness – a measure of cations such as calcium and magnesium in water; usually expressed as parts per million equivalents of calcium carbonate. Refers to the calcium and magnesium ion concentration in water on a scale of very soft (0-20 ppm $CaCO_3$), soft (20-50ppm), hard (50-500ppm), and very hard (500+ ppm).

IHNV – Infectious Hematopoietic Necrosis Virus.

Immunoblot – a lab test in which a small portion (blot) is tested against a labeled antibody.

IPNV- Infectious Pancreatic Necrosis Virus.

ISA – Infectious Salmon Anemia virus.

K/S – Kidney-Spleen (sample tissue type)

LMBV – Largemouth Bass Virus.

Myxobolus cerebralis – parasite responsible for causing Whirling Disease in salmonids.

OF – Ovarian Fluid (sample tissue type)

OMV – *Oncorhynchus masou* Virus.

PBS – Phosphate Buffered Saline

pH – an expression of the acid-base relationship, where the value of 7.0 expresses neutral solutions; values decreasing below 7.0 represent increasing acidity; and those increasing above 7.0 represent increasingly basic solutions.

PKD – Proliferative Kidney Disease

PRI – pathogen of regional importance.

PCR – polymerase chain reaction; a test that amplifies small quantities of pathogen-specific sequences of DNA, preformed with one or two ("nested") sets.

PTD – pepsin trypsin digest, a laboratory assay utilizing digestive enzymes to dissolve head cartilage, allowing the release and visualization of *Myxobolus cerebralis* spores.

Renibacterium salmoninarum – bacterial agent responsible for bacterial kidney disease.

Serum neutralization – a confirmation test for virology based on serology. Dilution series of viral suspensions grown in tissue cell culture are combined with specific neutralizing antibodies to test specificity and allow identification of the viral agent.

Service – U.S. Fish and Wildlife Service.

SVCV – Spring Viremia of Carp Virus.

Survey – National Wild Fish Health Survey (or NWFHS).

TYES – Tryptone Yeast Extract Salts – a low nutrient agar or broth for growth of certain types of bacteria.

USGS – United State Geological Survey.

VHSV- Viral Hemorrhagic Septicemia Virus

WF – Whole fish (sample tissue type)

WV – Whole Viscera (sample tissue type)

WSHV2 – White Sturgeon Herpesvirus 2.

WSIV – White Sturgeon Iridovirus.

Yersinia ruckeri – bacterial agent responsible for causing enteric redmouth disease.

Appendix 1.B
APHIS Memorandum No.567.6 regarding OIE Notifiable Diseases

***DISCLAIMER**: The procedures outlined in the USDA Animal and Plant Health Inspection Service (APHIS) Veterinary Services Memorandum No.567.6 (August 2002) are provided at the time of this publication as general information only. It is the responsibility of any user of this Manual to check directly with APHIS and/or OIE for current procedures for notifiable fish pathogens.*

NOTE: The list of OIE Reference Laboratories is updated by the International Committee of the OIE each year, and the revised list is published in each May issue of the OIE Bulletin. This information can be found on the OIE Web site at http://www.oie.int/eng/OIE/organisation/en_LR.htm

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Veterinary Services Memorandum No.567.6

August 27, 2002

I. PURPOSE

This memorandum describes the procedures for reporting OIE notifiable diseases.

II. BACKGROUND

As a member country of the OIE and in accordance with the International Aquatic Animal Health Code, the United States is obligated to report aquatic diseases that are on the list of OIE notifiable diseases. The Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), is the official contact point for reporting the OIE on notifiable animal diseases in the United States. Therefore APHIS-VS is required to report the following:

A. All new detections of notifiable diseases in fish, crustaceans, and mollusks;
B. The measures taken to prevent the spread of diseases; and
C. The movement restrictions and/or quarantine measures that are placed on the affected premises.

III. TO WHOM TO REPORT

To comply with our reporting obligations, we request that all diagnostic laboratories that identify or detect and of the OIE notifiable aquatic diseases listed below report them to the Federal Area Veterinary in Charge (AVIC) in their State. The AVIC should forward this information to all of the following:

A. Chief Staff Veterinarian, Emergency Programs;
B. Assistant Director, Sanitary International Standards Team; and
C. National Aquaculture Program Coordinator.

After evaluating the information, APHIS-VS will send a report to the OIE informing them of the detection.

IV. LIST OF NOTIFIABLE DISEASES

The following aquatic diseases are notifiable to the OIE:

A. Diseases of Fish

1. Epizootic hematopoietic necrosis
2. Infectious hematopoietic necrosis
3. Oncorhynchus masou virus (salmonid herpes virus type 2)
4. Spring viremia of carp virus
5. Viral hemorrhagic septicemia

B. Diseases of Mollusks

1. Bonamoisis (*Bonamia ostreae* and *Bonamia sp*)
2. Haplosporidiosis (*Haplosporidium nelsoni* and *H. costale*)
3. Marteiliosis (*Marteilia refringens* and *M. sydneyi*)
4. Mikrocytosis (*Mikrocytos mackini* and *M. roughleyi*)
5. Perkinsosis (*Perkinsus marinus* and *P.olseni*)

C. Diseases of Crustaceans

1. Taura Syndrome
2. White Spot Disease
3. Yellowhead Disease

Memo Signed by W. Ron DeHaven
Deputy Administrator
Veterinary Services

CHAPTER 2

Sample Collection and Submission

Norman P. Heil
USFWS – Warm Springs Fish Health Center
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I. Introduction

The purpose of this section is to provide guidance to laboratory staff and user groups regarding the proper sample collection and submission procedures. This is an absolute necessity to assure that samples received by the Centers are acceptable specimens for definitive pathogen identification. Each sample submission must include a NWFHS Submission Form (Appendix 2.B). Specimens will be stored, maintained and processed in accordance with protocols described in subsequent chapters of this Manual. Sampling procedures not found in this Manual will follow diagnostic procedures outlined in the AFS-FHS blue book *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*, (AFS-FHS 2004).

Additional, detailed information regarding sample collection and processing can be found for specific assays in the following chapters:

Chapter 3 - Sample Receipt and Laboratory Tracking
Chapter 4 - Standard Necropsy Procedures for Finfish
Chapter 5 - Bacteriology (*Aeromonas salmonicida, Yersinia ruckeri, Edwardsiella ictaluri*)
Chapter 6 - ELISA for Detection of *Renibacterium salmoninarum* Antigen in Fish Tissue
Chapter 7 - Corroboration Testing of Bacteria by Polymerase Chain Reaction (PCR)
Chapter 8 - Parasitology (*Myxobolus cerebralis*, *Bothriocephalus acheilognathi*, *Ceratomyxa shasta*)
Chapter 9 - Corroboration Testing for Parasites by PCR
Chapter 10 - Tissue Culture of Fish Cell Lines
Chapter 11 - Virology
Chapter 12 - Corroborative Testing of Viral Isolates
Chapter 13 - Histology of Finfish
Chapter 14 - Non-Lethal Methodology for Detection of Fish Pathogens
Chapter 15 - Procedure for Revision to the National Wild Fish Health Survey Laboratory Procedures Manual

II. Considerations for Sampling Fish Populations

Disease Recognition and Action – The majority of sampling conducted under the Survey will occur when no external signs of disease exist in wild fish populations. However, the Survey may also be helpful in determining the cause of fish kills or monitoring wild populations when abnormal behavior patterns, external abnormalities, or high mortality are reported for natural fish populations. In these cases, an immediate response is needed to determine the cause of mortality and determine if infectious agents are present or if adverse environmental conditions exist (low dissolved oxygen, elevated temperatures, toxic algal bloom, water contaminants, etc.). The following offers guidelines for sampling fish under various scenarios:

A. In clinical cases of disease (≥ 0.5% mortality/day) 10 moribund fish are generally sufficient to detect fish pathogens and make a disease diagnosis.

B. In survey or monitoring situations where no excessive mortality or clinical disease is apparent, a larger sample size of 60 animals may be necessary to detect infection rates below 5%. However, depending upon individual circumstances, sample sizes may vary between 10 and 60 fish. Samples should be examined from several stretches of a stream or body of water to ensure representation of the entire population. It is also extremely important that sampling techniques are optimal and laboratory assays as sensitive as possible to allow detection of fish pathogens, especially when sample numbers are small (<60 fish).

C. Donor populations – in some cases, natural populations will be used as donor broodstock to provide gametes to a hatchery program involved with a captive broodstock program or restoration activities. When a disease history for natural population is needed, a minimum of 60 samples from spawning adults is required to detect pathogens at 5% prevalence and a confidence interval of 95%. Samples of choice are from spawning or post spawning individual female fish consisting of ovarian fluid and kidney/spleen tissue.

D. Priority for acceptable samples submitted for Wild Fish Survey testing is as follows:

1. Live specimens or samples taken on site

2. Iced specimens or tissues

3. Preserved specimens or tissues

III. Sample Collection

Prior to collecting samples, the Center will contact sampling personnel with instructions on the appropriate types of samples and numbers of fish needed. Partners collecting specimens need to coordinate dates of collection with the work schedule of the receiving fish health center. If advance notice is not given, lab personnel may not be available to receive and process the submission after it is collected and shipped. Samples that are not in an acceptable condition (either substandard or improperly packaged) upon arrival cannot be processed due to the poor sample quality and unreliability of pathogen testing.

The following instructions are general guidelines. User groups and or individual collectors should be properly trained in the use of these sampling procedures by a fish health specialist, pathologist, or technician prior to sampling fish. Different procedures are followed in bacteriology, virology, parasitology, ELISA, PCR, and histological analyses.

Further details regarding the procedures below are addressed in the appropriate chapters of this Manual.

A. Live fish are preferred, and should be sampled immediately upon removal from the water. If this is not possible, fish should be held on ice and processed within 1-2 hours after collection. Whole fish can be packaged live, or freshly killed (iced) for shipment to the Centers for processing within 24 hours. When individual tissues are collected, these should be kept cold with ice or artificial icepacks but should not be allowed to freeze (insulated from direct contact with ice).

B. Samples for virology should be processed within 48 hours and inoculated into cell culture within 72 hours of sample collection. Upon specimen arrival determine the freshness of the fish. Criteria for this freshness are: smell, appearance of eyes, gills, internal organs and presence of postmortem stiffness (rigor mortis). If shipping is delayed beyond 24 hours, some tissues may not be suitable for processing.

C. Euthanize the fish or group of fish. Use clean sterile dissecting instruments. Clean dissecting tools with soap and water and disinfect with alcohol between the sampling of each individual fish (species) or species group. The use of disposable tools is recommended for ELISA samples, as Rs antigen is difficult to remove by standard disinfection methods. Disinfect hands between the sampling of each fish (species) or species group. Before taking tissue samples, wipe clean any mucus or debris from the fish and then disinfect the outer surface of the fish by flooding with 70% alcohol. Proceed with necropsy of fish tissues.

IV. Bacteriology

Bacteriological samples should be taken first to reduce the chance of contamination. If open sores or lesions are present swab these areas and streak the sample onto a BHIA agar slant. Discard swab and re-cap slant. The abdominal cavity is entered by cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors or scalpel. The cut is continued dorsally to just below the lateral line. Start again at the base of the pectoral fin and continue the incision towards the posterior of the fish along the ventral abdominal wall to the vent. Stay slightly above the intestinal tract when making the incision so that it is not punctured, thereby contaminating the abdominal cavity and target tissues.

Use the butt end of a sterile inoculating loop or forceps to pull back the internal viscera and air bladder to expose the kidney. Stab the posterior kidney with sterile forceps or loop and collect a large piece of kidney tissue or blood and streak it directly onto a BHIA agar slant or plate. The kidney tissue can be used for the virology or ELISA sample as well. Record sample number and date on tube or plate. Sample numbers can be any logical group of letters and numbers in order (RBT-1, RBT-2). Store tubes in a cool place such as cooler or cool room (15-20°C).

V. Virology

A. Kidney/Spleen

Dissect approximately 0.5 gm piece of kidney and/or spleen and place into a small WHIRL- PAK™ bag or snap-cap tube with a small amount of HBSS (Hank's Balance Salt Solution) to cover sample (Appendix 11.B.2-Transport Medium). Keep the HBSS cold at all times. Label bag with sample number, K/S and date. Up to 5 fish samples may be pooled in one bag or tube if appropriate. Keep samples on ice while in the field. Samples must be kept cold (5-18°C), but **do not freeze**, during shipment to the Center.

B. Ovarian Fluid:

Sexually mature females only. Remove a small amount of ovarian fluid from the oviduct using a pipettor or express ovarian fluid into a paper cup (approximately 1 mL per fish) Place the fluid in a small snap-cap or transport tube. Up to five (5) fish may be pooled into one sample. Label tube with sample number (OV-1, OV-2, etc.) and date. Keep samples cold (4°C) with pre-packaged ice packs, but **do not freeze** during collection or transport. Sample tubes should be placed within a WHIRL-PAK™ or zip- lock bag which is labeled with the stream or lake name, fish species, sample type, etc.

VI. ELISA for *Renibacterium salmoninarum* (BKD)

Remove the remaining amount of kidney or sizable portion using **individual,** or **disposable** instruments. Place the kidney tissue into a snap-cap tube or small WHIRL-PAK™ bag. Up to fish five can be pooled into one sample if necessary to obtain a minimum tissue weight of 0.08 g per sample (0.08 g diluted 1:8 during processing will yield 560 µL for the ELISA assay). Label with sample number, KD-ELISA and date (similar numbering scheme as with bacteriology slants). Keep samples on ice for transport to the lab. Samples can be frozen at -20°C for delayed processing, however if long-term storage is needed, -70°C is recommended.

VII. Parasitology

Myxobolus cerebralis (Whirling Disease):

Salmonid Fish Only. For small fish remove the entire head and gill arches. For larger fish, take a cranial core sample and/or gill arches. If taking a core sample, see Chapter 9 – Corroborative Testing of Parasites by PCR, for the target tissue site for *Myxobolus cerebralis*. Up to (5) fish may be pooled into one sample. Place samples in a WHIRL-PAK™ or zip-lock bag labeled with sample information. Pack samples on ice for transport to the CENTER.

Either during sample collection, or during processing in the laboratory, the head tissue is halved into two separate pieces to allow testing of one half by Pepsin-Trypsin Digest (PTD) and archiving of the second specimen for corroborative testing by PCR or histology. If tissue is halved during collection, label each half to allow correlation between archive and tissue to be used for PTD tissues. Recommend a notation of "PTD" and "ARC", as well as FISH ID NUMBER on each 5-pool sample (e.g., PTD 1-5, PTD 6-10, ARC1-5, ARC 6-10, etc). Tracking the fish identification for each sample pool will facilitate corroborative testing by PCR. In this way, only the PTD positive sample pool will require corroborative testing of the archive sample by PCR, rather than the entire sample set.

Bothriocephalus acheilognathi (Asian Tapeworm) and Ceratomyxa *shasta* (salmonid ceratomyxosis):

Remove the GI track of the fish from the esophagus to the anus. Place GI track into WHIRL-PAK™ or zip-lock bags. Small fish of the same species can be pooled (5 fish) if applicable. Label samples with appropriate information. Pack samples on ice for transport to the Center.

VIII. Histological Samples

Histological samples should be fixed in Bouin's solution, 10% buffered formalin, Davidson's fixative, or Prefer fixative. Fix live fish after anesthetizing. Fix tissues within **2 minutes** of removal from water and/or time of death. Fish rapidly autolyze (especially gill) and only freshly fixed tissues are worth processing for histological analysis. The volume of fixative must be at least 10 times the volume of tissue. For fish longer than 6 cm, slit the abdomen, detach the intestine at the anus, and pull the internal organ mass out slightly to allow penetration of fixative within the body cavity. For larger fish, send only specified organs in fixative. Cut tissues with a sharp blade or scissors - don't tear as this action creates artifacts.

> **SAFETY NOTE ! formalin-based fixatives are toxic and strong irritants.**
> Read the entire Material Safety Data Sheet. Avoid contact with skin and eyes by wearing gloves and a face shield. Use only in well-ventilated areas (outdoors or under a fume hood).
>
> **HAZARDOUS MATERIALS** - Fixatives and alcohol require special shipping procedures as Dangerous Goods, or Hazardous Materials. Refer to your local regulatory agency and commercial carrier for requirements for shipping these materials.

Place a paper **penciled** label (location, date, species, tissue type, and initials) **inside** the fixative container (alcohol and fixatives tend to wash off pen marks on the container).

Prevent spills during transport. Tightly cap the container, then wrap the cap with several layers of parafilm, and place the containers inside Ziploc bag(s). If samples fixed in Bouin's or Davidson's Fixative cannot reach the laboratory in 48 hrs, it will be necessary to transfer fixed

tissues to 70% ethanol after 24-48 hours and then transport the tissues to the Center (Samples fixed in 10% Buffered Formalin or Prefer fixative can be held in fixative indefinitely).

IX. Non-lethal Collection of Tissue Samples

Compliance with the Endangered Species Act (ESA) of 1973 requires special consideration regarding take of threatened or endangered (T&E) species if they occur in a proposed sampling site or watershed. All Centers and Partners in the Survey are responsible for obtaining appropriate collection permits, coordinating sample collection with Federal, State and local regulatory agencies, and fully complying with the regulatory statutes of the Endangered Species Act.

When lethal sampling of T&E species is prohibited, non-lethal sampling techniques should be considered. While non-lethal sampling methods are less sensitive than standard detection methods, they may provide limited fish health information when no other alternative exists. Refer to Chapter 14 -Non Lethal Methodology for Detection of Fish Pathogens for specific protocols for non-lethal sampling.

A. Bacteriology

1. Blood Samples

   Obtain blood via heart puncture or caudal vein or artery using a needle and syringe. Streak blood onto BHIA slant with sterile loop. Discharge appropriate amount of blood directly into the appropriate volume of PBS-T20 for ELISA. Heparinized blood may be used for delayed transport and processing.

2. Ovarian Fluid Samples

   If the fish is a sexually mature female, remove a small amount of ovarian fluid and inoculate a BHIA plate for bacterial growth. Also, the same sample can be placed into a centrifuge tube. Up to five (5) fish may be pooled into one sample. Keep samples cold for transport, but **do not freeze.** Following centrifugation and processing for virology, the Ovarian Fluid Pellet can be used to screen for *Renibacterium salmoninarum* by FAT.

3. Mucus Samples

   Pass a sterile swab along the lateral surface of the fish. Streak the sample onto a BHIA agar slant.

4. Vent Samples

   Place a sterile swab or loop approximately ½ to 1 inch into the anal vent and remove. Streak the sample onto a BHIA agar slant**.** (Discard the swab or loop).

5. External Lesions

   Pass a sterile swab along the surface of an external lesion. Streak the sample on selective media for the targeted bacteria. Plate serial dilutions of the inoculum to decrease the number of interfering bacteria and fungi that are likely to be present in this type of sample.

B. Virology

1. Blood Samples

   Obtain blood via heart puncture or caudal vein or artery using a needle and syringe. Discharge appropriate amount of blood directly into the appropriate volume of antibiotic-antimycotic incubation (anti-inc) solution. Keep samples cold for transport, but **do not freeze.** Follow normal processing protocol.

2. Ovarian Fluid Samples

   If the fish is a sexually mature female, remove a small amount of ovarian fluid and place into a centrifuge tube. Up to five (5) fish may be pooled into one sample. Keep samples cold for transport, but **do not freeze.** Follow normal processing protocol.

3. Mucus Samples

   Collect mucus by passing a blunt edge instrument along the lateral surface of the fish, head to tail. This is easily accomplished when the fish is removed from the water, holding the fish head to tail in a vertical position. Mucus is placed in a 15 (mL) or smaller graduated centrifuge tube with antibiotics used in viral sample processing. Keep the samples cold for transport to the Center, but **do not freeze.** In the lab, samples should be vortexed, and then a low dilution scheme (1:2, 1:5, 1:10) is set up using Hanks Balanced Salt Solution (HBSS). Samples are centrifuges at low speed, 2000-3000 rpm for ten to pellet cellular debris. Supernatant is inoculated into cell culture.

4 Fecal samples

   Feces are collected by aspiration with a syringe and small tubing catheter. Samples are placed into small collection tubes with antibiotics (may need to increase concentrations of antibiotics by 25-50% for fecal samples). Keep the samples cold

for transport, but **do not freeze.** Follow processing as in mucus samples (may require higher dilutions to avoid toxicity to cell lines).

5. External Lesions

   Pass a sterile swab along the surface of an external lesion. Place the swab in anti-inc solution pressing, or rolling the swab against the interior of the tube to release the material into solution. Keep samples cold for transport, but **do not freeze.** Follow normal processing protocol.

C. Parasites

1. Blood Samples

   Obtain blood via heart puncture or caudal vein or artery using a needle and syringe. Discharge appropriate amount of blood directly into a slide and prepare a thin blood film (Chapter 4, page 10). Blood films are air dried, and fixed in absolute methanol for 10 minutes. Blood parasites can be viewed following staining with Diff-Quick or a Gram stain (Chapter 4, page 9).

2. Mucus Samples

   Collect mucus by passing a blunt edge instrument along the lateral surface of the fish, head to tail. This is easily accomplished when the fish is removed from the water, holding the fish head to tail in a vertical position. Mucus is placed in a 15 (mL) or smaller graduated centrifuge tube. The tissue can be examined directly under microscopy for parasites or kept cold for transport and examined in the laboratory.

3. Fecal samples

   Feces are collected by aspiration with a syringe and small tubing catheter. Samples are placed into small collection tubes. The tissue can be examined directly under microscopy for parasites or kept cold for transport and examined in the laboratory.

4. External Lesions

   Pass a sterile swab along the surface of an external lesion. Place the swab in normal saline or PBS solution pressing, or rolling the swab against the interior of the tube to release the material into solution. The tissue can be examined directly under microscopy for parasites or kept cold for transport and examined in the laboratory.

5. Tissue Biopsy

   Gill filament can be removed from anesthetized fish with little injury to the fish. The tissue can be examined directly under microscopy for parasites, preserved for

histology, or frozen for examination using other diagnostic methods. See Chapter 14 for more a detailed protocol for gill biopsy.

Water and sediments sampling for virus, bacteria and parasites is also discussed in Chapter 14 - Non-Lethal Methodology for Fish Pathogens.

X. Shipping Samples – See Appendix 2.A for Shipping Addresses of Centers

A. Ship samples in small/medium (heavy duty) mailing cartons or plastic coolers lined with a plastic trash bag. Group the samples by type in separate Ziploc bags or racks that are labeled with the number of samples, location, species, and date. Position the samples upright and use packing material to hold samples in place.

B. Place an adequate amount of ice (or artificial gel packs) around the insulating layer of packing material. Seal the outer trash bag to prevent leakage.

C. Complete the NWFHS Submission Form (Appendix 2.B) for each species and enclose in a waterproof plastic bag within the cooler.

D. Close, seal and label the ice chest with laboratory address, be sure to include the Center contact's NAME AND PHONE NUMBER).

E. Appropriate shipping labels should also be affixed to ensure proper handling during shipment and upon receipt of containers.

"Live Fish – Do Not Freeze"	.....for live samples
"Keep Frozen"	.....for ELISA and/or Head tissue
"Refrigerate but DO NOT FREEZE"	.....for virology or sets of samples that include both Bacteriology and Virology samples.

NOTE: Temperature indicator strips can be placed with the samples. These strips will change color if 20°C has been exceeding during shipment.

F. Transport within 24 hours via overnight U.S. mail or Federal Express.

NOTE: Collectors should always include a Submission Form with samples to provide the collection information for the laboratory tracking and database entry. Samples submitted without this information may be refused or at least delayed for processing. When in doubt regarding collection and shipping instructions, consult the Center contact for the Survey.

Centers are prepared to provide all supplies needed for field sampling and training in sample collection. Transportation costs, personnel to assist with sampling and shipping costs may be provided if possible.

References

AFS-FHS. (American Fisheries Society – Fish Health Section). 2004. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2004 edition. AFS-FHS, Bethesda, Maryland.

Additional Reading

California-Nevada Fish Health Center, 1997. Histological sampling of fish tissues. S.O.P., California-Nevada Fish Health Center. Anderson, California.

Lasee, B. A., editor. 1995. Introduction to fish health management. 2nd edition. U.S. Fish and Wildlife Service, La Crosse Fish Health Center. Onalaska, Wisconsin.

Meyers, T. R., editor. 1997. Fish pathology section laboratory manual, special publication No. 12, Alaska Department of Fish and Game.

Mitchell, A. J., and G. L. Hoffman. Submitting samples for fish disease diagnosis. U.S. Fish and Wildlife Service, Fish Farming Experimental Station, Stuttgart, Arkansas.

Plumb, J. A., and P. R. Bowser. 1983. Microbial fish disease laboratory manual. Alabama Agricultural Experimental Station, Auburn University, Auburn.

Appendix 2.A

Shipping Addresses and Contacts for Fish Health Centers

Bozeman Fish Health Center 920 Technology Blvd, Suite G Bozeman, MT 59718	Crystal Hudson Kenneth Peters 406-582-8656 406-587-3998 (fax)
California-Nevada Fish Health Center 24411 Coleman Hatchery Road Anderson, CA 96007	Scott Foott Kimberly True 530-365-4271 530-365-7150 (fax)
Idaho Fish Health Center P.O. Box 272 Orofino, ID 83520 FOR SHIPPING: 4447 Ahsahka Ahsahka, ID 83520	Kathy Clemens Laura Kessel 208-476-9500 208-476-9741(fax)
La Crosse Fish Health Center 555 Lester Avenue, Suite 100 Onalaska, WI 54650-8552	Rick Nelson Becky Lasee 608-783-8444 608-783-8450 (fax)
Lamar Fish Health Center P.O. Box 155 Lamar, PA 16848 FOR SHIPPING: Hatchery Road Lamar, PA 16848	John Coll Patricia Barbash 570-726-6611 570-726-7379 (fax)

Lower Columbia Fish Health Center 201 Oklahoma Rd Willard, WA 98605	Susan Gutenberger Ken Lujan 509-538-2400 509-538-2404 (fax)
Olympia Fish Health Center 3859 Martin Way E., Suite 101 Olympia, WA 98506	Ray Brunson Sonia Mumford 360-753-9046 360-753-9403 (fax)
Region 2 Fish Health Unit P.O. Box 219 Dexter, NM 88230 FOR SHIPPING: 7116 Hatchery Rd. Dexter, NM 88230	John C. Thoesen Jason Woodland 505-734-5910 505-734-6130 (fax)
Warm Springs Fish Health Center 5151 Spring Street Warm Springs, GA 31830	Norm P. Heil Brian Hickson 706-655-3382 Ext.233 706-655-3389 (fax)

Appendix 2.B - NWFHS Submission Form

National Wild Fish Health Survey Submission Form

Submitter: Phone No:	Collection Date: Collection Time:	Lab Case No: (Completed by Lab)
Location: County: State:	GIS Coordinates (Datum NAD 83): Latitude: ____Deg____Min ___Sec Longitude:____Deg ___Min___Sec	
Site Description - Name of Water Body :		
Capture Method / Procedure:	Sample Type: ☐ Random (routine) ☐ Selective (diagnostic)	
Remarks:	Submitter Signature & Date:	

Species	No. FISH	Sample Type			Comments
		Whole	Tissue(s)	Other	
		☐	☐		
		☐	☐		
		☐	☐		
		☐	☐		
		☐	☐		

Comments:

Lab Use Only

Case Number / Laboratory ID:	Date Received: Time:
Remarks:	Received By:
Case Coordinator/inspector/pathologist Signature:	Date Finished:

Species and Sample Information

SPECIES:	No. FISH	No. Samples	Pool Size	Sample ID Numbers
Viral Tissue:				
Bact Cultures:				
R.sal Tissues (KD):				
Parasite Tissues: Type:				
Type:				
Comments:				

SPECIES:	No. FISH	No. Samples	Pool Size	Sample ID Numbers
Viral Tissue:				
Bacti Cultures:				
R.sal Tissues (KD):				
Parasite Tissues: Type:				
Type:				
Comments:				

SPECIES:	No. FISH	No. Samples	Pool Size	Sample ID Numbers
Viral Tissue:				
Bacti Cultures:				
R.sal Tissues (KD):				
Parasite Tissues: Type:				
Type:				
Comments:				

National Wild Fish Health Survey Submission Form: Sample Tracking

Species: Location: Date:

Fish No.	Length (mm)	Weight (g)	Sex (M/F)	BHIA #	Kidney # (ELISA)	Virology sample #	Head (pool #)	other	other	Comments
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										
14										
15										
16										
17										
18										
19										
20										
21										
22										
23										
24										
25										
26										
27										
28										
29										
30										
Total # samples (pools) submitted										

NOTICE

In accordance with the Paperwork Reduction Act (44 U.S.C. 3501), please be advised that:

1. The gathering of information is authorized by the Fish and Wildlife Act of 1956 (16 U.S.C. 742f), the Wildlife Coordination Act (16 U.S.C. 661-666c), and the Anadromous Fish Conservation Act (16 U.S.C. 757a – 757g).
2. Failure to provide all of the requested information is sufficient cause for the U.S. Fish and Wildlife Service to deny your request for Aquatic Animal Health Inspection under 713 FW 4.
3. You are not required to respond to a collection of information unless it displays a currently valid OMB control number.
4. This information collection has been approved by OMB and assigned clearance number 1018-XXXX.
5. The requested information may be subject to disclosure under provisions of the Freedom of Information Act (5 U.S.C. 552).

The public reporting burden for the information collected on this form is 15 minutes. This burden estimate includes time for reviewing instructions, gathering data, and completing and reviewing the form. Comments on this form should be directed to the Information Collection Officer, Mail Stop 222, Arlington Square, U.S. Fish and Wildlife Service, Washington, DC 20240.

CHAPTER 3

Sample Receipt and Laboratory Tracking

Becky Lasee
USFWS - LaCrosse Fish Health Unit
LaCrosse, Wisconsin

I. Introduction

Laboratory tracking is an important component of Quality Control because it ensures proper sample identification and accuracy in reported test results. This chapter describes the procedures required to retain sample identity and maintain quality assurance throughout the laboratory testing procedures.

II. Initial Documentation During Collection

All samples collected for the Survey will have a record of collection beginning at the time of collection. The record will describe the details of the collection process, as well as, the collector and their agency or affiliation. The collector will be identified sufficiently that any diagnostician at the receiving laboratory is able to contact that person if necessary.

III. Laboratory Case History Number

All samples will receive a unique identifying number or code upon receipt into the laboratory (in this document referred to as "case history number"). The case history number will be recorded on every sample and all subsequent paperwork generated in the laboratory. Labeling of all materials must be sufficient that any other diagnostician can identify the sample and its significance (see example Case History Record and Chain of Custody Form in Appendices 3.A and 3.B).

IV. Chain of Custody

The location of any sample must be determinable at any time. This is best accomplished by having a chain-of-custody record with all samples. As a person takes possession of any samples, they will mark the date of possession, their printed name, and signature on the next available space on the chain-of-custody record. Emphasis should be placed on the requirement for fixed responsibility upon a particular person for the care, custody, processing, and storage of all samples. This responsibility is initially placed upon the sample collector.

V. Designated Storage Areas

Storage facilities for samples will be determined for each laboratory. Such facilities include refrigerators, freezers, and incubators as appropriate (see sample-specific storage suggestions below). A list of all storage facilities used in a laboratory will be maintained in the lab and available to all responsible parties. The specific storage area used to store specific samples must be indicated in the main case history record (check-in sheet) and updated as samples are moved within the laboratory. The disposition of samples must be recorded from the time of sample check-in until the time that the samples are destroyed.

VI. Sample(s) Received Through a Third Party

Sample receipt through a third party is of utmost importance. The biologist who is the contact for each case should be notified immediately upon receipt of samples in the lab. That person is then responsible for unpacking, examining the samples, and notifying others what processing needs to be done on the samples. Samples must be properly stored to ensure pathogen viability. Samples should be checked in as soon as practical after they are received in the building, and should be completed before moving onto another task.

VII. Sample Receipt

Sample containers whether received in person, via U.S. Mail, Federal Express or other commercial carriers are all treated alike. If there is an address slip on the container (for instance, a Federal Express slip) for the current shipment, that tag is saved and marked with the case history number. If more than one container is received at a time, they are opened one at a time, and all the samples from one container properly put away before the next container is opened. This is to ensure that there is no opportunity for cross contamination of samples received from different locations. If it is clear that multiple containers are from the same site, they can be opened at the same time. All written information contained with the samples needs to be saved and filed with other case history documents. Each sheet of information must be marked with the Case History Number.

VIII. Sample Check-In

The case history number should be a unique number assigned to each case. One way to create a unique numbering system is to record the fiscal year with the number in succession from the previous case assigned from a logbook. The following is a list of minimum information that must be included on the Submission Form and/or the case history or check-in form for the Survey:

A. Date – The date samples are received at the Laboratory and the date collected

B. Case History Number – Assigned when samples are received

C. Sample Collection Site

D. Identification of the Sample Collector & Running Chain-of-Custody

F. Species – one Submission Form for each species sampled

G. Number of Fish Sampled – per species if multiple species were collected

H. Type and number of samples – Count the number of samples to ensure that the number reported on the sample collection form agrees with what is actually in the shipping container. Note any discrepancies or specific information about the shipment. Also, note if/how samples are pooled for each specific assay to be performed.

I. Latitude and Longitude of Collection Site

J. County of Collection Site

K. Tests Performed and Results

The sample numbers and types must be reconciled to keep proper chain of custody and to ensure that the reporting of the results is accurate. If sample containers (e.g., virology tubes) have leaked in transit, that must be noted, and the number still available for processing recorded. Even if the proper number were collected, obviously a broken tube will result in fewer fish actually being tested, and that is the correct number reporting.

IX. Sample Storage

After the reports and the samples are reconciled, the samples are distributed in the lab as appropriate. Processing of samples will be done according to Manual procedures.

A. Slants for Bacteriology

Each rack of slants is surface-disinfected, and then labeled with the case history number, date, and initials of the person who checked the samples in, and placed in the appropriate incubator.

B. Virology Samples - K/S, WF, WV, OF, other

Virology samples will generally arrive in whirlpak bags, which should be within a larger Ziploc bag. The larger bag should be surface disinfected, and the case history number and date received recorded on label and then stored until processing. If they are loose in the container, use a new Ziploc, record the case history number and date received on the outside, and store in assigned sample refrigerator until processing. Viral samples must be processed within 48 hours and plated on cell cultures within 72 hours from collection to ensure optimum sample quality for virus detection.

C. ELISA samples

ELISA samples can be processed immediately and stored frozen (-70°C) until complete analysis is completed. If samples cannot be processed immediately, they can be refrigerated and processed within 24 hours. If unable to process within 24 hours, unprocessed samples should be frozen (-70°C) and stored until a later date.

D. PCR Samples

PCR samples are frozen (-70°C) if they cannot be immediately processed. It is important to store PCR samples separately from areas of the laboratory used for DNA extraction, amplification, and storage of specific PCR primers. See Chapter 9 -Corroborative Testing of Parasites by PCR, for a full description of designated work and storage areas for PCR.

E. Heads and/or Gill Arches for Parasitology

Heads are usually received in a plastic Ziploc back. The bags are surface disinfected with 70% isopropyl alcohol, labeled with the case history number, initials, and date, and frozen (-70°C). If they can be processed the following day, they can be refrigerated overnight.

Heads may be halved during processing to create an archive tissue in addition to the tissue being tested by Pepsin-Trypsin Digest. This can be done upon receipt of samples or at a later date.

F. Intestinal Tracts for Parasitology

Gastrointestinal tracts will be kept in physiological saline in the refrigerator until they can be examined (within 24-48 h) for presence of *Ceratomyxa shasta* and/or *Bothriocephalus acheilognathi*, as appropriate. If samples cannot be processed within 24-48 hours, they can be frozen and examined at a later time.

G. Other Samples

If other samples are received (e.g., tissues preserved in Neutral Buffered Formalin or Davidson's Fixative) for other types of processing, notify the appropriate person to process them.

X. Notification of Laboratory Personnel

The person primarily responsible for each area of testing should be informed immediately of sample arrival. In that person's absence, their designated alternate should be notified. When each individual takes possession of any samples, he or she should initial and date the case history record to that effect. From that point until that person both completes testing and destroys the samples or until another staff member takes possession, the responsible party must be aware of the exact location and disposition of the samples. He or she is also responsible for noting the final disposition (discarded, archived, etc.) of the samples they are responsible for.

XI. Data Sheets / Worksheets

Individual datasheets or worksheets of testing performed are maintained differently in each laboratory. For each major division of work, all procedures performed, their dates, and the initials of the diagnostician should be noted. The main case history record must contain records of the logs kept for each procedure (See Appendix 3.A for example forms).

Appendix 3.A – Fish Health Center Case History Record

CASE #:_____ **DATE REC**:_____ **REC BY**:_____

FACILITY:_____**STATE:**_____

Type: ☐ NFH ☐ SFH ☐ TFH ☐ WFS ☐ FERAL ☐ CFH

☐ OTHER:_____

SERVICE:

Type: ☐Complete ☐Virology ☐Bacteriology ☐Diagnostic

☐OTHER:_____

FISH SPECIES:

1)_____ 2)_____ 3)_____ 4)_____

5)_____ 6)_____ 7)_____ 8)_____

MEMO:_____

_____

_____

TYPE & NUMBER OF SAMPLES:

_____
_____
_____
_____
_____
_____
_____

ALL DIAGNOSTICIANS NOTIFIED: Initials_____

Possession Assumed/Initials:

☐Virology_____ ☐Bacteriology_____ ☐Parasitology_____ ☐DFAT_____

☐ELISA_____ ☐PCR_____ ☐Other_____

LABORATORY FINDINGS:

☐ VIROLOGY:_____

_____

_____

Completed/Date/Initials:_____ Sample Disposition:_____

☐BACTERIOLOGY:_____

_____

_____

Completed/Date/Initials:_____ Sample Disposition:_____

☐PARASITOLOGY/plankton centrifuge, digest,
PCR:_____

_____

_____

Completed/Date/Initials:_____ Sample Disposition:_____

☐(BKD) *R. salmoninarum* /DFAT:_____

_____

ELISA:_____

_____

PCR: _____

Completed/Date/Initials:_____ Sample Disposition:_____

Results and Recommendations (if applicable):_____

_____

_____

Appendix 3.B - Chain of Custody Form

U.S. Fish & Wildlife Service
Wild Fish Health Survey
Chain of Custody Record

Source of Samples_____
Case History Number (to be filled in by receiving lab)_____

FROM: (print name, agency) TO: (print name, agency)	RELEASE SIGNATURE: RECEIPT SIGNATURE:	RELEASE DATE: RECEIPT DATE:	DELIVERED VIA: ☐ U.S. Mail ☐ Fed Ex ☐ UPS ☐ In person ☐ Other:
FROM: (print name, agency) TO: (print name, agency)	RELEASE SIGNATURE: RECEIPT SIGNATURE:	RELEASE DATE: RECEIPT DATE:	DELIVERED VIA: ☐ U.S. Mail ☐ Fed Ex ☐ UPS ☐ In person ☐ Other:
FROM: (print name, agency) TO: (print name, agency)	RELEASE SIGNATURE: RECEIPT SIGNATURE:	RELEASE DATE: RECEIPT DATE:	DELIVERED VIA: ☐ U.S. Mail ☐ Fed Ex ☐ UPS ☐ In person ☐ Other:

☐ **Continuation of Chain-of-Custody on Further Sheets**

CHAPTER 4

Standard Necropsy Procedures for Finfish

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I. Introduction

This chapter describes the general procedure for performing a diagnostic necropsy on finfish. While the majority of sampling conducted for the Survey will be performed on normal fish, identification of characteristic behaviors and clinical signs of infected or diseased fish are important components of any pathogen screening program. Proper dissection techniques during necropsy will optimize pathogen detection and subsequent determination of disease status.

Live fish should be examined for behavioral abnormalities (spiral swimming, flashing, flared gill opercula, prostration, etc.) then anesthetized to avoid tissue artifacts caused by alternate methods of euthanasia such as pithing or a blow to the head. Some external abnormalities (whitened or eroded fin tips, cloudy cornea, body discoloration, excessive mucus) are best observed while the fish is submerged in water. In many cases postmortem change in fish that are received dead will prevent this latter opportunity.

II. General Necropsy Procedure

(Figure 1 gives an example of the general anatomy of salmonids)

A. Necropsy subjects should first be examined for external abnormalities or lesions that could include: pugheadedness or otherwise poor body condition; exophthalmia; cloudy cornea or lens opacity; hemorrhaging within the anterior chamber of the eyes, fins, body surface or body orifices (anus, nares, mouth, gill chamber), frayed or missing fins; gas bubbles within the fin rays or connective tissues of the eyes; ulcerations, abscesses, abrasions; body discoloration; excessive mucus; trailing fecal casts or rectal prolapse; external foreign bodies such as fungus, metazoan or protozoan parasites, cysts or tissue growths; potbelly or other protrusion or body malformations (spinal deformities, cranial swelling, shortened opercula, pugheadedness, microeye).

B. External lesions such as ulcerations or abrasions should be inoculated onto BHIA (see Chapter 5 - Bacteriology). Use of BHIA with 1% NaCl may be necessary depending upon case information and whether fish are in saltwater and a halophilic bacterial pathogen is suspected.

C. A peripheral blood smear can be made by excising the caudal peduncle (for small fish) and allowing a drop of blood to be deposited near the frosted end of a clean glass slide. The blood is smeared <u>before clotting</u> with a second glass slide by touching the drop with the slide at a 45° angle to the first slide and pushing the angled slide to the end of the first slide. Capillary action draws the smear across the first slide and the narrower the angle the thinner the smear (Figure 2, page 10). Stain the smears in Diff-Quik® (see staining procedures in section V.) and observe on the microscope at 1000X for bacteria, erythrocytic inclusion bodies (EIB) and viral erythrocytic (VEN) cytoplasmic inclusions, necrobiotic bodies (IHNV) and erythroblastosis or other blood abnormalities in cell composition and morphology. Larger fish may be bled by caudal vein puncture into a heparinized syringe or Vacutainer® and blood expressed onto a slide for subsequent smearing. For blood collection, the needle should be inserted at the location just below

the lateral line that intersects with the rear margin of the anal fin. The needle should be inserted until just penetrating the vertebra (hemal canal) as indicated by slight resistance. Blood will automatically begin to flow when the Vacutainer® is punctured by the needle base or when the plunger of the syringe is pulled back.

D. Fish should be placed on their right sides for performance of the remaining necropsy procedures. Skin scrapes of normal and lesion areas mounted with a drop of PBS and coverslip on a glass slide should be made by using either the edge of the coverslip as the scraping instrument, or a scalpel. Bacteria or fungus from lesion areas or protozoan parasites such as *Ichthyobodo* and *Trichodina* are common subjects to look for beginning at 40X and then at 200-400X on a compound microscope (if phase contrast is not available on the microscope, the condenser and diaphragm can be adjusted to increase contrast).

E. Wet mounts of gill filaments are made by using a small pair of surgical scissors to Remove a portion of one gill arch. Gill filaments should be slightly teased apart for good viewing of filament and lamellar profiles and mounted in PBS with or without a coverslip. These should be examined immediately since branchial epithelium rapidly deteriorates causing postmortem artifact. Look for gas bubbles in the capillaries, telangiectasia, hyperplasia, external parasites (bacterial, protozoal, fungal, metazoan), or other foreign bodies. Should bacteria be observed or suspected the coverslip may be removed and used to mince the gill tissue. This is allowed to air dry for later Gram staining (see Chapter 5). After staining, the gill tissue is removed with forceps for viewing of the stained slide for bacteria by oil immersion.

F. Disinfect the outer surface of the fish by flooding with 70% ethanol. Disinfect a pair of scissors, forceps and scalpel by immersion in 100% ethanol and passing the instruments through a Bunsen flame allowing the alcohol to ignite and burn off. Repeat one or two more times. Wipe instruments clean of any organic matter beforehand for effective disinfection.

G. The abdominal cavity is entered by pulling the pectoral fin with sterile forceps while cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors. The cut is continued dorsally to just below the lateral line where resistance is encountered. Start again at the base of the pectoral fin and continue the incision towards the posterior of the fish along the ventral abdominal wall to the vent. Stay slightly above the intestinal tract when making the incision so that it is not punctured, thereby contaminating the tissues. At the vent continue dorsally to just below the lateral line and continue cutting anteriorly to connect with the first incision. Remove the flap of abdominal tissue, thus exposing the internal viscera and cavity. When done correctly on a moribund specimen the air bladder should remain inflated and the GI tract completely intact. Instruments may need wiping of organic material and flaming repeatedly during this procedure.

H. Visually examine viscera (heart, liver and gall bladder, kidney, pancreas, adipose tissue, spleen, air bladder, pyloric caecae and entire GI tract) for abnormalities such as:

discoloration or mottled appearance; enlargement (hypertrophy); hemorrhage or erythema; abscesses or cysts; fluid in the abdominal cavity (ascites causing potbelly); foreign bodies such as fungus, metazoan parasites or tissue growths, etc.

I. If bacteria samples are to be taken they should be inoculated onto BHIA or other appropriate growth medium (i.e., TYES from the kidney, spleen, visceral lesion, or other tissues if indicated. See Chapter 5 – Bacteriology).

J. Tissues to be taken for viral assay of larger fish (kidney/spleen pool) should also be placed into sterile tissue culture fluid for refrigeration and homogenization at a later time. Fry are generally processed whole for virology (see Chapter 11 - Virology).

K. Kidney smears for FAT and tissues (kidney or kidney/spleen) for ELISA detection of the *Renibacterium salmoninarum* should be taken at this step. Generally, bacterial problems due to Gram-negative bacteria such as furunculosis and ERM agents can be detected more efficiently by isolation on prepared media.

L. If the spleen has not been completely removed for virus assay, a spleen squash can be made by placing a cut section of the tissue with a drop of PBS on a glass slide and covering with a coverslip. Whole spleen squashes will be necessary when small fish are examined. Look for the presence of motile or non-motile bacterial rods and fungal hyphae. The coverslip may be removed and the squash Gram stained for confirmation of bacteria as described for gill tissues.

M. A squash of a small section of the lower intestine (rectum) should also be made on a glass slide using PBS and a coverslip. Look for presence or absence of food and *Hexamita* or amoebae. Bacteria should obviously be abundant as part of the normal gut flora. Also look for fungal hyphae within the gut wall.

N. A squash of lesion material from a visceral organ or organs may be warranted if present and if its cause is not readily discernible. Gram stains (Chapter 5) and/or Diff-Quik® stains (Section V) of this material may also be warranted. An example would be stained impression smears of kidney tissue to examine for possible BKD, PKD or *Enterocytozoon salmonis.*

O. If the cause of mortality or morbidity is in question as to whether or not the above procedures will provide an answer, histology samples should be taken as a backup measure, but only if moribund fish are available. Fish that have been dead for several hours or longer are generally not suitable for histology due to postmortem tissue autolysis. If fry are involved, whole fish may be dropped into Davidson's, or a non-formalin based fixative. Fingerlings should have the abdomens opened with scissors for better fixative penetration (refer to Chapter 13 – Histology, for more information on fixing tissues).

P. If clinical signs suggest a central nervous system disorder the top of the cranial cavity should be opened and the brain included in bacteriologic sampling using BHIA and

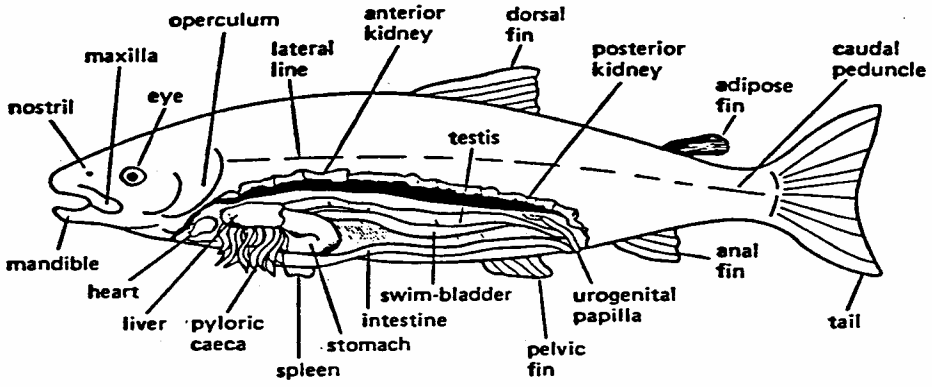
TYES agar. Heads from additional affected fish should be severed behind the gill opercula and placed into whirlpak bags for later testing for *Myxobolus cerebralis.* Heads can be halved for PTD and an archive sample for corroborative testing by PCR or histology.

Q. During necropsy, occasional serial sectioning of skeletal muscle using a razor blade may be necessary should a lesion within that tissue be suspected. Examples would include abscesses, hematomas, neoplasms or encysted parasites causing a protrusion of the musculature. Depending upon the nature of the lesion, bacteriological sampling, Gram staining or fixation for histology may be necessary.

If clinical signs are present, or fish are moribund, include at least 5-10 moribund for proper diagnosis. Control or healthy fish should also be examined and compared to determine whether abnormalities perceived in the population are real, or not. The number of control fish processed will depend upon availability and the particular case and may range from 10 to none.

Necropsies are best performed as a 2-3 person team effort in which a microbiologist and/or technician can make gross external and internal observations and the bacteriologic and tissue preparations. The pathologist in charge can devote his or her time to interpreting the sample preparations on the microscope. In this approach a case can be processed in a minimum amount of time and provides further pathology experience to the support staff.

Figure 1 – Salmonid Anatomy



III. Results and Report of Findings

In summary, a standard necropsy should include all the information contained on the NWFHS Submission Form (Case History Number, location, species, examination date and number of tissues/samples submitted). In addition to the Submission Form, a complete Necropsy Form, containing the following information, should be attached:

A. External and internal gross observations recorded on a necropsy worksheet.

B. Wet mounts or squashes of:

   1. Gills
   2. Skin
   3. Spleen
   4. lower gut
   5. lesions (if any)

C. Peripheral blood smear stained with Diff-Quik® (Optional)

D. Gram Stain – Type and Number of tissues

E. Overall evaluation / findings

IV. Fish Diseases: Causative Agents and Signs

A. Bacteria

1. **Bacterial Kidney Disease (*Renibacterium salmoninarum)***

   External signs: exophthalmia; abdominal swelling; sometimes blisters in skin filled with clear amber to cream colored purulent fluid. In advanced disease, large muscle lesions may be present.

   Internal signs: kidneys pale and swollen; abscesses in kidney, liver or spleen; may have ascitic fluid in abdomen; intestine distended, fluid filled.

2. **Cold Water Disease (*Flavobacterium psychrophilum*)**

   External signs: tail darkening, white or bluish areas behind dorsal or adipose fins; loss of epidermis on dorsal or posterior surface; erosion of the dermis on the peduncle exposing skeletal muscle; loss of caudal peduncle; erosion of jaw or snout; gill hemorrhages and anemia. In some cases, no external signs are observed.

   Internal signs: generally not remarkable but sometimes has enlarged spleen with myriad number of filamentous rods; petechial hemorrhages of adipose tissues.

3. **Columnaris (*Flavobacterium columnare*)**
   External signs: white to yellow lesions that may have a red periphery on the head, jaw, back (saddleback lesion), and/or fins, especially caudle fin. Gills may also be infected; disease begins at the tips of the lamellae and causes a progressive necrosis that may extend to the base of the gill arch. Bacteria are gliding and often form clumps that appear like a column or "haystack."

4. **Edwardsiella tarda Septicemia (*Edwardsiella tarda*)**
   External signs: small cutaneous lesions that become large abscesses within the muscle, and become necrotic. May also have loss of dermal pigmentation.
   Internal signs: generalized septicemia, ascitic fluid in abdominal cavity, protruding hemorrhaged anus, opaqueness in eyes; small white nodules may be present in the kidney, liver, spleen, and gills.

5. **Enteric Redmouth (*Yersinia ruckeri*)**
   External signs: hemorrhaging or erosion around mouth; pale gills; exophthalmia; swollen abdomen; reddened opercula and fin bases; inflamed hemorrhagic vent.
   Internal signs: inflammation and hemorrhaging in most visceral organs; edema in spleen, liver and kidney; liver may be pale; fluids may accumulate in abdominal cavity, stomach and intestine; inflamed, hemorrhagic lower intestine with bloody diarrhea.

6. **Enteric Septicemia (*Edwardsiella ictaluri*)**
   External signs: Fish refuse feed and swim at the surface. External lesions with hemorrhage around the mouth and lateral and ventral portions of the body and fins; pale gills; exopthalmia; and small ulcerations on the body. Ulceration in the fontanelle of the frontal bones.
   Internal signs: generalized septicemia with petechiae throughout the visceral mass, in the peritoneum and musculature. Ascites and enlargement of the liver, kidney and spleen.

7. **Furunculosis (*Aeromonas salmonicida)***
   External signs: skin blisters or furuncles which may ulcerate; erythemia of eyes, base of fins and anal vent. In acute cases, bleeding from the gills may be seen.
   Internal signs: kidney necrosis; petechiae in mesenteries around pancreatic tissue; localized hemorrhages in intestine and liver; dark, hypertrophied spleens.

8. **Citrobacter infection (*Citrobacter freundii*)**
   External Signs: Ulcerative lesions may be seen on skin, eye and base of fins.
   Internal Signs: Hemorrhaging in the peritoneum and gastro intestinal tract; swollen kidney with multiple lipoid granuloma in some fish species.

B. Parasites

1. **Asian tapeworm (*Bothriocephalus acheilognathi*)**
   External signs: abdominal swelling if heavily infected.
   Internal signs: little abdominal fat due to starvation, presence of tapeworm in stomach.

2. **Ceratomyxosis (*Ceratomyxa shasta*)**
   External signs: loss of appetite, hemorrhaging and swelling of urogenitial opening.
   Internal signs: ascites; swelling and hemorrhaging of the intestine; swollen vent. Developing parasites incite a diffuse granulomatosis in many host tissues, including intestine, liver, kidney, spleen, gonads, and muscle. The abdomen is often distended because of granulomatous peritonitis.

3. **Whirling Disease (Myxobolus cerebralis)**
   External signs: black tail in 3-6 month old fish; impaired balance and a frenzied, tail-chasing behavior. Older fish that survive often develop spinal curvature, pug-headedness, or an undershot jaw from cartilage damage.
   Internal signs: none except histological.

C. Viruses

1. **Infectious Hematopoietic Necrosis Virus (IHNV)**
   External signs: exophthalmia, body darkening, abdominal distension, pale gills, trailing white fecal cast; lethargic swimming, riding high in the water column.
   Internal signs: ascites, viscera paleness, anemia, petechial hemorrhages, pale kidney; little or no food in the intestinal tract.

2. **Infectious Pancreatic Necrosis Virus (IPNV)**
   External signs: whirling, agonal swimming, anorexia, dorsal darkening, abdominal distension, and/or trailing white feces.
   Internal signs: petechial hemorrhages and yellow exudate in gut of older fish; fry will have pale viscera with few petechiae.

3. **Infectious Salmon Anemia Virus (ISAV)**
   External signs: appear 2-4 weeks after infection, few external signs other than exopthalmia, pale gills and lethargy. Mortality of up to 3% per day in some cases. Infected facilities may see a predictable rise in mortality by 0.05% per day for three consecutive days.
   Internal signs: hemorrhaging on the kidney and other organs; swollen eyes; fluid in body cavity; swelling of kidney; darkening of posterior gut and swollen spleen.

4. **Largemouth Bass Virus (LMBV)**
   External signs: moribund fish loose equilibrium and float at the surface due to enlarged swim bladder.

Internal signs: Gas gland excessively red; air bladder lesions consist of a yellow to brown waxy residue in the lumen of the air bladder.

5. **Oncorhynchus Masou Virus (OMV)**
   External signs: epithelioma (tumors of the epithelial layer) occurring mainly around the mouth, ulcers on skin.
   Internal signs: intestinal hemorrhages, white spots on liver.

6. **Spring Viremia Carp Virus (SVCV)**
   External signs: lethargy, sluggish breathing, concentration in slow waters. A darkening of skin and gills, bloody mucus or fecal casts from hemorrhaging vent.
   Internal signs: Fluid in the body cavity, swollen spleen, blood in swim bladder and a general hemorrhagic condition.

7. **Viral Hemorrhagic Septicemia Virus (VHSV)**
   External signs: lethargy, avoid current, listless, hang suspended or drop to bottom; body darkening, exophthalmia with hemorrhaged orbit, external hemorrhage especially base of fins and in roof of mouth, pale gills with focal hemorrhage.
   Internal signs: empty gastrointestinal tract; scattered hemorrhages of connective tissue, adipose tissue, swim bladder, and intestine; kidney red and thin in acute stage, gray and swollen in chronic stage.

8. **White Sturgeon Iridovirus (WSIV)**
   External signs: emaciation, go off feed: swollen gills.
   Internal signs: hyperemic areas on abdomen, necrosis of epidermis.

9. **White Sturgeon Herpesvirus (WSHV-2)**
   External signs: few to severe hemorrhagic signs in young fish; hemorrhages around ventral scutes and mouth; small ulcers with petechial hemorrhaging. Chronic form will have reoccurring blisters, especially after stress, often starts on the head.
   Internal signs: none, except for ulceration in the mouth area.

V. Staining Procedures

A. Gram Stain - See Chapter 5 – Bacteriology, for Gram Staining procedure.

B. Diff-Quik® Stain:

1. Make smears of blood, fluids, or tissues and air-dry

2. Dip 5 times in Diff-Quik® solution 1, one second each time, and drain

3. Dip 5 times in Diff-Quik® solution 2, one second each time, and drain

4. Dip 5 times in Diff-Quik® solution 3, one second each time, and drain

5. Rinse in tap water and drain

6. Air-dry and examine using 10x, 40x, or 100x objective lens

   NOTE: Diff-Quik® solution 3 can become weakened with age or use. Check stains intensity on slides periodically. Slides may be re-stained with fresh solution 3 if necessary. Periodically pass solutions 2 and 3 through separate 0.45-µm filters to remove precipitates and contaminating bacteria.

Figure 2. Preparation of a Thin Blood Smear

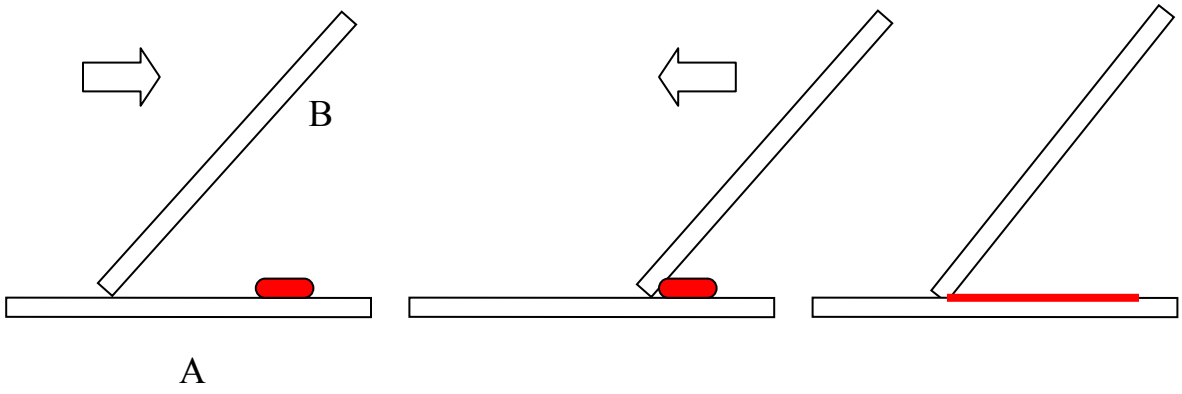
1. On slide "A" express a drop of blood about one-half inch from the end.

2. The edge of a second slide "B" is placed on the surface of slide "A" at about a 45° angle and is moved backward (to the right in the diagram) until contact with the drop of blood.

3. Contact with the blood will cause the drop to spread along the edge of slide "B" due to capillary action. Slide "B" is then pushed forward (left in the diagram), being careful to keep the edge pressed uniformly against the surface of slide "A".

4. The size of the drop of blood and acuteness of the angle formed between the slides will determine the thickness of the film. A more acute angle results in a thicker film.

5. The smear is allowed to air dry for transport in a slide box and later staining.



CHAPTER 5

Bacteriology

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I. Introduction

This section defines the procedures and techniques used to correctly identify the target bacterial pathogens identified for the Survey (*Yersinia ruckeri*, *Aeromonas salmonicida* and *Edwardsiella ictaluri*). Proper identification relies on bacterial growth characteristics, appropriate biochemical tests, and corroboration by serological and molecular (Chapter 7) techniques.

Pathogens of Regional Importance (PRIs) include: *Citrobacter freundii, Edwardsiella tarda, Flavobacterium columnare* and *Flavobacterium psychrophilum.* The later two bacteria have special requirements for culture and serological confirmation. Molecular Techniques are also available for confirmation of *Flavobacterium psychrophilum*. Several excellent sources are listed in the Reference section for identification of PRIs and other bacteria that may be isolated from fish sampled for the Survey. Additional media formulas are also provided for PRIs in Appendix 5.A.

II. Media Preparation

A. Plate Media

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely dissolve agar. Common media recipes are given in Appendix 5.A.

2. Cover beaker with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 15 minutes at 15 pounds pressure.

3. Cool media to 50°C.

4. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C, then proceed to step 5.

5. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface.

6. Label the plates or plate storage tins with the type of medium, preparers initials, and date made.

7. Remove bottle cap and pour plates or dispense with a Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per 100×15 mm petri dish. Replace lids as soon as the plate is poured.

8. Immediately after use, rinse the automatic pipettor in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

9. Invert plates when the media has cooled completely (~ 30-60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid. Do not use the UV light because it can denature the proteins in the media.

10. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

11. Follow manufacturer's recommendation for storage period of prepared media.

B. Tube Media

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely dissolve the agar.

2. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.

3. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.

4. Use an automatic pipettor or Pipet-aid™ to dispense the medium. If using the Brewer or Cornwall pipette prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few dispenses of medium. Dispense approximately 5 to 10 mL media in 16×125mm or 20×125mm tubes. Close caps loosely.

5. Immediately after use, rinse the automatic pipettor in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

6. Follow manufacturer's recommendation for autoclave time and temperature.

7. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. short butt and long "fish-tail" slant for TSI or a standard slant over ¾ of the tube length for BHIA).

8. Tighten caps when tubes can be easily handled but still warm to the touch. Cool completely to room temperature in the slanted position.

9. Label the tubes or the tube rack with type of medium, preparer's initials, and date made.

10. Store at 2-8°C, following manufacturer's recommendation for period of long-term storage.

III. Media Formulations

Numerous differential media and biochemical tests can be used to determine identification of bacterial cultures. Some common bacteriological media are listed in Appendix 5.A, but this list is incomplete and a good bacteriology media reference such as Difco Manual (1998), McFaddins Biochemical Media Used for Detection of Bacteria (McFaddins 1980 and 1985), or Atlas's Handbook of Microbiological Media (Atlas, 1997) should be used for additional information. The purpose and use of various media are described in the testing section.

IV. Bacterial Culture Isolation

A. Aseptically inoculate samples onto BHIA tubes or plates labeled with pertinent case history information.

B. Incubate aerobically for 24-48 hours at 20-24°C (room temperature). If no growth occurs at 24 and 48 hours, record this information on the data sheet. If no growth occurs after 96 hours, samples are discarded.

C. When growth does occur on field collection tubes or plates, use a sterile loop or needle to select a single colony to subculture onto fresh BHIA. If colonies are not well isolated, the plate will have to be re-inoculated on BHIA and thoroughly struck over the entire plate surface to achieve isolation of bacteria.

D. Incubate at 20-24°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.

1. Using a sterile needle or small loop, pick individual distinct bacterial colonies. Use of a dissecting scope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

2. Begin initial testing to determine presumptive identification of pure strain bacterial cultures by CO, motility, catalase, and Gram stain.

3. Based on preliminary tests, follow the Flowchart (Appendix 5.D for major pathogens & Appendix 5.E for PRIs) to determine which biochemical tests are needed to determine identification.

E. Inoculate biochemical tubes and label with pertinent case history information.

1. Follow the directions for interpretation of biochemical tests in the next section.

F. Treat all bacterial cultures as potential human pathogens. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave.

V. Gram Stain

Gram stain pure strain cultures to determine whether Gram-negative or Gram-positive. Gram staining detects a fundamental difference in the cell wall composition of bacteria. Kits are available commercially, or formulas for reagents are listed in Appendix 5.B.

A. Prepare a bacterial smear from a pure culture

1. Put a drop of saline, distilled water, or PBS on a clean glass slide
2. Using a sterile loop or needle touch an isolated colony and mix in the water drop.
3. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).
4. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.
5. Allow to cool.

B. Flood the slide with crystal violet, and allow to remain on the slide for 60 seconds

C. Wash off the crystal violet with running tap water.

D. Flood the slide with Gram's iodine, and allow to remain on the slide for 60 seconds.

E. Wash off with running tap water.

F. Decolorize with 95% alcohol and 5% acetone solution until the solvent flows colorless from the slide (approximate 5-10 seconds). Excessive decolorization should be avoided since it may result in a false gram-negative reading.

G. Rinse immediately with running tap water.

H. Counter stain with Safranin for 60 seconds.

I. Rinse with tap water and allow to air dry.

J. Results

1. Gram-negative cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counterstained with safranin.

2. Gram-positive cells retain the crystal violet and remain purple to dark blue.

K. Quality Control

Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC® and maintained at 2-8°C for long term use). Additionally Fisher Scientific has gram control slides (Catalog# 08-801)

1. Positive: *Staphylococcus sp.*

2. Negative: *Yersinia ruckeri*

VI. Alternative test for Gram Reaction – 3% Potassium Hydroxide

A. Add a heavy inoculum of pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 grams KOH per 100 mL distilled water) on a clean glass slide.

B. Stir for about one minute, occasionally lifting the loop to look for thickening and "stringing" of the slurry.

C. Results:

1. Gram positive bacteria will not appear to change the viscosity of the KOH solution.

2. Gram negative bacteria will cause the KOH solution to become stringy or mucoid in appearance and consistency.

D. Quality Control:

1. Gram Positive: *Staphylococcus sp*. (ATCC® any isolate)

2. Gram Negative: *Yersinia ruckeri*

VII. Presumptive Identification of Gram Negative Bacteria

Refer to Flow Chart in Appendix 5.D and 5.E then perform the following series of tests. All the following tests should be incubated aerobically unless stated otherwise.

A. Carbohydrate Utilization (MacFaddin 1980)

The following carbohydrates are utilized to aid in bacterial species identification: Arabinose, Rhamnose, Mannitol, Salicin, Sorbitol, and Sucrose (saccharose). The procedures to be followed for each of these media are identical.

1. Inoculate carbohydrate tube (Appendix 5.A "Carbohydrate Utilization Medium") with growth from an 18 to 24 hour pure culture.

2. Incubate with loosened cap 18 to 24 hours at 20-24°C. A prolonged incubation of up to four days may be necessary for some negative results.

3. Results

   a. Positive - Acid is produced from fermentation, which turns media yellow.

   b. Negative - No fermentation of carbohydrate, media remains green.

   c. Aerogenic - Gas bubbles are present within the media.

4. Quality Control

Carbohydrate	**Positive Control Isolate**	**Negative Control Isolate**
Arabinose	*Escherichia coli* (ATCC® 25922)	*Yersinia ruckeri*
Sorbitol	*Escherichia coli* (ATCC® 25922)	*Y. ruckeri* Type I
Rhamnose	*Enterobacter aerogenes* (ATCC® 13048)	*Yersinia ruckeri*
Salicin	*Enterobacter aerogenes* (ATCC® 13048)	*Yersinia ruckeri*

5. Precautions

   a. Difficulty in interpreting test results may occur with slow growing bacteria. Prolonged incubation may be required.

   b. Heavy bacterial growth throughout the media can offset the color of a negative (green) reaction, giving the appearance of a weakly positive (yellow) reaction. This is especially true with yellow-pigmented bacteria. These tubes should be retested if a true yellow color is not noted within several days.

B. Catalase

This test determines bacterial production of catalase enzymes.

1. Place a drop of hydrogen peroxide (3% H_2O_2 - reagent grade) on a microscope slide or in the concave surface of a hanging drop slide.

2. With a sterile loop, collect a sample of 18 to 24 hour old pure bacterial culture.

3. Place the loop in the hydrogen peroxide.

   a. If the test is positive, there will be immediate bubbling or foaming, and liberation of O2 gas.

b. Record results.

C. Cytochrome oxidase

This test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. Add an inoculum of pure 18 -24 hour old bacterial culture to the test strip impregnated with reagent.

2. Results

   a. Positive: purple color within 5-10 seconds (reactions that occur after 10 seconds are negative).

   b. Negative: no purple color.

3. Quality Control:

   a. Positive: *Pseudomonas aerugenosa* (ATCC® 10145)

   b. Negative: *Yersinia sp*.

D. Decarboxylase Test (Lysine and Ornithine)

A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity.

1. For each isolate to be tested, it is necessary to inoculate a decarboxylase control tube and lysine or ornithine test tube (Appendix 5.A "Decarboxylase Medium Base"). Use light inoculum from 18 to 24 hour pure culture.

2. Add 1 to 2 mL oil overlay to each tube.

3. Incubate 24 hours at 20-24°C. A prolonged incubation of up to four days may be necessary.

4. Results

Test Result	Lysine or Ornathine Tube	Control Tube
Positive	Turbid to faded purple (glucose fermented, decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented, decarboxylase not produced)	Yellow (glucose fermented)
Negative	Purple (glucose not fermented, decarboxylase not produced)	Purple (glucose not fermented)

5. Quality Control

   a. Positive - *Enterobacter aerogenes* (ATCC® 13048)

   b. Negative - *Proteus vulgaris* (ATCC® 13315)

6. Precautions

   a. At the end of incubation, the lysine tube might show a layer of purple over yellow. Gently shake the tube before interpreting the result.

   b. An indistinct yellow-purple color may be difficult to interpret. Use the control tube for comparison. Any trace of purple color after a 24-hour incubation in the amino acid tube denotes a positive result.

   c. Do not interpret tests prior to 18 to 24 hours. During the first 12 hours, only glucose is fermented which produces a yellow color. Decarboxylase enzymes do not form until the acidic environment is established by the fermentation of glucose.

E. Esculin Test

To determine the ability of an organism to hydrolyze the glycoside esculin (aesculin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%).

1. Inoculate the surface of the bile esculin slant (Appendix 5.A "Bile Esculin Agar") with inoculum from an 18 to 24 hour old pure culture.

2. Incubate 20-24°C for 24 to 48 hours.

3. Results

   a. Positive - Presence of a black to dark brown color on the slant.

   b. Negative - No blackening of the medium.

4. Quality Control

   a. Positive - *Enterobacter aerogenes* (ATCC® 13048)

   b. Negative - *Yersinia ruckeri*

5. Precautions - False positives may occur with hydrogen sulfide producing organisms, such as *Shewanella putrefaciens*. Neither of the target organisms for these protocols will, however, produce hydrogen sulfide.

F. **Gelatinase**

A test to determine bacterial production of gelatinase enzymes that liquefy gelatin.

1. Inoculate by stabbing ½ to 1 inch deep into the nutrient gelatin media (Appendix 5.A "Nutrient Gelatin") with a heavy inoculum from an 18 to 24 hour pure culture.

2. Inocubate 18 to 24 hours at 20-24°C.

3. Results

   a. Positive – Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

   b. Negative – No liquefaction occurs in media.

4. Quality Control

   a. Positive - *Proteus vulgaris* (ATCC® 8427)

   b. Negative - *Escherichia coli* (ATCC® 25922)

5. Precautions

   a. The liquid will generally appear turbid due to bacterial growth.

   b. Nutrient gelatin softens at temperatures above 20°C. Keep refrigerated until ready to inoculate, and do not let tubes reach room temperature or warmer. This will make interpretation of results difficult. Tests, which are incubated at 35°C, should be refrigerated prior to recording results.

G. Indole Test

A test to determine bacterial ability to split indole from the tryptophan molecule. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole), and indoleacetate. Indole, pyruvic acid, ammonia, and energy are principle degradation products of tryptophan. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent. The reagent is not a dye or stain, but reacts with indole to produce an AZO dye.

1. Inoculate tryptone broth (Appendix 5.A"Tryptone Broth") with a light inoculum from an 18 to 24 hour pure culture.

2. Incubate 24 to 48 hours at 20-24°C

3. At the end of 24 hours incubation do the following:

a. Aseptically remove 2 mL of media and place in an empty sterile test tube. Save extra tube for 48-hour incubation, if necessary.

b. Add about 5 drops of Kovac's reagent (Appendix 5.B "Kovac's Indole Reagent") to one of the tubes and agitate tube.

c. If a positive reaction is observed, the test is complete.

d. If the 24 hour incubated sample is negative, incubate the remaining tube for an additional 24 hours, and test again for the presence of indole with Kovac's reagent.

4. Results

a. Positive - Within 1 to 2 minutes, a cherry red ring will form at the surface of the media.

b. Negative - No color formation is observed at the surface; the color remains that of the reagent – yellow.

c. Variable - An orange color may develop. This indicates the presence of skatole, which may be a precursor of indole formation.

5. Quality Control

a. Positive - *Escherichia coli* (ATCC® 25922)

b. Negative - *Pseudomonas aerugenosa* (ATCC® 27853)

6. Precautions

a. Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.

b. Tests for indole should be conducted after both 24 and 48 hours of incubation before a test can be declared negative. Split the broth culture prior to performing the 24-hour test. If negative, incubate the untested tube (without Kovac's) for another day and try again.

c. Do not eliminate the 24-hour test, because some organisms may have produced indole by 24 hours, but have broken it down by 48 hours. DO BOTH!

d. Kovac's reagent should be fresh. A color change from yellow to brown indicates aging and results in reduced sensitivity of the test.

e. The procedure described here produces more reliable results than those obtained from MIO (motility-indole-ornithine) medium.

H. Malonate Test

A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon.

1. Inoculate malonate media (Appendix 5.A "Malonate Broth") with a light inoculum from an 18 to 24 hour pure culture.

2. Incubate 24 to 48 hours at 20-24°C.

3. Results

   a. Positive - Light blue to deep blue color throughout the media.

   b. Negative - Color remains the same as un-inoculated tube - green.

4. Quality Control

   a. Positive - *Enterobacter aerogenes* (ATCC® 13048)

   b. Negative - *Yersinia ruckeri*

5. Precautions
   The test tube must be incubated for at least 48 hours before it may be called negative. Since some bacteria produce only slight alkalinity, it is useful to compare the test to an un-inoculated tube. Any trace of blue color denotes a positive reaction.

I. Motility

This test determines if a bacterial isolate is motile by means of flagella.

1. Place a drop of distilled water or sterile PBS onto the center of a clean microscope cover glass. Place an additional tiny drop in one corner of the cover glass (to adhere the cover glass to the depression slide when it is inverted). Inoculate the center drop from a pure strain culture that is 24 to 48 hours old using a sterile loop. Carefully invert the cover glass and place over the concave portion of a hanging drop slide. Observe for motility using phase contrast at 400X magnification on a compound microscope. Care should be taken to not interpret "drift" or "Brownian motion" as motility. Record results as motile or non-motile.

2. If this method fails to show motility then:

   a. Inoculate a nutrient broth with the isolate and incubate at room temperature until growth is obtained, usually 24 hours. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover glass. Place a tiny drop of distilled water in one corner of the same cover glass. Continue as above.

b. Semi-solid motility test medium can also be used. Stab the medium with a small amount of inoculum. Incubate overnight at room temperature. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

3. Quality Control

a. Positive: *Escherichia coli* (ATCC® 25922)

b. Negative: *Aeromonas salmonicida*

J. Nitrate Reduction

To determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or further reduced products.

1. Inoculate the broth (Appendix 5.A "Nitrate Broth") with 18 to 24 hour old pure culture.

2. Incubate 24 to 48 hours at 20-24°C aerobically.

3. After incubation add about 5 drops of *a*-naphthylamine and sulfanilic acid (Appendix 5.B "*a*-naphthylamine", "sulfanilic acid") to the medium and shake gently to mix reagents..

4. If there is no color development after addition of *a*-naphthylamine and sulfanilic acid, add a small amount of zinc dust.

5. Results

a. Positive – Formation of a pink or red color in the medium within 1-2 minutes following the addition of *a*-naphthylamine and sulfanilic acid. or no color development within 5-10 minutes after adding zinc dust.

b. Negative – No pink or red color development within 1-2 minutes following the addition of *a*-naphthylamine and sulfanilic acid. or red color development within 5-10 minutes after adding zinc dust.

6. Quality Control

a. Positive – *Escherichia coli* (ATCC® 25922)

b. Negative – *Acinetobacter sp.* (ATCC® 33304)

6. Precautions – Make sure to watch for the color change as the color may fade quickly. Before final determination of results are made be sure to add a small amount of zinc dust.

To much zinc dust can reduce the nitrate to quickly resulting in a false negative reaction. Strongly reducing bacteria may exhibit a brown precipitate.

K. Glucose Fermentation (OF Basal with 1% Glucose)

Bacteria metabolize carbohydrates by oxidative and/or fermentative pathways. Oxidation occurs in the presence of atmospheric oxygen (aerobic), whereas fermentation takes place in an anaerobic environment. Metabolism of the carbohydrate dextrose by either an aerobic or anaerobic pathway results in acid production. The resulting acidic environment causes the Brom Thymol blue pH indicator in the medium to turn from green to yellow. The presence of bubbles in the tube indicates gas production (aerogenic). If no reaction occurs, the medium can remain unchanged or become alkaline (blue at the surface).

1. A deep butt tube (~7 mL in 16 × 125mm) is used for this test.

2. With a sterile needle take a small inoculums from an isolated colony and stab to the bottom of the tube of the glucose fermentation medium (Appendix 5.A "Glucose Fermentation Medium).

3. Incubate at 20-24°C for 24 to 48 hours. Check tubes at 24 hours for acid and/or gas production.

4. Results: A = acid(yellow); AG = acid + gas, N = no change or alkaline

	Top of Tube	Bottom of Tube
Oxidative	A	N
Fermentative	AG or A	AG or A
Non-reactive	N	N

5. Quality Control: Fermentative: *Aeromonas* sp.
   Oxidative: *Pseudomonas* sp.

L. Simmons Citrate

Organisms that are able to use citrate as the sole source for metabolism and growth are able to grow on Simmons citrate agar. By metabolizing citrate by the bacteria alkaline conditions are formed in the medium. The pH indicator in Simmons citrate agar, bromothymol blue, will turn from green from acidic conditions to a royal blue when the medium becomes alkaline.

1. Inoculate the agar (Appendix 5.A "Simmons Citrate Agar") by making a streak onto the surface of the slant with a 18 to 24 hour old pure culture.

2. Incubate for up to 4 days at 20-24°C.

3. Results

a. Positive – growth and medium color change to a blue-green or royal blue.

b. Negative – little or no growth and no color change in the medium, remaining dark green.

5. Quality Control

a. Positive – *Enterobacter aerogenes* (ATCC® 13048)

b. Negative – *Escherichia coli* (ATCC® 25922)

6. Precautions – Inoculation with a large amount of bacteria may produce a yellow to tan color on the slant. This does not signify a reaction.

M. Triple Sugar Iron (TSI)

This medium can determine the ability of an organism to utilize specific carbohydrates incorporated in a basal growth medium, with or without the production of gas, along with the determination of hydrogen sulfide (H_2S) production. TSI agar contains the three sugars in varying concentrations: glucose (1X), lactose (10X), and sucrose (10X). It also contains the pH indicator phenol red. If sugar fermentation occurs, glucose will be initially used and the butt of the tube will be acidic (yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube will become acidic. Certain bacteria are unable to utilize any sugars and will breakdown the peptone present. Peptone utilization causes an alkaline (red) shift in the medium that causes a color change from orange to red. Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

1. With a sterile needle inoculate the TSI slant (Appendix 5.A "Triple Sugar Iron Agar") by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

2. Incubate at 20-24°C. Read after 18 to 24 hours.

3. Results

A = Acid; K = Alkaline; H2S = Hydrogen sulfide produced; N =No change

Slant/Butt	Color/Reaction	Interpretation
K/N or K/A	red/orange (oxidative) or red/yellow (fermentative)	only peptone utilized or only glucose-fermented
A/A	yellow/yellow	glucose, plus lactose and/or sucrose-fermented
gas	splitting or bubbles	gas production
H_2S	black butt	Hydrogen sulfide produced

4. Quality Control

   A/A: ± gas: *Aeromonas* spp., *Escherichia coli* (ATCC® 25922)
   K/A w/gas H_2S: *Edwardsiella tarda, Salmonella typhimurium*
   K/A: *Proteus spp., Shigella flexneri* (ATCC® 12022)
   K/N: *Pseudomonas spp.*
   K/N w/H_2S: *Shewanella putrefaciens*

N. O/129 Discs – this test determines the sensitivity of a bacterial organism to the vibriostatic agent 2,4-diamino-6,7 di-isopropylpteridine (O/129).

1. Suspend bacteria in sterile saline or PBS (Appendix 5.B "Saline Solution"or "Phosphate Buffered Saline Solution").

2. Streak suspension on plate in three planes with a cotton swab.

3. Aseptically place sensitivity disc in the center of inoculum.

4. Incubate at 20-24°C for 24 hours.

5. This test can be done on the same plate as the antibiotic sensitivity test (Appendix 5.A "Mueller-Hinton Agar").

6. Results

   a. Sensitive: Zone of inhibition around disc

   b. Resistant: Growth adjacent to disc

7. Quality Control

   a. Positive: *Vibrio anguillarum*

   b. Negative: *Aeromonas hydrophila*

O. Commercial Identification Systems

Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C - room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. A good approach to this problem is to develop a library of known fish pathogen profiles based on serological testing. Referring to this information will assist with interpretation when these commercial test strips are used at room temperature (see Appendix 5.C for some common API profiles based on known bacterial isolates).

1. API 20E™ – The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of Enterobacteriaceae and other Gram-negative bacteria. It is a ready-to-use, microtube system designed for the performance of 23 standard biochemical tests from isolated colonies on plating medium. The test strip consists of microtubes containing dehydrated substances which are reconstituted by adding a bacterial suspension, incubated so that the organisms react with the contents of the tubes, and read when the various indicator systems are affected by the metabolites or added reagents, generally after 18 to 24 hours of incubation. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179).

2. Bionor™ – There are three Bionor Aqua™ rapid agglutination tests that are useful in fish pathology labs; Mono-As™ for *Aeromonas salmonicida* (product # DD 020), Mono-Va™ for *Vibrio anguillarum* (product # DE 020), and Mono-Yr™ for *Yersinia ruckeri* Type I (product # DC 020). These kits contain a test reagent and a control reagent. The test reagents consist of mono-dispersed particles coated with antibodies that form a granular particle agglutination pattern when mixed with the homologous bacteria. When the bacterial isolate is mixed with the control reagent, no agglutination will appear. See instructions enclosed with each test for complete directions. These tests appear to be very specific. For example, the Mono-As will not agglutinate with *Aeromonas hydrophila* and the Mono-Yr™ is type I specific. The test kits can be purchased from Bionor, Strømdaljordet 4, P.O. Box 1868 Gulset, N-3701 Skien, Norway.

3. Biolog – MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. All organisms can be identified/characterized using one of four standardized MicroPlates™; GN2-MicroPlate™ for gram-negative bacteria; GP2- MicroPlate™ for gram-positive bacteria; AN-MicroPlate™ for anaerobic bacteria; and YT-MicroPlate™ for yeasts. Each of these MicroPlates™ contains a pre-selected group of carbon sources as well as an indicator dye. When the appropriate MicroPlate™ is inoculated and incubated, a characteristic pattern (fingerprint) of the organism develops based on which carbon sources the organism can utilize. The resulting pattern can be read either visually or with Biolog's MicroStation Reader™. The results are then compared by the system software to the extensive organism database for final identification. Biolog sells both manual and automated versions of its popular identification/characterization system. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website www.biolog.com).

VIII. Characteristics of Target Bacterial Pathogens

A. ***Aeromonas salmonicida*:** Gram-negative small rod, non-motile, brown diffusible pigment* on TSA or BHIA, cytochrome oxidase positive**, ferments OF basal glucose. Additional biochemical testing on API and corroboration by serological methods (agglutination or FAT) is recommended.

B. ***Edwardsiella ictalur*i**: Gram-negative small rod, motile, cytochrome oxidase negative, ferments OF basal glucose, produces alkaline slant and acid butt with gas (K/Ag) on TSI. A differential medium, *Edwarsiella ictiluri* medium (Appendix 5.A "*Edwarsiella ictiluri* Medium"), is also available to aid in identification. Further biochemical testing on API, and serological testing for corroboration is recommended.

C. ***Yersinia ruckeri*:** Gram-negative small rod, motile, cytochrome oxidase negative, alkaline/acid (K/A) on TSI, ferments OF basal glucose. Biochemical tests with API and corroboration by serological testing are recommended.

D. ***Renibacterium salmoninarum*:** Gram-positive rod, extremely slow growing (4-6 weeks) on KDM-2 or other types of specialized growth agar. Identify with FAT or ELISA. Corroboration with PCR.

* Some strains of *A. salmonicida* do not produce brown diffusible pigment, or pigment production is delayed.

** An Oxidase-negative *A. salmonicida* has been reported (Chapman et al. 1991)

IX. Characteristics of Bacterial Pathogens of Regional Importance

A. ***Citrobacter freundii:*** Gram-negative, rod shaped (1.0µm x 2.0-6.0 µm), motile, cytochrome oxidase negative, ferments OF basil glucose, alkaline slant and acid butt with gas (K/Ag) or the entire tube can become acidic with gas production on TSI., and also produces H_2S on TSI, and lysine and ornathine decarboxylase negative. Further biochemical testing on API, and serological testing for corroboration is recommended.

B. ***Edwarsiella tarda*:** Gram-negative, small rods (1.0 µm x 2.0-3.0 µm),motile cytochrome oxidase negative ferments OF basil glucose, alkaline slant and acid butt with gas (K/Ag) or the entire tube can become acidic with gas production on TSI., and also produces H_2S on TSI, and lysine and ornathine decarboxylase positive. Further biochemical testing on API, and serological testing for corroboration is recommended.

C. ***Flavobacterium columnare:*** Gram-negative, gliding motility, long rods, yellow pigmented bacteria. For additional characteristics see Appendix 5.F.

D. ***Flavobacterium psychrophilum:*** Gram-negative, gliding motility, long rods, yellow, yellow pigmented. For additional characteristics see Appendix 5.F.

X. Corroboration Methods

In most cases corroboration is performed by either direct or indirect FAT. However antisera is not always available for all bacterial pathogens. If antisera is not available, there has been recent developments of PCR methods to aid in bacterial identification. See Chapter 7 (Corroborative Testing of Bacteria by Polymerase Chain Reaction (PCR)) for additional corroborative methods for *Flavobacterium psychrophilum*, *Yersinia ruckerii*, and *Aeromonas salmonicida*. In particular the methods for corroboration of PRIs are not covered in this chapter. Refer to the AFS-FHS Blue Book (2004) for additional information on PRIs and Chapter 7 for corroboration methods by PCR for *Flavobacterium psychrophilum*. In the case that antisera or PCR is not available for corroboration, determination of bacterial identification can be made on biochemical and morphology characteristics alone.

A. Slide Agglutination Test

Slide, or serum agglutination, test confirms bacterial identification by agglutination of a pure bacterial culture with its specific antiserum (e.g., *Aeromonas salmonicida*, *Yersinia ruckeri, Edwardsiella ictaluri* as well as PRIs such as *Flavobacterium psychrophilum*, and *Flavobacterium columnare*).

1. Procedure

   a. Put 0.5-1.0 mL PBS into a 12x75mm tube. (**A. salmonicida* may work better with deionized water.)

   b. Sterilize a loop and collect a sample of a 24-48 hour pure bacterial culture.

   c. Suspend the bacteria in the PBS by thoroughly vortexing.

   d. Heat *F. columnare* and *F. psychrophilum* cultures 5 minutes at 50-55°C to prevent auto agglutination.

   e. Using a ring plate, put one drop of bacterial suspension onto each of three wells.

   f. Negative Control - on the first well, place a drop of PBS (*or water).

   g. On the second well, place a drop of antiserum for the appropriate bacterium, and on the third well, place a drop of normal rabbit serum (NRS).

   h. Mix the drops together by gently rocking the plate back and forth. You can also use a rotating plate set on slow speed.

   i. Allow 5-10 minutes for agglutination to occur. It may be helpful to observe the plate on a light stand or with an illuminated magnifier to see agglutination of bacteria and antibody.

j. Interpretation

i. Agglutination in the well with bacteria and antiserum is a positive test; it is often referred to as a somewhat flaky 'dissolved aspirin' appearance.

ii. Negative controls should appear turbid, but without agglutination.

B. Fluorescent Antibody Technique (FAT) for bacteria.

The Fluorescent Antibody Test is one serological method for corroboration testing of Gram-negative bacterial isolates such as *Aeromonas salmonicida, Citrobacter freundii, Edwarsiella tarda,* and *Yersinia ruckeri*. It is also often used for the Gram-positive bacterium *Renibacterium salmoninarum* (Rs), the causative agent of bacterial kidney disease (BKD) in very small fish where the quantity of kidney tissue is inadequate for ELISA.

In general, there are two types of staining procedures that utilize fluorescent antibodies; the indirect (IFAT) and direct (DFAT) techniques. The principle and techniques are similar, however the indirect utilizes a second antibody, which is often biotinylated for increased sensitivity. The direct FAT is used more commonly for bacterial corroboration testing and the indirect method, IFAT, is most often used for corroboration testing of viral isolates by staining cell cultures infected with virus. This section describes the DFAT only; see Chapter 12 "Corroborative Testing of Viral Isolates", for the IFAT protocol.

There are three basic steps for DFAT: preparing and fixing bacterial cultures or kidney tissue on glass slides; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the slides (two methods described here for pure bacteria or tissues):

a. Pure Bacterial Cultures (corroboration testing of pure isolates of *Aeromonas salmonicida, Citrobacter freundii, Edwarsiella tarda,* or *Yersinia ruckeri*). Pure isolates of bacteria are diluted in sterile PBS and applied to a FAT slide. Air-dry and fix in absolute methanol for 5-10 minutes. Proceed to step #2.

b. Kidney Smears from small fish - DFAT for *Renibacterium salmoninarum.* Kidney samples for FAT most often are collected in the field during necropsy. Kidney tissue is collected from each fish using sterile tools between lots to avoid cross-contamination. The kidney tissue is squashed onto the slide in a manner to provide a thin smear (thick smears tend to adhere poorly to the slide and are difficult to view microscopically).

c. Ovarian Fluid pellet smears – DFAT for *Renibacterium salmoninarum*,
In the case of sampling wild fish that are too valuable to sacrifice for lethal sampling or will only be used as brood fish, then ovarian fluid is collected.

i. Ovarian fluid sample is collected in individually or pooled (up to five fish) using approximately 1 mL per fish.

ii. Transfer two 1.5 mL aliquots from each original pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL microcentrifuge tubes (see Note). Freeze the remainder of the sample at -20°C for PCR corroboration.

iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes (see Note).

iv. The pellet is carefully removed with a small amount of supernatant using sterile pipette and a thin smear is prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

Note: Elliot and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL of ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mL from a 5-fish-pool). Most 15 mL polystyrene centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal for (rcf) as high a 10,000g. It is, therefore, necessary to use polypropylene tubes or aliquot the appropriate amount of liquid into microcentrifuge tube suitable for the required rcf.

d. After the tissue has completely dried (air dried or heat fixed), slides are fixed in acetone for approximately 5-10 minutes.

e. At the lab (or receiving facility), the slides are checked for completeness of labeling (case number and fish identification) and then stained immediately or refrigerated for staining on a subsequent day.

2. Staining - Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or kidney tissue), however a counter stain is usually not required for bacterial cultures.

a. Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for DFAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

b. Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate on each well of the sample slide and control slides.

c. Incubate for 30-60 minutes at room temperature, according to manufacturer's recommendation.

d. Using a squirt bottle or transfer pipette, GENTLY rinse the slides with PBS (or FTA Buffer) by flooding the solution over all wells.

e. If Rhodamine counter stain has not been incorporated in the FAT stain, place the slides in a staining rack and GENTLY rinse in the following order:

   i. PBS for 30 seconds

   ii. Evan's blue counter stain for 3-4 minutes for *Renibacterium salmoninarum*. (A counter stain is not necessary for corroboration testing of pure bacterial cultures, Rhodamine can also be used if desired.)

   iii. Final rinse/soak in PBS or FTA buffer for 5-10 minutes.

f. Air-dry completely.

g. Add a very small drop of FA Mounting Fluid, pH 9 (Difco #3340-57) to each well or target area. Place a 24x50 mm cover glass over the slide using care not to trap air bubbles.

h. Spread the mounting fluid by gently pressing the cover glass with the blunt end of a pen or lab marker evenly over the target area. The fluid should just cover the target area (If the mounting fluid spreads out from under the cover glass, too much mounting fluid was applied).

i. Add one drop of immersion oil to the cover glass over every other well and examine at 1000X using the epiflourescent filter.

3. Reading and Rating: Slides are read at 1000X on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluoresence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells.

   a. Bacterial Corroboration Testing: Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control.

   b. Kidney and Ovarian pellet smears tested for *Renibacterium salmoninarum* - bacterium will have a distinctly **apple-green fluorescent cell wall**; be the

appropriate **size - 1 micron long by 0.5 micron**; and be the **proper shape (bean shaped or pear shaped with one end appearing slightly pinched)**. Compare any suspect bacteria to the control slide to be sure all three of the above criteria are met for *Renibacterium salmoninarum*.

4. Hints for Good Results:

   a. Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (see page 24).

   b. Filter all conjugated antibody reagents (0.45 um filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.

   c. Prepare thin smears; thick smears will not stain or fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.

   d. Evenly distribute the kidney material in PBS, or use a very light inoculum of pure bacterial culture (excess bacteria will stain poorly).

   e. Heat-fix slides prior to fixing in acetone. If there is not an adequate way available to heat-fix the slides they can be air-dried and sent to the lab without fixation.

   f. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process.

   g. Use acetone to fix slides; the acetone reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.

5. FAT Material Suppliers

   a) Kirkegaard and Perry Laboratories, Inc
      2 Cessna Court
      Gaithersburg, MD 20879-4145 USA
      Phone: 800/638-3167, 301/948-7755
      Web Site: http://www.kpl.com

      Antibodies available: Polyclonal antibodies available in FITC-conjugated and other preparations for *Renibacterium salmoninarum* only. KPL also provides positive control material for FAT.

b) Microtek International, LTD (Bayotek)
6761 Kirkpatrick Crescent
Saanichton, B.C.,CA
Phone: 250-652-4482
Web Site: http://microtek-intl.com

6. Reagents

a. Phosphate buffered saline, pH 7.2 (PBS)

NaCl (sodium chloride, MW 58.44)	7.20 g
Na_2HPO_4 (sodium phosphate, anhydrous dibasic, MW 141.96)	1.48 g
KH_2PO_4 (potassium phosphate, anhydrous, monobasic, MW 136.1)	0.43 g

Bring components to 1 L with distilled water. Adjust pH to 7.2 with 1 M NaOH or HCl.

7. Determination of Antiserum and Conjugate Working Dilutions for FAT.
In most cases commercially prepared antisera and conjugates are lyophilized in a concentrated state. Each should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 mL can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome. Generally the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or testing dilutions on either side of the recommended concentration. The following example shows preparation of a direct FAT conjugate where the manufacturer recommends a working dilution of 1:40.

a. Using the stock solution dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended.

b. The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).

XI. Antibiotic Sensitivity Testing

The antibiotic sensitivity test determines the sensitivity or resistance of a bacterial isolate to specific antibiotics. Filter paper discs, each saturated with a different antibiotic, are evenly spaced on an agar plate surface inoculated with a lawn of the bacterial isolate to be tested. The antibiotics diffuse into the surrounding medium, and create a decreasing gradient of the antibiotic concentration. If sensitive, a zone of bacterial growth inhibition (clear zone) will be present around the antibiotic disc. The following antibiotics can be routinely tested: oxytetracycline, sulfadimethoxine with ormetaprim (Romet 30), erythromycin, penicillin, polymyxin, O/129, and novobiocin.

A. Suspend cells from a pure bacterial culture in log phase (24 to 48 hour culture) in sterile saline to obtain a turbidity equivalent to a 0.5 McFarland standard.

B. Streak a Mueller-Hinton agar plate (Appendix 5.A "Mueller-Hinton Agar") with a sterile cotton swab soaked with the bacterial suspension. Swab the plate in three separate planes.

C. Aseptically place antibiotic discs to be tested onto a freshly inoculated plate. Press onto agar surface lightly.

D. Invert the plates and incubate at 25°C for 24 to 48 hours. Observe and record results by measuring the diameters of the zone of inhibition around each disc.

E. Results: The following table lists zone of inhibition for each compound

1. Sensitive: A specific diameter zone of inhibition around the disc.

2. Resistant: Bacterial growth within the zone of inhibition or adjacent to the disc.

Antimicrobial	Disc Content	Resistant	Intermediate	Sensitive
Erythromycin [1]	15ug	No zone	< 15 mm	≥ 15 mm
Novobiocin [2]	30 ug	No zone	< 10 mm	≥ 10mm
Oxolinic Acid [1]	2 ug	No zone	< 15 mm	≥ 15 mm
Oxytetracycline [1]	30 ug	No zone	< 15 mm	≥ 15 mm
Penicillin G [3]	10 U	≤ 11 mm	12-21 mm	≥ 22 mm
Romet 30 [1]	25 ug	No zone	< 15 mm	≥ 15 mm
O/129 [2]	0.1% (W/V)	No zone	< 7 mm	≥ 7 mm

[1] Model Comprehensive Fish Health Protection Program. Pacific Northwest Fish Health Protection Committee, September 1989.

[2] CFMD pathology case materials (Myers 1997).

[3] "Performance Standards for Antimicrobial Disc Susceptibility Tests," NCCLS 1981.

F. Quality Control

Control cultures should be included with each sensitivity test. Cultures are available from American Type Culture Collection (ATCC®) or National Collection of Industrial, Food and Marine Bacteria (NCIMB) of the United Kingdom (www/ncimb.co.uk.)

An excellent reference for antibiotic sensitivity testing is the Provisional Antimicrobial Susceptibility Testing Protocols – 27 December (1998), produced by the Workshop on MIC Methodologies in Aquaculture. Jerry Tjernagel (www/gtbugs@mnic.net) of MicroBiologics is attempting to make all the NCIMB strains available in the United States for use by fish health professionals. Also contact Tom Bell, Bozeman INAD office for a copy of the Provisional Antimicrobial Susceptibility Testing Protocols or further information.

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Appendix 5.A - Media Formulations

I. Common Growth Media

A. Brain Heart Infusion Agar (BHIA)

A basic agar for most bacterial cultures, better for warm water fish.

Suspend 52 g of dehydrated medium (Difco #0418) in 1 L distilled water and heat to boiling. Boil for one minute to completely dissolve agar. Sterilize and dispense as required.

B. Tryptic Soy Agar (TSA)

A basic agar for most bacterial cultures.

Suspend 40 g of dehydrated medium (Difco #0369) in 1 L distilled water and heat to boiling. Boil for one minute to completely dissolve agar. Sterilize and dispense as required. Store at 2-8°C. Final pH = 7.3±0.2 at 25°C.

C. Tryptone Yeast Extract Salt Agar (TYES)

A nutrient poor agar to help select for Flavobacterium.

Tryptone	5.0 g
Yeast Extract	0.4 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.2 g
Agar	10.0 g
d-H2O	1 L

Mix ingredients and heat to dissolve completely. Sterilize and dispense as required. Store at 2.8°C. Final pH = 7.1±0.2.

D. Cytophaga Agar

Another medium for isolating Flavobacterium. Differences in cell and colony morphology aid in distinguishing the numerous bacteria that grow on this partially selective agar. Streak for isolation on two plates, incubate one plate at 20°C and the other at 25°C for 3-5 days.

1. Formula

Tryptone	0.5 g
Yeast Extract	0.5 g
Sodium Acetate	0.2 g
Beef Extract	0.2 g
Agar	10.0 g
d-H_2O	1 L

a. Suspend the above ingredients in distilled water and heat to dissolve.

b. Adjust pH to 7.2-7.4. Sterilize and dispense as required. Store at 2-8°C.

2. Results

a. Flavobacterium psychrophilum – No growth at 25°C. Growth of bright yellow colonies at 20°C with convex center, and spreading periphery.

b. Flexibacter (marine) – Orange or yellow colonies with uneven edges.

c. Flavobacterium columnare – Light or no growth at 20°C. Greater growth of yellow convoluted centered colonies with rhizoid edges at 25°C.

d. Flavobacterium sp. – Growth range 10-25°C, with best growth at 18°C. Light yellow round colonies, transparent and smooth.

E. Nutrient Broth

To use when a liquid growth medium is preferred.

a. Dissolve 8 g of the dehydrated medium (Difco #0003) in 1 L of distilled water.

b. Dispense 5-7 mL/tube and autoclave for 15 minutes at 15 pounds pressure.

c. Final pH = 6.8 at 25°C. Store at 2-8°C.

II. Commonly Used Media to Identify Growth and Biochemical Characteristics

A. Bile Esculin Agar

A commercially prepared dehydrated media (Difco #0879) used for the esculin test. The esculin agar is prepared according to manufacturer's recommendations.

B. Carbohydrate Utilization Medium

A basal media for carbohydrate utilization tests, available in a commercially prepared dehydrated powder (DF #0688). The OF basal is prepared according to manufacturer's recommendations prior to the addition of individual carbohydrates as described below:

1. Preparation of Final Medium

a. Aseptically add 10 mL of a filter-sterilized (0.45 µm) 10% carbohydrate solution to autoclaved and cooled (50oC) medium resulting in a 1% final concentration, with the exception of salicin, which should be made as a 5% solution resulting in

a 0.5% final concentration (see below). Only one carbohydrate is added to the basal medium for each test to be run.

10% Arabinose (1 g Arabinose to 10 mL in d-H2O)
10% Rhamnose (1 g Rhamnose to 10 mL in d-H2O)
10% Sucrose (1 g Sucrose to 10 mL in d-H2O)
10% Sorbitol (1 g Sorbitol to 10 mL in d-H2O)*
5% Salicin (0.5 g Salicin to 10 mL in d-H2O)

b. Mix flask thoroughly and aseptically dispense into sterile tubes.

c. Store at 2 to 8°C. Final pH = 6.8 + 0.2 at 25°C.

*A sorbitol utilization slant media can also be prepared and utilized as described in Cipriano and Pyle (1985).

C. Cytochrome Oxidase Spot Test

Individual test strips can be purchased from Remel (1-800-255-6730, catalog #38-191), or can be prepared in the laboratory with the following procedure.

1. Cut Whatman #1 filter paper into strips and autoclave

2. Spread filter paper on a glass petri dish.

3. Put a few drops of oxidase reagent on each strip to saturate it.

4. Oxidase reagent: Tetramethyl-p-phenylenediamine dihydrochloride (Eastman Organic Chemicals). Prepare 1% aqueous solution.

5. Dry completely, then store in brown bottle. Freeze surplus until needed.

D. Decarboxylase Medium Base

A commercially prepared dehydrated basal media (Difco #0872) for use in Lysine and Ornithine test. The decarboxylase medium base is prepared according to manufacturer's recommendations. The basal media, without addition of Lysine or Ornithine, serves as the control.

1. L-Lysine Medium

   a. Add 5 g L-Lysine to 1 liter of prepare basal decarboxylase media.

   b. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool.

   c. Final pH = 6.8 + 0.2 at 25°C. Store at 2 to 8°C.

2. L-Ornithine Medium.

   a. Add 5 g L-Ornithine to 1 liter of prepare basal decarboxylase media.

   b. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool.
   c. Final pH = 6.8 + 0.2 at 25°C. Store at 2 to 8°C.

E. Glucose Fermentation Medium

1. Suspend 9.4 g of dehydrated medium (Difco #0688) in 1 L of distilled water and heat to boiling.

2. Distribute in 100-mL amounts, autoclave for 15 minutes at 15 pounds pressure (121°C).

3. To prepare final medium aseptically add 10 mL of a filter-sterilized (0.45 µm) 10% glucose solution.

4. Mix flask thoroughly and aseptically dispense 5.0 mL into sterile tubes.

5. Store at 2-8°C. Final pH = 6.8 + 0.2 at 25°C.

F. MacConkey agar (for use with API)

1. Suspend 50 g of the dehydrated medium (Difco #0075) in 1 L distilled water and heat to boiling.

2. Dispense into bottles and autoclave at 15 pounds pressure for 15 minutes.

3. Pour into petri plates.

4. Final pH = 7.1 ± 0.2. Store at 2-8°C.

G. Malonate Broth

A commercially prepared dehydrated media (Difco #0395) used for the malonate test. The malonate broth is prepared according to manufacturer's recommendations.

H. Motility Test Medium

1. Suspend 20 g of dehydrated medium (Difco #0105) in 1 L distilled water and heat to boiling.

2. Dispense 5 mL/tube and autoclave at 15 pounds pressure for 15 minutes.

3. Cool medium in an upright position in a cold water bath.

4. Final pH = 7.2 + 0.2 at 25°C. Store at 2-8°C.

I. Mueller-Hinton Agar

1. Suspend 38 g of dehydrated medium (Difco #0252) into 1 L distilled water and heat to boiling to dissolve completely.

2. Autoclave for 15 minutes at 15 pounds pressure (121°C).

3. Aseptically dispense into sterile petri dishes.

4. Store at 2-8°C. Final pH = 7.3 + 0.1 at 25°C.

J. Nitrate Broth

A dehydrated medium, available commercially (Difco #0268, (Remel® 061532) for use in testing nitrate reduction. The nitrate broth is prepared according to manufacturer's recommendations.

K. Nutrient Gelatin

A dehydrated medium, available commercially (Difco #0011) for use in testing presence of Gelatinase. The Nutrient Gelatin is prepared according to manufacturer's recommendations.

L. Simmons Citrate Agar

A dehydrated medium, available commercially (Difco #0091, Remel® #060496) for use in testing. The Simmons citrate agar is prepared according to manufacturer's recommendations.

M. Triple Sugar Iron Agar (TSI)

1. Suspend 65 g of the dehydrated medium (Difco #0265) in 1 L of distilled water and heat to boiling.

2. Dispense 7 mL/tube.

3. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool in slanted position.

3. Store at 2-8°C. Final pH = 7.4 at 25°C.

N. Tryptone Broth
For use with indole test.

Tryptone	10 g
Distilled water	1000 mL

1. Heat gently to dissolve and then dispense into tubes.

2. Autoclave at 15 pounds pressure (PSI) for 15 minutes and allow tubes to cool.

3. Store at 2-8°C.

III. Differential and Selective Media Used to Identify Bacteria Pathogenic to Fish

A. *Edwarsiella ictiluri* Medium *****Shotts and Waltman (1990)
A differential medium used to help in identification of Edwarsiella ictiluri, Edwardsiella tarda, Aeromonas hydrophila and other species of bacteria.

1. Preparation of *Edwarsiella ictaluri* Medium

   a. Mix the following ingredient in a two-liter flask:

      1 L deionized water
      17 g agar (omit for enrichment medium)
      10 g Bacto-tryptone
      10 g yeast extract
      3.5 g mannitol
      1.25 g phenylalanine
      1.2 g ferric ammonium citrate
      5 g sodium chloride
      1 g bile salts
      0.03 g bromothymol blue

   b. Adjust pH to 7.0 – 7.2 with 1.5 – 2.0 mL 3.0N NaOH.

   c. Autoclave media without removing stirring bar.

   d. Cool to 45-48 C while mixing the following in a small beaker:

      i. 0.010 g colistin

      ii. 0.5 mg (2 mL of 250 µg/mL) fungizone

   e. Swirl to dissolve colistin.

f. Pour into 5-mL syringe, and filter sterilize solution through a 0.45 µm syringe filter directly into autoclaved medium.

g. Add 4 mL deionizer water to syringe.

h. Filter sterilize into medium.

i. Recover flask, and place on stirring platform for a few minutes. Ideally, agar will be 46 - 48 C and then can be aliquoted into petri dishes.

j. Once inoculated, plates should be incubated at 30 C for 36 h.

2. Results

a. *Edwarsiella ictiluri* colonies appear light green, 0.5 – 1.5 mm.

b. *Edwarsiella tarda* colonies appear light green with dark green (H_2S) centers, 1.5 – 2.5 mm.

c. *Proteus sp.* colonies appear brown-green and may swarm.

d. *Serratia marcescens* colonies appear red, 1.0 – 2.0 mm.

e. *Aeromonas hydrophila* colonies resistant to inhibitors appear yellow-green, 0.5 – 5.0 mm.

Appendix 5.B – Reagents

I. Gram stain reagents

These stains can be ordered as a complete kit from VWR (#l5204-004) or can be reconstituted as follows:

A. Crystal violet

crystal violet (90% dye content)	20.0 g
ethanol (95%)	200 mL
ammonium oxalate	8.0 g
dH_20	800 mL

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining ingredients and filter before use.

B. Gram's Iodine

iodine crystals	1.0 g
potassium iodide	2.0 g
dH_20	300 mL

C. Decolorizer

acetone	40 mL
ethanol (95%)	60 mL

D. Safranin

safranin O	2.5 g
95% ethanol	100 mL
dH_20	900 mL

Filter safranin solution before use.

II. Kovac's Indole Reagent

Isoamyl alcohol	30 mL
p-Dimethyl aminobenzaldehyde	2 g
Hydrochloric acid (HCl) 10mL	

Dissolve the aldehyde in the alcohol. Slowly add the acid to the mixture. Store solution at 2-8°C in amber dropper bottle.

III. *a*-naphthylamine Reagent (Remel® "Nitrate Reagent A TI No. 21242)

N-N-Dimethyl-1-naphthylamine	6 mL
Glacial Acetic Acid	286 mL
Deionized Water	714.0 mL

IV. Oxidase reagent

Tetramethyl-P-Phenylenediamine Dihydrochloride	1.0 g
d-H_2O	100 mL

V. O/129 Discs

0.1% solution of pteridine dissolved in acetone

OR

0.1% solution of pteridine phosphate dissolved in sterile distilled water.

A. Autoclave at 15 pounds per square inch (psi) at 121°C for 15 minutes.

B. Place sterile blank discs (Difco, Bacto Concentration Discs ¼") in pteridine or pteridine phosphate solution, remove and dry at 37°C.

C. Store at 2-8°C in a dark screw-cap bottle.

D. Use on agar plates (usually Mueller-Hinton Agar, Appendix 5.A) to test the sensitivity of cultures to this vibrio-static agent.

VI. Phosphate Buffered Saline Solution

A dehydrated saline reagent, available commercially (Difco # DF2314). The Phosphate Buffered Saline Solution is prepared according to manufacturer's recommendations.

Standard 1X concentration formula (0.15 M NaCl, 0.01 M phosphate; make 1 L)

NaCl	8.50 g
Na_2HPO4 (anhydrous)	1.07 g
$NaH_2PO_4 \bullet H_2O$ (monohydrate)	0.34 g
d-H_2O	to 1 L

Adjust pH to 7.1 with 1 N hydrochloric acid or 0.1 N sodium hydroxide.

VII. Saline Solution (0.85%) - sterile

Sodium Chloride	8.5 g
Distilled water	1000 mL

A. Mix the above thoroughly and then aliquot about 7 mL into glass culture tubes (16×125mm or 20×125mm tubes).

B. Loosely place lids on the tubes and autoclave for 15 minutes at 15 pounds pressure at 121°C.

C. After the tubes have been autoclaved allow the tubes to cool and then tighten the caps.

VIII. Sulfanilic acid (Remel® "Nitrate Reagent A TI No. 21239)

Sulfanilic acid	8 g
Glacial Acetic acid	286.0 mL
Demineralized Water	714.0 mL

Appendix 5.C –Profiles Obtained with API-20E for Known Fish Pathogens

I. Yersinia ruckeri

The following table represents API20E profiles for *Yersinia ruckeri* when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35-37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database.

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	**5307500**	1.) *Serratia mercescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	**5107500**	(Same ID as 5307500 above) 1.) *Serratia mercescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
Unknown source – Isolate confirmed by biochemical and serological testing.	**5144100**	1.) *Escherichia coli* 2.) *Yersinia ruckeri*
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	**5107100**	"Unacceptable profile"
Coho, Quinault River, WA	**5106100**	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	**5105100**	1.) *Hafnia alvei* "Acceptable ID"
Notes from ERM archived files – previous testing	**5104500**	"Questionable ID"

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	**5104100**	1.) *Yersinia ruckeri* "Very good ID"
Coho, Quilcene R., WA (11/88)	**5104000**	1.) *Yersinia ruckeri* "Very good ID"
Unknown source	**5100100**	1.) *Yersinia ruckeri* "Excellent ID"
Unknown source	**4105100**	1.) *Hafnia alvei*
Unknown source	**4104100**	1) *Yersinia ruckeri* 2) *Salmonella gallinarum*
Unknown source	**4104000**	1.) *Yersinia ruckeri* "Acceptable ID"
Unknown source	**0104100**	1.) *Yersinia ruckeri* "Acceptable ID"

*** *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22-25°C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles.**

II. *Aeromonas salmonicida*

The following represents API20E profiles for *Aeromonas salmonicida* isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	**API PROFILE***	**API Manual or Computer Identification**
spp not identified, Makah NFH, WA (8/88)	**0006104**	1.) *Pseudomonas pseudomaleae* "Acceptable ID"
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH , WA (12/89)	**0006104**	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	**0006104**	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	**0006104**	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	**2006104**	1.) *Aeromonas salmonicida*
Profiles given in **API MANUAL** for *Aeromonas salmonicida*	**6006104** **6006504** **4006104** **2006104**	*Aeromonas salmonicida* "Good to Excellent ID"

***Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22-25°C).**

Profiles provided in the API Manual are based on positive reactions for some or all of these first 3 biochemical tests, therefore the first digit of the "acceptable" profiles for A.sal include the values 2, 4, or 6. More often, a zero value is obtained after 24 to 48 hours incubation at room temperature. Longer incubation periods are required for these tests.

III. *Edwarsiella ictiluri*

The following represents a API20E profile for *Edwarsiella ictiluri* isolates following manufacturer's instructions but incubating test strips at room temperature (25°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
Catfish	**4004000**	"unreliable identification"

***Edwarsiella ictiluri* generally fails to produce a positive lysine reaction when incubated at room temperature (22-25°C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles.**

IV. *Edwarsiella tarda*

The following represents API20E profiles for *Edwarsiella tarda* isolates following manufacturer's instructions but incubating test strips at room temperature (25°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

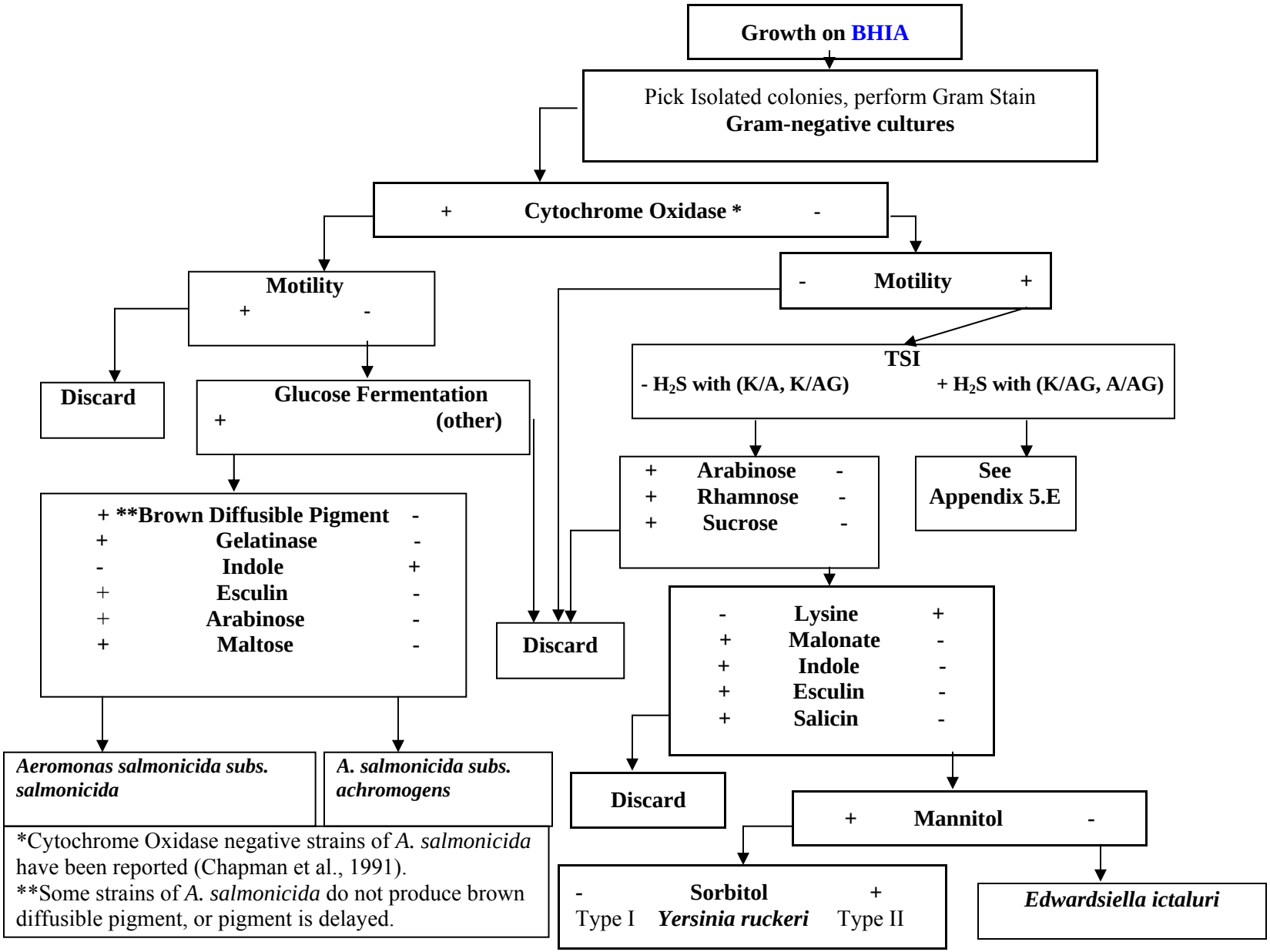
Bacterial Isolate ID (collection date)	API PROFILE*	API Manual or Computer Identification
Catfish	**4544000**	*Edwarsiella tarda* "Excellent Identification"
Catfish	**4144000**	*Edwarsiella tarda* "very good ID"
Catfish	**4744000**	*Edwarsiella tarda* "very good ID"
Catfish	**4145000**	"unacceptable profile"

V. *Citrobacter freundii*

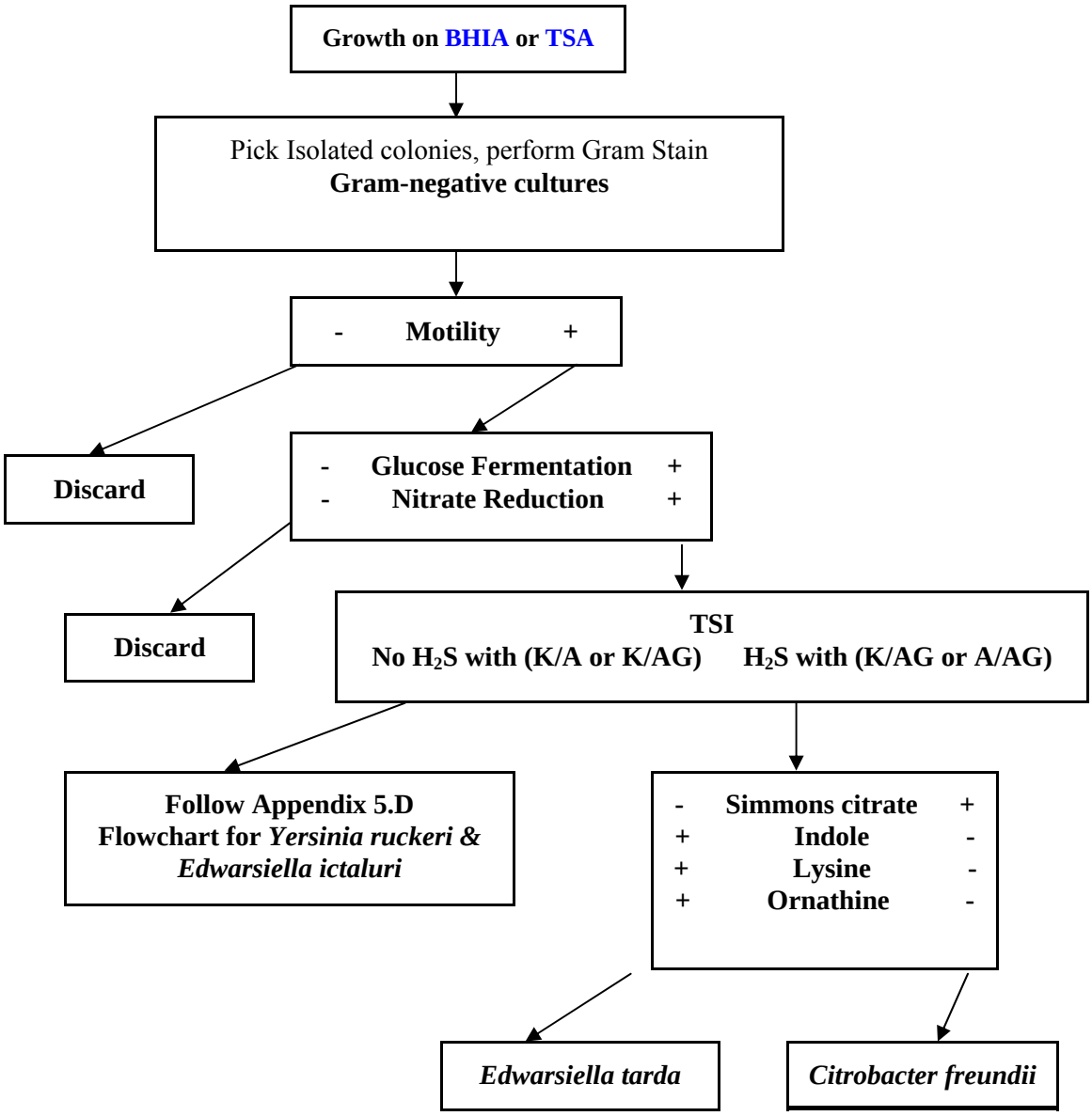
The following represents API20E profiles for *Citrobacter freundii* isolates listed by the manufacturer.

Bacterial Isolate ID (collection date)	API PROFILE*	API Manual or Computer Identification
Profiles given in **API MANUAL** for *Citrobacter freundii*	**160457257** **160457357** **160477357** **160477257** **360477357** **360457357** **360457257**	*Citrobacter freundii* "Good to Excellent ID"

Appendix 5.D – Flowchart for Targeted Major Gram-Negative Fish Pathogens



Appendix 5.E – Flowchart for Targeted Pathogens of Regional Importance Gram-Negative Fish Pathogens



Appendix 5.F – Some Characteristics of Long Gram-negative Bacteria

Flavobacterium columnare	*Flavobacterium psychrophilum*
Morphology and Growth Characteristics	
Growth on TYE or Cytophaga agar	Growth on TYE or Cytophaga agar
Little or no growth at 20°C, optimum growth at 25°C.	No growth at 30°C; optimum growth at 15-18°C.
Yellow dry colonies, rhizoid margin, spreading, adhere to agar.	Moist yellow colonies, entire margin, spreading slowly.
Thin gram-negative rods 0.4 x 2-20 um	Thin rods, 0.5 x 2-7um
Motility by gliding, or flexing motion: forms "haystacks" of aggregate cells on wet mount.	Gliding motility

Additional information on the characterization of *Flavobacterium columnare* and *Flavobacterium psychrophilum* can be found in the AFS-FHS Blue Book (2004).

Biochemical Reactions

No acid from simple or complex carbohydrates (glucose, lactose, galactose and sucrose)

Does not reduce nitrates, produces hydrogen sulfide

Weak catalase positive

Proteolytic for gelatin, casein, albumin

Flexirubin pigments present; colonies turn orange-brown on addition of 20% KOH.

Corroborative Tests

Serum agglutination with specific antisera. If antisera is not available there are PCR methods available for aiding identification of *Flavobacterium psychrophilum* (Chapter 7 "Corroborative Testing of Bacteria by Polymerase Chain Reaction (PCR)").

CHAPTER 6

Enzyme Linked Immunosorbent Assay (ELISA) for Detection of *Renibacterium salmoninarum* Antigen in Fish Tissue

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Enzyme Linked Immunosorbent Assay (ELISA) for Detection of *Renibacterium salmoninarum* Antigen in Fish Tissue

I. Introduction

The following section contains a detailed protocol for the double antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for detection of the *Renibacterium salmoninarum* (Rs) antigen in fish tissues. The protocol, appendices, and figures are adapted from the methods of Ron Pascho and Dr. Diane Elliott (Pascho and Mulcahy 1987, Pascho et al, 1987 and 1991), at the USGS-Western Fisheries Research Center in Seattle, Washington, with additional revisions by Dr. Theodore Meyers (1993a,1993b) of Alaska Department of Fish and Game, and U.S. Fish and Wildlife Service – California/Nevada Fish Health Center (unpublished data).

The ELISA is a highly sensitive and complex protocol that requires careful preparation of assay reagents and strict adherence to protocol. Each major section of the protocol contains a general overview of the process in the first paragraph, then detailed step-by-step instructions to perform the assay. Read through each section prior to performing the assay, so the overall concepts as well as the individual steps are clear. Additional procedures, included in Appendices 6.A-D, are part of the overall methodology of ELISA and ensure quality control of reagents, consistent test results, and optimum performance of the assay. The instructions described here are specific for the reagents and equipment described in Appendix 6.D; other equipment and reagents may perform as well, but will require modifications to this protocol and validation through appropriate testing of the assay.

The ELISA was selected as the most sensitive preliminary testing method for *Renibacterium salmoninarum* (Rs) antigen in both salmonid and non-salmonid fish species tested for the Survey. As a corroborative method, subsets of samples containing the highest OD values from each ELISA assay will be tested for the presence of Rs DNA by the Polymerase Chain Reaction, see Chapter 7 – Corroborative Testing of Bacteria by PCR. The combination of these two methodologies assures the highest sensitivity in detection, as well as the most specific corroborative test available for detection of this pathogen.

An integral part of the Survey includes a Quality Control program for ELISA (Appendix 6.A) that ensures comparable test results and consistent data management through national standardization of the ELISA protocol among all nine Fish Health Centers. The Quality Control program includes the use of standardized reagents and controls; standard methods for conducting the assay; consistent and comparable interpretation of data; and an annual testing program to monitor assay performance among all laboratories. Specifically, the ELISA methodology includes all of the following:

A. Standardization of Reagents

1. Standardization of Antibody Reagents
   Antibodies used in ELISA, (Coating and HRP-labeled), are rehydrated and pooled into large reagent batches, then tested using a Checkerboard Titration to determine

optimum dilutions for each antibody reagent. Antibodies are aliquoted into small working volumes and this standardized source of reagent is used for 1-2 years. This pooling and standardization of antibody reagents ensures consistency in reagent quality, prevents reuse of reagents that may be degraded or stored in unfavorable conditions, and reduces variability between assays over time. Follow the Standardization of Antibody Reagents in Appendix 6.B.

2. Optimization of Antibody Dilutions
   The Checkerboard Titration Assay ensures optimum assay performance. Antibody testing, using a dilution matrix, determines the optimum antibody dilution for pooled lots of antibody reagent. This step ensures consistent ELISA results over time regardless of potential changes in antibody quality supplied by a manufacturer. Follow the Checkerboard Titration Assay in Appendix 6.B prior to reagent allocation and use.

B. Tissue Collection and Processing

Fish tissues are collected and processed with care to avoid contamination of kidney tissue with foreign material or gastrointestinal contents, which could give false-positive reactions in the ELISA. Sterile, individual instruments are used for each fish tissue collected to prevent cross-contamination between positive kidney tissues and negative samples. Established tissue collection and transportation procedures are followed to ensure sample integrity.

C. Performing the Assay

The assay is performed following a detailed protocol. Special care is taken to ensure accurate dilution of antibody reagents, placement and isolation of control wells on each plate, and adherence to precise incubation temperatures and periods. Dedicated equipment such as pipettors, reagent dispensing cassettes, and glassware are an additional precaution to ensure accurate and consistent test results.

D. Interpretation of Data

Interpretation of data and determination of a positive optical density (OD) threshold is the most critical aspect of the ELISA (see Appendix 6.A - Quality Control Program for ELISA). Established guidelines for a Control Plate, control wells on each subsequent plate, and the application of the standardized Negative Control Tissue (NCT) are included in the protocol to ensure optimum assay performance, consistent test results, and comparable analysis of data.

For data interpretation, two standard deviations above the mean OD value of the Negative Control Tissue is used to establish the positive-negative threshold. The Negative Control Tissue consists of chinook kidney tissue that was tested and found to be below detection limits for Rs antigen by ELISA, and negative for Rs DNA by the highly sensitive PCR assay.

E. Quality Control

The Quality Control program for ELISA among USFWS Fish Health Centers includes: optimization and standardization of antibody dilutions, use of standardized Negative

Control Tissue, standard data analysis and reporting methods, corroborative testing with Rs-PCR and annual testing of each laboratory to ensure consistent and comparable testing across the United States.

II. ELISA - Day 1

The afternoon prior to the ELISA, the sample template, or sample placement map, is prepared using computer software such as Lotus or Excel, or it can be handwritten in a format that corresponds to the 96 well plates. It is very important to follow the sample template both on Day 1 when plates are coated, and during the entire assay to ensure the proper reagents and kidney tissues are placed into the appropriate wells.

Prepare the Coating Solution used for diluting the Coating Antibody (CAb). Set 5-10 mL aside for Chromogen Control (CC) and Substrate Control (SC) wells. Add the CAb to the remaining coating Solution and dispense 200 µL into appropriate wells.
Incubate coated plates in a humid chamber at 4C for 16 hours.

NOTE: Add the CAb at the appropriate dilution determined by Checkerboard Titration; this protocol refers to 1:1000 (1 ug/mL) as a standard dilution for CAb as an example only.

Refer to Table 6.1 for proper placement of reagent controls at each step of the assay. The Control Plate Template (Figure 1) also provides a detailed illustration of sample and control placement in plate wells.

It is also helpful to prepare the Wash solution and centrifuge the processed kidney samples the afternoon before the assay so plates can be loaded early the next morning and the assay completed within eight hours.

A. Coating Immunoplates with Antibody

Determine number of samples to be run, and number of plates as follows:

Number of samples		Plate	Notes
1-32	**(32)**	**1**	(First plate contains FULL CONTROLS and 32 samples)
33-76	(44)	2	(Additional plates accommodate 44 samples)
77-120	(44)	3	
121-164	(44)	4	
165-196	**(32)**	**5**	(Add a CONTROL plate every fourth plate, i.e. #1, #5, #9)
197-240	(44)	6	
241-284	(44)	7	
285-328	(44)	8	
329-360	**(32)**	**9**	(Add a CONTROL plate every fourth plate, i.e. #1, #5, #9)
361-404	(44)	10	

1. Calculate Coating Antibody (CAb) needed based on the number of plates - use **25 mL per plate** as a guideline (200 µL per well x 96 = 19.2 mL). This allows extra volume for priming dispensing cassettes without significant waste of antibody reagent.

Number of samples	Number of plates	CAb Required (1:1000)
1-32	1	25mL
33-76	2	50mL
77-120	3	75mL
121-164	4	100mL
165-196	5	125mL

2. Prepare a 1X Coating Solution from 10X concentrate:

   a. Prepare an extra 5 mL to be used in Conjugate and Substrate (CC & SC) negative control wells.

      EXAMPLE: For example, for 3 plates prepare 80 mL of coating solution.

   b. Remove the 5mL and place in a reagent reservoir or clean test tube. Add 200 µL to each CC and SC well of column #1 of the Control plate(s) (i.e. plate #1 and #5). Reagent reservoirs should be dedicated for each reagent and control dispensed manually or with the Multi-drop machine (i.e., one reagent reservoir for Coating Sln, CAb, PBS-Tween20, Milk Diluent, HRP, ABTS, and Stop Sln).

3. Dilute CAb to 1:1000 (or appropriate dilution determined by Checkerboard Titration) in Coating Solution:

   a. Gently mix or vortex CAb prior to diluting.

   b. Pipette CAb into the remaining coating solution at 1:1000 or µL/mL.

      EXAMPLE: 75 µL CAb to remaining 75 mL of Coating Sln.

   c. Swirl or vortex gently to mix (avoids foaming antibody reagents when mixing).

4. Add CAb Solution to plates using Multi-drop Dispenser:

   CONTROL or PLATE #1

   a. Sequentially number the microplates.

   b. Place the CAb dispensing cassette on the Multi-drop unit and place the tubing in the bottom of the CAb Solution.

   c. Set volume for 200 µL and 12 columns (use the same volume setting for all reagents, except the last step using STOP SLN = 50 µL)

d. Press the **PRIME** button to draw the solution through the tubes and into the dispensing tips (will take 3-4 prime steps to completely fill the tubes and dispensing tips of the cassette).

e. Place the first plate on the carriage, and press the **STEP** button **TWO TIMES** to advance the plate forward to the second column (first column received Coating Sln only and does not receive CAb).

f. Press **START** and the unit will dispense reagent into the remaining columns (not necessary to set the number of columns to less than 12 as the Multi-drop will stop automatically when it reaches the last column of the plate).

Table 6.1 Sample and Control Placement used in ELISA

STEPS> **Incubation time & temp>**	**STEP I** COATING ANTIBODY **(16 hr, 4^0C)**	**STEP II** SAMPLES/ CONTROLS **(3hr, 25^0C)**	**STEP III** CONJUGATE **(2hr. 25^0C)**	**STEP IV** CHROMOGEN **(ABTS)** **(20 min 37^0C)**	**STEP V** STOP SOLUTION **Immed Read**
Negative Control Tissue (NCT)	Coating Ab	Negative Control Tissue (NCT)	HRP-Ab	ABTS Chromogen	Stop Solution **Volume=50µL**
Blank (BLK)	Coating Ab	PBS-Tween20	HRP-Ab	ABTS Chromogen	Stop Solution
Conjugate Control (CC)	**Coating Solution**	PBS-Tween20	HRP-Ab	ABTS Chromogen	Stop Solution
Substrate Control (SC)	**Coating Solution**	**PBS-Tween20**	**Milk Diluent**	ABTS Chromogen	Stop Solution
Positive Controls	Coating Ab	KPL Positive Controls	HRP-Ab	ABTS Chromogen	Stop Solution
Tissue Samples	Coating Ab	Tissue samples	HRP-Ab	ABTS Chromogen	Stop Solution

ADDITIONAL PLATES

g. Place each additional plate on the carriage and press **START**; all 12 columns will be filled with CAb.

(If running more than four plates, repeat steps 4a-f for each additional control plate).

h. Watch the volume level and tubing placement for the last plate to be sure the tubing does not come out of solution and introduce air bubbles that will affect volume accuracy.

i. Seal each plate with a plate sealer, place in a humid chamber, and incubate at 4°C for 16 hours.

5. Rinse Dispensing Cassette with dH_20

a. Place an empty plate on the carriage (can reuse a designated plate labeled "Rinse Plate" for this purpose).

b. Place the cassette tubing in a 50 mL conical tube, and add dH_20.

c. Press **PRIME**, then **START** to dispense water and flush the dispensing tubes.

d. Discard the liquid from the plate and repeat with approximately 50 mL of dH20.

e. Remove the tubing from the water and press **PRIME** to remove the remaining liquid in the tubes (or press **EMPTY** to reverse the flow of liquid in the dispensing cassette, which will empty the contents back into the 50mL conical tube).

f. Remove the dispensing cassette from the Multi-drop and air dry overnight.

g. Repeat this procedure for each dispensing cassette after its use.

B. Preparing Wash Solution and Washer Reservoirs

*(Bio-Tek Reservoir and Vacuum system for Model EL540 Washer)

1. Dilute Wash Solution Concentrate 1:20 in dH_20. Prepare ~750 mL for each plate and place in WASH reservoir.

2. Refrigerate Wash Solution overnight, but bring to room temperature (RT) prior to running the assay on day 2.

3. Fill RINSE reservoir with dH_20.

4. Screw caps down tightly on all reservoirs (O-rings must seat down evenly for proper vacuum operation).

5. Connect all tubing between reservoirs and to the vacuum.

* Protocol specifically designed for the Bio-Tek Model EL540 Washer.

C. Thaw and centrifuge Test Samples

1. Remove samples from freezer and thaw completely.

2. Spin samples at 11,000 x g (14, 000 rpm) for 10 minutes.

3. Set up sample racks in the same order as plate templates, this organizes samples and prevents errors when samples are added to individual plates.

4. Refrigerate samples overnight. (May need to re-centrifuge individual sample tubes on the morning of day 2 if supernatant and tissue pellet does not remain separated).

III. Running the ELISA - Day 2

The morning of the second day, plates are washed and sample/antigen is loaded onto the coated plates following a sample template map. Plates are incubated for 3 hours at RT, washed again, and the secondary HRP-labeled antibody (HRP-Ab) is added. Plates are incubated for 2 hours at RT. Next the ABTS color developer is added and plates are incubated at 37˚C for exactly 20 minutes. Stop Solution is added and plates are read immediately using a Spectrophotometer (Plate Reader) set at 405 nm wavelength.

Reagents should be brought to RT prior to their addition to the plates. Aliquot separate volumes of ABTS Substrate and Solution B needed for the day, and bring these to RT (mix these together just before use). Milk Diluent is diluted in dH20 and Stop Solution is stored at RT, so these reagents can be added directly to plates following their preparation.

A. Washing Plates

Remove unbound antibody by running plates through a Plate Washer with pre-programmed wash cycles. The cycle dispenses 250 µL of wash solution, soaks for 30 seconds, aspirates all liquid from the well, and repeats this process 5 times. Visually inspect the dispensing and aspirating syringes on the washer periodically to ensure all wells are receiving wash solution and all liquid in wells is being aspirated completely. The plates should be free of excess moisture following the wash cycle. The first wash requires priming of the Bio-tek Washer (Model EL540). Following initial priming of the washer, the same WASH PROTOCOL is used throughout the assay.

1. Procedure for INITIAL SET-UP AND PRIMING OF THE WASHER (Bio-tek Model EL540)

   a. Turn on the vacuum and allow pressure to reach 1.5 to 3.0 psi on the vacuum gauge (manufacturer suggests that 1.5-1.75 is optimal, vacuum settings may vary with vacuum equipment reservoir system used).

   b. Turn the WASH and RINSE valves to the correct position:

i. Turn the RINSE valve to the closed (perpendicular) position.

ii. Turn the WASH valve to the open (parallel) position.

c. Place a designated "WASHER PLATE" on the plate carriage (this plate can be reused repeatedly for machine priming).

d. On the control panel, press **RINSE** and allow the cycle to run (display will read [Done Rinsing] when complete).

e. Press the **STOP** button, then press **PRIME** to begin the prime cycle.

f. Repeat the PRIME cycle. The machine is now ready to for use throughout the remainder of the assay.

2. Wash Protocol

a. Place Plate #1 on the carriage and press WASH and START to begin washing cycle. Each wash cycle takes approximately 3-4 minutes to complete. Plate should be dry, and can be set aside for the next step; loading of test samples and controls.

b. Wash the additional plates in order.

c. Use the "Day Maintenance" mode of washer to keep dispensing syringes in a "wetted condition" between plates.

d. Turn vacuum off when finished with last plate.

B. Loading Tissue Samples and Controls

Samples, reagent controls (NCT, Blanks, CC, and SC) and KPL Positive Controls are loaded onto washed plates following the Control Plate Template (for Plate #1) and subsequent plate templates for additional plates. The first plate contains all the controls, subsequent plates have controls (NCT and Blank) in column 1 and samples in all remaining columns. Carefully pipette the PBS-Tween (negative control reagent) into the appropriate wells and seal this area of the plate with a strip of plate sealer film. Add KPL positive controls and seal these columns as well. The test samples are added last, following the template and notations are made of any pipette errors, tissue debris, or other problems that could affect assay results. Incubate sealed plates at RT (22-25°C) for 3 hours.

<u>PLATE # 1 - Full Control Plate</u> (Refer to Table 6.1 on page 8, and Figure 1 on page 13):

1. Pipette 200 µL of PBS-T20 into BLANKS, CC and SC wells.

2. Dispense 200 µL of Negative control tissue (NCT-98) into appropriate wells. Seal this area of plate.

3. KPL Positive Controls:

   a. Cover the sample area of the plate with a plate lid or plate sealer prior to loading Positive Controls.

   b. Vortex each Positive Control dilution prior to dispensing into wells.

   c. Add 200 µL of each dilution (1:100, 1:1000, 1:2000, and 1:5000) into the appropriate wells.

   d. Seal these wells with plate sealer film.

4. Load all samples into their appropriate wells last, using the following matrix for each column:

Sample		**Well Address**
#1	into well	A & E
#2		B & F
#3		C & G
#4		D & H

5. Place a plate sealer over the entire plate, write the time on the flap of the plate sealer film and record it on the plate template as well.

6. Incubate in humid chamber at room temperature for 3 hours.

Figure 1 - Control Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	NCT	Pos 1:100	Pos 1:2000	1	5	9	13	17	21	25	29
B	CC	BLK	Pos 1:100	Pos 1:2000	2	6	10	14	18	22	26	30
C	CC	NCT	Pos 1:100	Pos 1:2000	3	7	11	15	19	23	27	31
D	CC	BLK	Pos 1:100	Pos 1:2000	4	8	12	16	20	24	28	32
E	SC	NCT	Pos 1:1000	Pos 1:5000	1	5	9	13	17	21	25	29
F	SC	BLK	Pos 1:1000	Pos 1:5000	2	6	10	14	18	22	26	30
G	SC	NCT	Pos 1:1000	Pos 1:5000	3	7	11	15	19	23	27	31
H	SC	BLK	Pos 1:1000	Pos 1:5000	4	8	12	16	20	24	28	32

C. Washing Unbound Antigen

Prior to washing plates, carefully remove each plate sealer without cross-contaminating contents of the wells. Follow the **WASH PROTOCOL (Step III.A.2 on page 11).** All plates are washed of unbound sample/antigen. Plates should be dry and can be set aside following washing.

D. Add Secondary HRP-Labeled Conjugate (HRP-Ab)

This step is an opportunity to coordinate the plate incubation times more closely. Closely synchronized incubation times for the 2 hour HRP incubation step allows more efficient performance of the assay at the next wash step, during addition of ABTS Substrate, and at the plate reading step. It also decreases the chance of errors that can occur if plate incubation periods do not follow the plate sequence (some plates required more time to load test samples).

To synchronize incubation periods of plates, simply pace the addition of HRP-Ab to each plate at 3-4 minute intervals when dispensing the HRP-Ab with the Multi-Drop unit. Incubation period begins when the last column is filled. For the next wash step, plate incubation periods will be as close together as possible (the washer requires 3-4 minutes to wash each plate) and the assay can proceed without unnecessary delays.
Prepare Milk Diluent and set a small volume (5 mL) aside for Substrate Control (SC) wells. Prepare the conjugated HRP-Ab (0.5 ug/mL or 1:2000) in remainder of Milk Diluent and dispense 200 µL of solutions to appropriate wells. Incubate sealed plates in humid chamber at RT for 2 hours.

NOTE: Add the HRP-Ab at the appropriate dilution determined by Checkerboard Titration; this protocol refers to 1:2000 (0.5 ug/mL) as a standard dilution for HRP-Ab as an example only.

1. Prepare 1X Milk Diluent from 20X concentrate

   a. Similar volumes of Milk Diluent are prepared as was used for Coating Solution (25 mL per plate).

   b. Dilute the Milk Diluent 1:20 prior in dH_20.

      (Example: Add 5 mL Milk Diluent to 95 mL dH_20).

   c. Remove 5 mL to be used in SC wells as a negative reagent control, and place in a reagent reservoir.

   d. Manually pipet 200µL of MILK DILUENT ONLY to each SC well on Plate #1 (and any additional control plates).

2. Dilute HRP-Conjugate 1:2000 (0.5 µL/mL) in the remaining volume of Milk Diluent

   a. Pipette HRP-Ab into the remaining Milk Diluent at 1:2000
   (For example, 45 µL HRP-Ab to 90 mL of Milk Diluent)

   b. Mix well with gentle vortexing.

3. Manually pipette HRP-Ab into the CC wells immediately prior to dispensing the HRP-Ab into the remaining columns with the Multi-drop.

   a. Pipette 200 µL of HRP-Ab into each CC well on Plate # 1 and any additional Control Plate(s).

4. Add HRP-Ab to plates using Multi-Drop

   a. Prime the dispensing cassette with HRP-Ab

   b. Place Plate #1 on carriage.

   c. Press **STEP** TWO TIMES to advance plate to column #2.

   d. Press **START** to load the remaining columns on the plate.

   e. Seal with plate sealer and write time on sealer flap,

   f. Incubate at RT for 2 hours in a humid chamber.

g. Dispense HRP-Ab in remaining plates, seal each plate, record the time and incubate for 2 hours.

5. Rinse dispensing cassette with dH_20

6. Turn on incubator and set temperature for 37˚C at this time. (Most incubators will not require two hours to reach and hold a stable temperature of 37˚C, but it is better to plan ahead than wait until just before the ABTS step.)

E. Wash Plates (Wash Protocol)

All plates are washed of unbound HRP-Ab. Plate incubation periods remain approximately 3-4 minutes apart and can be loaded into the washer one after another at each appropriate time. Hold washed plates for next ABTS Substrate step.

F. ABTS Substrate – Color Development

Allow the Plate Reader lamp to warm sufficiently (approximately 20 minutes) prior to reading the first plate. Prepare substrate-chromogen (ABTS Substrate System) by mixing equal portions (1:1) of ABTS Peroxidase Substrate and the Peroxidase Solution B (prepare just prior to dispensing). Synchronize the final ABTS incubation period by dispensing 200 µL to all wells of each plate at 1-2 minute intervals. This allows efficient reading of the plates. Cover each plate with a plastic plate lid and incubate at **37˚C FOR EXACTLY 20 MINUTES**.

1. Turn on the Reader and prepare computer software for reading the first plate.

2. Calculate the total volume of ABTS Substrate needed for the number of plates (approximately 25 mL per plate). Mix 1 part ABTS Peroxidase Substrate to 1 part Peroxidase Solution B.

3. Prime the appropriate dispensing cassette in the Multi-Drop unit.

4. Place the first plate on the carriage and press **START** (all wells of all plates receive 200 µL of ABTS reagent).

5. SET TIMER FOR EXACTLY 20 MINUTES after reagent is delivered to the last column.

6. Quickly cover plate with plastic plate lid and place in 37˚C incubator.

7. Allow approximately 1 minute, then proceed with the next plate. The minute between plates allows adequate time to add Stop Sln and read each plate before the next plate has completed the 20-minute incubation period.

8. Rinse the dispensing cassette with dH_20.

G. Stop Solution and Plate Reading

During the final 20-minute incubation period, prepare the SDS Stop Solution from concentrate. Prime the Stop Solution dispensing cassette with diluted (1:2) Stop Solution, remove plates from incubator at exactly 20 minutes and dispense **50** µL **of Stop Solution to all wells**. The plate bottom is wiped dry, and plates are read immediately.

The manufacturer supplies Stop Solution at a concentration of 5% sodium dodecyl sulfate, and recommends a 1:5 dilution that brings the solution to a 1% concentration. Further dilution is recommended by adding an equal volume of the 1% solution to an equal volume in the sample well resulting in a final concentration of 0.5% sodium dodecyl sulfate in the well. Because most 96-well plates cannot accommodate a volume of 400 µL, the volumes can be adjusted as long as **the final concentration of Stop Solution when combined with the well volume equals 0.5% sodium dodecyl sulfate.**

The easiest way to achieve this is perform a 1: 2 dilution of Stop Solution in dH_20 (5% diluted 1:2 = 2.5% concentration), then deliver 50 µL of this diluted Stop Solution to each well, which contains 200µL of ABTS Substrate from the step F.4. This provides a second dilution of 1:5 (2.5% is further diluted to a final concentration of 0.5% sodium dodecyl in each well) and gives a total well volume of 250 µL.

1. Prepare stop solution by mixing 1 part Stop Solution Concentrate to 1 part dH_20. The volume needed for a single plate is 4.8 mL (50 µL per well x 96 wells), but prepare extra solution to prime the dispensing head and to avoid running out of reagent during this critical assay step. The following is a guideline based on number of plates:

Number of plate	Stop Sln	:	dH_20	Total Volume(mL)
1	7		7	14
2	10		10	20
3	12		12	24
4	15		15	30
5	17		17	34
6	20		20	40
7	22		22	44
8	25		25	50
9	27		27	54
10	30		30	60

2. Add STOP SOLUTION to plates using Multi-Drop

   a. Prime the dispensing cassette.

   b. CHANGE THE VOLUME SETTING TO 50 µL.

c. Remove each plate from the incubator at exactly 20 minutes, remove lid and place plate on carriage.

d. Press **START** (all wells receive Stop Solution).

e. Remove the plate and wipe any condensation off the bottom of the plate with a lint free tissue. Avoid marking the bottom of the plate with fingerprints, scratches or other effects that may interfere with optical density values.

3. **Read Plate immediately**

a. Place plate on reader carriage and read immediately (using computer software or manually using reader controls).

b. Repeat dispensing of Stop Solution (steps 2c-e) for each additional plate, and read immediately.

H. Equipment Maintenance upon completion of assay

1. Multi-Drop:

a. Rinse Stop Solution from dispensing cassette with dH_20. Remove dispensing cassette and air-dry overnight.

b. Turn Multi-drop power off and replace dust cover.

2. Plate Washer (Biotek model EL540):
Follow manufacturer's procedures for either DAY MAINTENANCE described below or LONG TERM STORAGE if the washer will not be used for 2-3 days (refer to operator's manual).

a. Turn on the vacuum and allow pressure to reach 1.5 – 1.75 psi on the vacuum gauge.

b. Turn the WASH and RINSE valves to the correct position:

i. Turn the RINSE valve to the open, parallel position.

ii. Turn the WASH valve to the closed, perpendicular position.

c. Place the "WASHER PLATE" on the plate carriage.

d. On the control panel, press **RINSE** and allow the cycle to run.

e. Repeat the Rinse cycle.

f. Run the **PRIME** cycle twice.

g. Press **MAINTENANCE** - display reads [day1].

h. Press **START** - display reads [unit primed?].

i. Press **START/YES** again (plate is loaded and liquid dispensed, display reads [Wetted condition].

j. Turn the power to the vacuum and washer off, and replace dust cover.

k. Empty all reservoirs. Clean the Collection reservoir with 500 ppm Chlorine. The Wash Solution and Rinse reservoirs can be cleaned with soapy water. Rinse all reservoirs with dH_20 thoroughly and air dry.

3. Reader

   a. Turn power to reader off and replace dust cover.

IV. Interpretation of Data

Reader files are downloaded into computer spreadsheets (Lotus™ or Excel™) or other appropriate software to analyze the data. All OD values from positive, negative, and reagent controls are examined for variability between replicates and overall range of values. If the assay control parameters are acceptable, the positive-negative threshold is determined by calculating 2 standard deviations (STD) above the mean OD value of the Negative Control Tissue. Test samples are scored appropriately, and reports are printed.

A. Quality Control

Optical density (OD) values for control reagents may vary between laboratories using different equipment or manufacturer models, reagents and/or protocols. These values are intended as a guideline only, and each laboratory will need to test and optimize the polyclonal ELISA in their laboratory setting before determining the expected values for each reagent control. In general, the following assessments should be made:

1. Verify that variations between replicates are acceptable (coefficient of variance should be < 10).

2. Verify low values for the BLANK (negative control) wells. Typical OD values for BLANK wells range from 0.068-0.072.

3. Verify absence of irregularities in Substrate Control and Chromogen Control values. These control wells should all be very similar to each other and typical OD values range from 0.060-0.065 for Conjugate Control (CC) and 0.055-0.060 for Substrate Control (SC)

B. Negative - Positive Determination

1. Negative-Positive threshold is determined by adding two standard deviations (STD) to the mean of replicate OD values of the Negative Control Tissue. Use of a spreadsheet to calculate the sample mean and standard deviation avoids rounding errors that could alter the calculated threshold value.

   EXAMPLE: Mean OD is 0.078 for all plates in a particular assay, and the standard deviation is 0.002 (these are typical values for NCT98 kidney tissue currently in use). The negative-positive threshold is set as follows:

   $$0.078 + 2(0.002) = 0.082$$

2. All test sample values above a mean OD value of 0.082 are reported as positive for Rs antigen for this assay. Because of variation in the quality of reagent lots, assay conditions, and subtle differences between individual operators, the mean OD and STD will fluctuate slightly between assays. Therefore the positive-negative threshold will also vary between assay runs. When the quality control program outlined in Appendix 6.A is implemented completely, and the protocol is followed precisely, positive-negative thresholds should be consistent and relatively comparable between laboratories.

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Appendix 6.A – Quality Control Program for ELISA

The ELISA is a highly sensitive and complex protocol which requires careful preparation of assay reagents and strict adherence to detailed protocol. Several additional procedures are included in the overall methodology of the ELISA to ensure quality control of assay reagents, sample integrity, optimum assay performance, and consistent test interpretation. This appendix provides an overview of the quality control program, while additional appendices B-D provide detailed instructions for each aspect of the methodology used for ELISA.

I. Standardization of Antibody Reagents

Antibody reagents used in ELISA (CAb and HRP) are reconstituted following manufacturers instructions and pooled into large volumes of concentrated antibody. This pooling of antibody reagents ensure consistent and high quality reagents for use over a 1-2 year period. This step allows long-term consistency in reagent quality and concentration, prevents degradation of reagents through proper long-term storage, and reduces variability between assays over time. Follow the procedure in Appendix 6.B - Standardization of Antibody Reagents.

II. Checkerboard Titration – Optimization of Antibody Dilutions

Following standardization of antibody reagents, the Checkerboard Titration Assay (CTA) tests the larger pool of antibodies for optimum working dilutions. This assay uses a dilution matrix to test each antibody's performance in the ELISA at various working concentrations. Taken together, the optimum working dilution of the two antibodies (Coating and HRP) are determined for the assay. Follow the Checkerboard Titration Assay (CTA) in Appendix 6.B.

> **NOTE:** If all antibodies being pooled are from the same lot number, the reagents can be pooled first, then tested by Checkerboard Titration. When reagents from several lots are pooled, a CTA should be performed on each antibody lot number (see NOTE in item A.3, Appendix 6.B).

III. Tissue Collection and Processing

Fish tissues are collected and processed with care to avoid contamination of kidney tissue with foreign material or gastrointestinal contents (feather meal in fish feed), which could give false-positive reactions in the ELISA. Kidney, or kidney-spleen is standardized to a single dilution of 1:8 (w/v) for ELISA testing performed for the Survey. A single dilution of 1:8 (versus 1:8 for juvenile fish and 1:4 for adults) is necessary to compare data among various species and life stages of fish. A 1:4 dilution was not selected because there would not be an adequate quantity of tissue for juvenile fishes unless samples pools were greater than 5 fish. The Negative Control Tissue (NCT), which establishes the negative/positive threshold, is also diluted at 1:8.

Sterile, individual instruments are used for each tissue collected to prevent cross-contamination between positive and negative kidney tissue samples. Standard procedures are followed as outlined in Appendix 6.C – Collection and Processing of Tissue Samples for ELISA.

IV. Performing the Assay

The assay is performed following a detailed protocol. Special care is taken to ensure accurate dilution of antibody reagents, placement and isolation of positive and negative control wells on each plate, and adherence to precise incubation periods. Dedicated equipment such as pipettes, reagent dispensing cassettes, and glassware is an additional precaution that ensures accurate and consistent test results.

V. Interpretation of Data and Quality Control

The most critical aspect of quality control is interpretation of data and determination of the negative-positive OD threshold for kidney tissue tested by ELISA.

A. Controls

Established guidelines for the control plate, control wells on each subsequent plate, and the application of the standardized Negative Control Tissue (NCT) are included in the protocol to ensure optimum assay performance and consistent data analysis (see Figure 1 – page 13).

1. One full Control Plate is included for every four (4) plates tested in an ELISA. The Control Plate contains at least four replicates of the following reagent controls:

    a. KPL Positive Control – *Renibacterium salmoninarum* whole cell preparation at each dilution of 1;100, 1:1000, 1:2000 and 1:5000. Positive controls ensure predictable absorbance values are produced by certain levels of antigen.

    b. Negative Control Tissue (NCT) - Negative kidney tissue, tested by ELISA and PCR. Statistically establishes a negative-positive threshold for evaluating test results.

    c. Blanks (BLK) - wells receive PBS-T20 diluent (in place of test tissue) to determine background absorbance levels in the absence of test sample.

    d. Conjugate Control - wells receive Coating Solution only (without Antibody) to ensure that non-specific binding of the secondary HRP-Ab to well surfaces has not occurred.

    e. Substrate Control - wells receive Milk Diluent only (without HRP-Ab) to test for non-enzymatic production of the color reaction.

2. Each subsequent plate also contains one column of control wells: 4 wells of Negative Control tissue and 4 Blank wells. The Control Plate(s) and control wells within subsequent plates allow close monitoring of assay performance between plates of a single assay, and between assays performed on various dates.

B. Positive Threshold

For data interpretation, two standard deviations above the mean OD value of the Negative Control Tissue (NCT) establish the negative-positive threshold for each assay. The NCT consists of chinook kidney tissue (1:8 dilution) tested by ELISA and PCR and found to be negative, or below detection limits of these highly sensitive assays. The mean OD value of replicate test samples is compared to the threshold value to determine the positive or negative status of each test tissue.

EXAMPLE: The negative-positive threshold is determined for an assay by calculating the mean and the standard deviation of Negative Control Tissue (NCT) wells on all plates. Two standard deviations above the NCT mean OD determines the positive-negative threshold and subsequent rating of the test samples. Calculations are performed using spreadsheets to avoid rounding errors. The final OD positive-negative threshold value is rounded to three significant figures for convenience and ease of reporting.

Individual Plates	NC OD values	OD MEAN for all Plates
Plate 1 (Control Plate)	.076, .075, .072, .078	
Plate 2 (4 wells)	.074, .073, .076, .074	
Plate 3 (4 wells)	.076, .075, .079, .072	.07525 (n=12)

Two Standard Deviations :	.00196 x 2	= .00392...
THRESHOLD:	.07525 + .00392	= .07918...
ROUNDED OD THRESHOLD		**= .079**

Samples with mean OD values above .07918 are reported as positive for Rs antigen.

VI. Corroborative Testing By PCR

Subsets of ELISA positive samples will be further tested by PCR utilizing specific primers for *Renibacterium salmoninarum.* The three highest OD values from each case history will be tested by PCR to confirm the presence of Rs DNA in the test sample. ELISA samples that are not corroborated by PCR will not be included in the National Wild Fish Health Survey Database.

VII. Annual Testing Program

The Quality Control program for Fish Health Centers using ELISA includes an annual testing program. Sample sets with known OD values will be distributed and tested by each laboratory. Results will be summarized and any discrepancy from acceptable ranges will be investigated for potential sources of variation and appropriate corrective measure will be taken. An annual testing program will ensure consistent and comparable test results from all USFWS Fish Health Centers across the United States.

VIII. Equipment Calibration and Maintenance

All equipment used in the ELISA should be operated and maintained following the manufacturer's recommendation. Manual pipettors and automated dispensing equipment will be checked for accuracy and calibrated on a regular basis. Major equipment such as the Plate Washer, Multi-drop dispenser and the Reader will be checked for proper operation and calibrated on a biannual basis following the instructions provided in the manufacturer's operating manual. Logs of all equipment maintenance and calibration will be maintained at each Fish Health Center.

Appendix 6.B – Standardization of Reagents

I. Overview

Antibody reagents used in ELISA (CAb and HRP) are reconstituted following manufacturer's instructions and pooled into large volumes of concentrated antibody. Pooling of antibody reagent ensures consistent and high quality reagents for use over a 1-2 year period.

Following pooling of antibody lots, the reagents are tested by Checkerboard Titration assay (Step 2), and then distributed into small working volumes for use in each assay. Working volumes are stored in microcentrifuge tubes or other vials suitable for cryopreservation. It is important to dispense at least 100 µL in each tube, as smaller volumes are less stable during long-term storage. Individual tubes of CAb are then used for a single assay, or reused a second time and discarded. This limits repeated freeze-thaw cycles that can degrade antibody protein.

The KPL Positive Control, a whole cell preparation of *Renibacterium salmoninarum*, is also pooled in large batches, diluted to specified concentrations of 1:100, 1:1000, 1:2000, and 1:5000 in Phosphate Buffered Saline (PBS). Then each dilution is aliquoted into small volumes for use with each assay. All these reagents are stored at -70˚C to ensure long-term stability and individual aliquots are only removed when they are ready to be used. All glassware used for preparation of Positive Controls is acid washed to thoroughly remove any Rs antigen.

Negative Control Tissue (NCT) with a known OD value is run on every plate to establish the negative-positive threshold for each assay. The Negative Control Tissue (NCT) also allows monitoring of plate-to-plate variation within a single assay, as well as assay performance over a period of time. Kidney tissues were collected from chinook salmon and tested by ELISA, culture on SKDM, and by PCR. Negative kidney tissues (by all 3 assays) were pooled and further diluted to 1:8 (w/v) in PBS-T20. Negative Control Tissue (lot # NCT-98) was aliquoted. and distributed to each Fish Health Center. NCT is used to: establish the negative-positive threshold for each ELISA; monitor assay performance between runs; compare ELISA results between laboratories; and to make relative comparisons of OD values when equipment, reagents, or control reagents change over time.

Negative Control Tissue (Lot # NCT98): The specific NCT in use currently was obtained from 50 Fall chinook salmon from the Coleman National Fish Hatchery. Entire kidneys were removed from female fish, homogenized and cultured on SKDM media and monitored for 6 weeks for growth of *Renibacterium salmoninarum* cells. An additional subset of tissue was submitted to the USGS - Western Fisheries Research Center in Seattle, WA for PCR testing. The remaining tissue was diluted 1:4 (w/v) with PBS-T20 and homogenized, heated to 100°C for 15 minutes and centrifuged at 10,000 rpm for 10 minutes. Supernatant from each test sample was tested at 1:4 and 1:8 dilutions by ELISA at the Ca-Nv Fish Health Center (Ca-Nv FHC). Following ELISA, supernatant from all negative kidney tissues were pooled into one large batch and frozen at –70°C.

Periodically, these steps will be repeated for new lots of NCT tissue as needed. Comparison testing will be completed between previous NCT lots and newly developed batches to evaluate changes in expected OD values for the ELISA.

II. Standardization of Antibody and Control Reagents

A. Pooling of Antibody Reagents

1. 50% Glycerol Solution for reconstituting Antibodies

    a. Prepare a 50% solution of glycerol by adding one part glycerol to 1 part sterile dH_20.

    b. Optional: For each mL of the above solution prepared, add 10 µL of 1% (w/v) Thimerosal (this gives a final concentration of 0.01% of Thimerosal as a preservative).

2. Reconstitute Antibodies - Coating Antibody (CAb) and Secondary HRP-labeled Antibody (HRP-Ab)

    a. Rehydrate lyophilized antibodies with 1 mL of 50% glycerol.

3. Pool Antibodies

    a. Select 4-5 vials of each antibody and pool the contents into a larger batch.

    b. Aliquot 100 µL volumes of CAb and HRP-Ab into microcentrifuge tubes and freeze at -70°C.

    c. Tubes are removed as needed.

    NOTE: Many laboratories have reported decreased antibody activity in recent production lots of unlabeled Coating and HRP-conjugated antibodies received from KPL. Due to this problem, individual antibody lots that are suspect for decreased antibody activity may need to be tested by Checkerboard Titration prior to pooling. This additional step of testing individual lots of antibody could preclude the inclusion of these lots in the standardized reagent pool and prevent subsequent dilution of reagents with higher antibody activity.

    I strongly recommend purchasing several vials of the same lot number when antibody standardization is planned. While this will not prevent the reported problems with antibody quality, it will save a tremendous amount of time and labor that would be required to test several individual antibody lots prior to pooling.

B. Positive Control Antigen

1. For Positive Control Antigen, reconstitute and pool several vials of *Renibacterium salmoninarum* whole cells.

   a. Prepare accurate dilutions in PBS-Tween 20 as follows: 1:100, 1:1000, 1:2000, 1:5000.

   b. Aliquot 500 µL into 1.5mL microcentrifuge screw-cap tubes (label each tube and cap with the dilution).

2. Heat in a water bath at 100°C for 15minutes. Allow to cool and then freeze at -70°C.

3. Positive Controls are thawed and vortexed thoroughly prior to use.

C. Negative Reference Tissue

1. Negative Control Tissue (lot # NCT98) has been diluted 1:8, boiled for 15 minutes, centrifuged, supernatant removed. Aliquots were stored frozen at -70°C. The NCT98 control tissue is ready to use after thawing; no further processing is required.

2. The source of this control tissue is Coleman NFH Fall Chinook, a stock historically negative for Bacterial Kidney Disease (BKD) and having a low incidence of *Renibacterium salmon*inarum antigen by ELISA and Rs-PCR.

3. Other Reference Tissue can be developed for other species of salmonids or non-salmonids; the main objective is to locate and test stocks with consistently low or negative OD values and use this tissue as an assay standard to monitor assay performance and help determine a negative-positive threshold.

III. Checkerboard Titration Assay (CTA)

Checkerboard Titration testing of antibodies determines the optimum working concentrations of Coating antibody and Conjugated HRP antibody used in the ELISA to detect *Renibacterium salmoninarum.*

Each time new lot numbers of Coating Antibody (CAb) or HRP-Conjugated secondary Antibody (HRP-CAb) are rehydrated and pooled, slight variations may occur in these critically important ELISA reagents. This is due to differences in reagent quality between manufacturer's antibody lots, the rehydration and pooling procedure, and accuracy of dilutions and/or pipette errors that can occur when large quantities of reagents are dispensed. In addition to changes in reagents used, other factors involved with equipment changes may also affect the values obtained by ELISA. These include use of new equipment such as dispensing cassettes (Multi-Drop unit) or filter replacement (Reader) and the proper maintenance and calibration of equipment. Therefore, each modification to the ELISA system (new equipment and/or calibration of existing

equipment) should be tested by Checkerboard Titration assay. Test results and overall assay performance should be monitored closely to detect significant changes that may alter positive-negative thresholds and the test results.

The Checkerboard Titration assay determines the optimal (working) concentration of antibody reagents by evaluating them together in a dilution matrix using a prescribed set of controls and test samples. From these data one can choose a combination of Coating antibody and Conjugate concentrations that produces optimal OD absorbance values and ensures consistent performance of the ELISA.

A. Reagents and Test Samples

1. Test the pooled antibody reagents prepared in step (II) A.1. Be sure the antibody pool is well mixed and remove a subset to test by titration assay.

2. Determine Antibody concentrations to test in Checkerboard Titration assay

   a. Test the Coating antibody at 0.5, 1.0, and 1.5 µL/mL (1:500, 1:1000 and 1:1500 (v/v) dilutions)

   b. Test the HRP Conjugate at dilutions of 1.5, 2.0 and 2.5 µL/mL (1:1500, 1:2000, and 1:2500 (v/v)dilutions).

3. Test samples and Negative-controls

   a. The controls include Blank (B), the Negative Control Tissue (N), the Conjugate Control (CC), and the Positive Controls at four concentrations; 1:100 , 1:1000, 1:2000, 1:5000.

   b. The negative control Blank (B) receives PBS-T20 diluent only and checks for background absorbance levels in the absence of test sample.

   c. The Conjugate Control (CC) receives Coating Solution only (no CAb) and ensures there is no nonspecific binding of the HRP-Ab to the well surface.

B. Microplate Format

The microplate format for evaluating concentrations of the CAb with three concentrations of the HRP-Ab is shown below. A separate microplate is used for each concentration of Coating Antibody tested. Each plate is divided into three sections to test each of the three conjugate dilutions. The top and bottom rows (A and H) are not used in order to have an equal number of replicates (4) for each test sample at each conjugate dilution (CC and SC have 2 replicates per conjugate dilution)

Plate #1 - Cab at 1:500, each conjugate dilution is added to 2 rows; Negative controls and KPL positive controls are tested at various dilutions with 4 replicates per conjugate dilution (Samples #1-4).

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B	B	N	CC	SC	1	1	2	2	3	3	4	4	Conjugate 1:1500
C	B	N	CC	SC	1	1	2	2	3	3	4	4	
D	B	N	CC	SC	1	1	2	2	3	3	4	4	Conjugate 1:2000
E	B	N	CC	SC	1	1	2	2	3	3	4	4	
F	B	N	CC	SC	1	1	2	2	3	3	4	4	Conjugate 1:2500
G	B	N	CC	SC	1	1	2	2	3	3	4	4	
H													

C. Checkerboard Titration Procedure

1. For each CAb concentration, coat the appropriate wells of a separate microplate.

   a. Determine volume of Coating Solution and CAb needed for each concentration group - there are only 60 wells per group, however the dispensing cassette delivers to entire columns, so calculate the volume needed for an entire plate plus the additional volume needed to prime the dispensing cassette. 25 mL per plate is a good guideline.

   b. Prepare 25 mL of Coating Solution by diluting the 10X Coating Solution 1:10 in dH_20.

      EXAMPLE: 2.5 mL Coating Solution: 22.5 mL diH_20

   c. Remove 3 mL of Coating Solution and set aside for the twelve (12) CC and SC wells.

d. Calculate the amount of CAb to add to the remaining 22 mL of Coating Solution for the appropriate Coating Antibody concentration.

EXAMPLE: The first plate receives CAb at 2.0 ug/mL (or 1:500 dilution).
1/500 = X/22,000 µL
X = 44.0 µL

The second plate will receive CAb at 1.0 ug/mL (or 1:1000 dilution)
1/1000 = X/ 22,000 µL
X = 22.0 µL

The third plate will receive CAb at 0.67 ug/mL (or 1:1500 dilution)
1/1500 = X/ 22,000 µL
X = 14.6 µL

e. Carefully prepare and label each CAb concentration.

f. Label three microplates with the CAb concentration they are to receive and coat each plate with its corresponding antibody concentration. Follow the Microplate format (CC and SC wells receive Coating Solution only, not CAb).

g. Rinse the CAb dispensing head thoroughly with deionized H_20 between CAb concentrations.

h. Coat the second and third plate, again using each plates corresponding CAb concentration and by following the Microplate format.

i. Incubate in a humid chamber at 4 ˚C for 16 hours.

2. On Day 2 - Proceed with ELISA following the standard protocol steps III.B.

3. Proceed with the addition of HRP-Ab as follows

a. Calculate the amount of Conjugate (HRP-Ab) that will be needed for all three plates following the Microplate format. Each plate receives a specific HRP-Ab concentration in 2 rows, or 24 wells. Therefore, the approximate volume of each concentration needed is 4.8 mL x 3 plates = 14.4 mL.

b. Prepare the 20 mL of 1X Milk Diluent, for each HRP-Ab concentration, from the 20X concentrate. Remove 2 mL for use in the SC wells (SC wells receive Milk Diluent only).

c. Calculate the amount of HRP-Ab to add to the remaining 18 mL of Milk Diluent for each HRP concentration specified on the Microplate format.

EXAMPLE: To the remaining 18mL of Milk Diluent add HRP-Ab as follows:

For the HRP-Ab concentration of 1:1500 (0.67 ug/mL)

1/1500 = X/18,000 µL
X = 12.0 µL

For the HRP-Ab concentration of 1:2000 (0.50 ug/mL)

1/2000 = X/18,000 µL
X = 9.0 µL

For the HRP-Ab concentration of 1:2500 (0.40 ug/mL)

1/2500 = X/18,000 µL
X = 7.2 µL

d. Manually pipet 200 µL of Milk Diluent into the corresponding SC wells.

e. The HRP-Ab is added to the remaining wells for that particular concentration by following the Microplate format. A single pipettor set at 200 µL can be used for each individual well, or a multi-tip pipettor (Octapette ™) with 6 tips in place can be used to dispense HRP-Ab in each ROW.

f. Proceed with the two-hour incubation step and the remainder of the ELISA protocol.

D. Determination of Antibody Dilutions

Determine a combination of antibody dilutions that give optimum OD values for ELISA by examining the values obtained in the checkerboard titration matrix and determine which combination of CAb dilution and HRP-Ab dilution give the range of OD values that are expected. The following is a **guideline only** of the expected values:

Test Sample or Buffer	Expected OD Values
Blank (B)	.065-.075
Substrate Control (SC)	.055-.060
Conjugate Control (CC)	.055-.060
Negative Control (NCT98)	.072-.078
KPL Positive Controls	
1:100	**2.20-2.80**
1:1000	**0.90-1.50**
1:2000	**.400-.550**
1:5000	**.200-.250**

Appendix 6.C – Collection and Processing of Tissue Samples for ELISA

I. Sample Collection, Transport and Storage

A. Field Collection Supplies and Guidelines

1. Field containers should be sterile, labeled bags with seal able top closure. Or for small tissues from juvenile tissues, use 1.5-2.0 mL microcentrifuge tubes (preferably graduated with labeling space).

2. All samples should be kept cold during sample collection; this is important for protein stability of samples for ELISA, but more important to maintain the quality of DNA for subsequent testing by PCR. ELISA samples should be frozen (-20 to -70 ˚C) if stored for any extended period of time prior to processing for ELISA, or PCR testing.

3. Care is taken to avoid rupturing the stomach, or gastrointestinal tract, or cross-contaminating samples.

   Commercial fish food frequently contains antigens of Rs that can be detected by the ELISA, resulting in a false positive reading.

4. Use disposable instruments for each kidney tissue, or have a sufficient number of pre-cleaned instruments to allow single use for each fish.

B. Samples Collected from Adult Fish

1. Samples are usually taken following spawning, care is exercised to avoid cross-contamination between fish and contamination of tissue sample with body fluids.

2. Collect a 2-5 gram sample; when sampling the kidney, it is recommended that this sample consist of a pool of small tissue pieces from the anterior, mid, and posterior kidney.

3. Keep tissue cool during collection.

C. Samples Collected from Juvenile Fish

1. Juvenile fish are often collected as whole fish and dissected upon return to the laboratory.

2. Remove the entire kidney from each fish. It is preferable to test tissues from individual fish, but a tissue pool may be made if the fish are small (Two-three fish pools are acceptable).
   If these guidelines cannot be followed regarding pool size and dilution, then the fish are too small for testing.

3. A minimum sample weight of 0.07 g is needed for testing.

4. Keep fish or tissues cool during collection and processing.

II. Sample Processing

A. PBS-T20 Dilution Buffer (see Appendix 6.D – Reagents, for PBS formula)

1. All buffers should be prepared and stored in acid-washed glassware, or disposable containers. Many of the antigens of Rs adhere to glassware, are heat stable, and will not be removed or destroyed by detergent washing and autoclaving.

B. Instruments

1. The cleaning of laboratory instruments is important. The requirements for the FAT and ELISA are different due: the former detects Rs cells while the ELISA detects a soluble antigenic fraction of the bacterium. *Soaking instruments in 100% bleach, or flaming them for at least 5 seconds can disrupt Renibacterium salmoninarum cells* (Idaho Fish and Game – Eagle Laboratory, unpublished data)

2. When tissues are removed in the laboratory, instruments should be flamed between fish in a given group. Mechanical cleaning to remove Rs antigen is as important as bactericidal disinfection: if sufficient quantities of antigen from nonviable cells is carried over on instruments during sample, a false-positive reaction from ELISA can occur.

C. Tissue Dilution and Processing both adult and juvenile kidney tissues are standardized to a dilution of 1:8 (w/v) in PBS-T20, homogenized, boiled for 15 minutes, and frozen in labeled microcentrifuge tubes. A minimum of 0.08 g tissue (560 µL homogenate) is required for sufficient supernatant to inoculate 2 replicate wells.

1. Collect kidney tissue in whirlpak or stomacher type bags with top closures.

2. Tare balance with empty collection bag. Keep samples cold during processing.

3. Dilute kidney 1:8 (w/v) with PBS-T20 (1 part kidney : 7 parts PBS-T20 (Table 2C.1 – page 36)).

4. Homogenize until well mixed.

   NOTE: individual wooden sticks can be used to macerate small samples collected directly into microcentrifugetubes. Follow the procedures for tarring (tubes must be consistent in weight) and diluting. Macerate the tissue by grinding the kidney tissue against the tube with the wooden stick until the tissue is homogenized).

5. Dispense or pour into labeled microcentrifuge tubes.

6. Boil samples in a water bath (100°C) for 15 minutes. Sample tubes should be submerged deep enough in the water bath to have all tissue in the tube heated thoroughly.

7. After samples are cooled, freeze in a labeled rack until ready to test. If long-term storage is required freeze at -70°C.

   NOTE: A higher short-term storage temperature (-20°C) does not affect the OD values significantly for ELISA (USFWS-California/Nevada Fish Health Center, unpublished data), however temperature can affect DNA degradation and subsequent PCR testing if samples are not kept cold during ELISA processing. Interfering proteases and nucleases will be denatured once the samples are boiled (100°C for 15 minutes).

Table 2C.1 - ELISA Dilution Table for Kidney Tissue (1:8 w/v in PBS-T20)

Tissue WT(g)	1:8		Tissue WT(g)	1:8		Tissue WT(g)	1:8
0.07	0.49		0.30	2.10		0.50	3.50
0.08	0.56		0.31	2.17		0.51	3.57
0.09	0.63		0.32	2.24		0.52	3.64
0.10	0.70		0.33	2.31		0.53	3.71
0.11	0.77		0.34	2.38		0.54	3.78
0.12	0.84		0.35	2.45		0.55	3.85
0.13	0.91		0.36	2.52		0.56	3.92
0.14	0.98		0.37	2.59		0.57	3.99
0.15	1.05		0.38	2.66		0.58	4.06
0.16	1.12		0.39	2.73		0.59	4.13
0.17	1.19		0.40	2.80		0.60	4.20
0.18	1.26		0.41	2.87		0.61	4.27
0.19	1.33		0.42	2.94		0.62	4.34
0.20	1.40		0.43	3.01		0.63	4.41
0.21	1.47		0.44	3.08		0.64	4.48
0.22	1.54		0.45	3.15		0.65	4.55
0.23	1.61		0.46	3.22		0.66	4.62
0.24	1.68		0.47	3.29		0.67	4.69
0.25	1.75		0.48	3.36		0.68	4.76
0.26	1.82		0.49	3.43		0.69	4.83
0.27	1.89					0.70	4.90
0.28	1.96						
0.29	2.03						

Appendix 6.D – Reagents, Supplies and Equipment Lists

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service and/ or the United States government. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified in this list.

I. Reagents

A. Kirkegard and Perry Laboratories (KPL) Assay Reagents

The following are available from Kirkegaard & Perry Laboratories (1-800-638-3167):

50-63-00	Wash Solution Concentrate, 4x200 mL
50-84-00	Coating Solution Concentrate, 2x25 mL
01-96-91	Affinity purified antibody to *R. salmoninarum*, 1.0mg (Coating Antibody)
50-82-01	Milk Diluent/Blocking Solution Concentrate, 2x100 mL
04-96-91	*Peroxidase*-Labeled Affinity Purified Antibody to *R. salmoninarum,* 0.1 mg (HRP Antibody)
50-62-00	ABTS Peroxidase Substrate System, 6x100 mL
50-85-01	ABTS Peroxidase Stop Solution (5X)
50-96-91	*R. salmoninarum* Positive Control (whole cell prep)

B. Additional Reagents (many sources exist for these common chemicals)

Glycerol AR, VWR/Baxter #5092-500
Thimerosal, Sigma #T-5125
Sodium Chloride, Sigma #S-9888
Potassium Phosphate (monobasic), Sigma #P-0662
Sodium Phosphate (dibasic), Sigma #S-9390
Potassium Chloride, Sigma # P-3911
Tween-20, Sigma # P-1379
Distilled or deionized water (reagent grade)

C. Formula for Phosphate Buffered Saline (PBS) with Tween-20

Phosphate-buffered saline (PBS), pH 7.4 supplemented with 0.05% (v/v) Tween-20, and 0.01% (w/v) Thimerosal as a preservative. To 1 L of reagent grade, sterile water, add:

NaCl	8.00 gram (g)
KH_2PO_4	0.20 g
Na_2HPO_4	1.09 g
KCl	0.20 g
Thimerosal	0.10 g
Confirm pH = 7.4	
Tween-20	0.5 mL

II. Supplies

Disposable, polystyrene pipettes
96-well Immunoplates (Nunc C96 Maxisorb #446612 or Corning #62407-867)
Humid chamber (Rubbermaid or Tupperware containers)
Reagent reservoirs (for dispensing: PBS-Tween 20, Coating Sln, and Milk Diluent)
Pressure sensitive plate sealers (VWR/Baxter # B1190-32)
Dedicated glassware (for preparing: Coating Ab, HRP-Ab, Substrate-Chromogen, and Stop Solution. Acid washing is required if glassware is not dedicated and/or comes in contact with Rs antigen).
Adjustable micropipettors and tips, dedicated for specific reagent use:

(1) Positive controls, 40-200 µL
(1) Sample Loading, 40-200 µL
(2) Antibody Reagents, 5-50 µL

III. Equipment

Major Equipment for ELISA

Spectrophotometer Plate Reader (this protocol refers to Biotek Model EL340) Automated Dispensing Equipment (ICN/Flow Multi-drop unit or equivalent model).
4 Dispensing Cassettes for Multi-drop, 1 for each reagent: Cab, HRP, ABTS, Stop Sln.
Plate Washer (this protocol refer to Biotek Model EL403).
Vacuum and Reservoir system for washer
Computer, appropriate software and cables to communicate with Plate Reader.
Variable temperature incubator (capacity for 37°C incubation).

Other Laboratory Equipment

Balance with accuracy of 0.01grams
Stomacher 80 Tissue Homogenizer and bags
PBS-Tween 20 (PBS-T20) Buffer (see Appendix 6.D – Reagents, for formula)
Wooden stick applicators (for homogenizing small tissue samples)
Screw-cap microcentrifuge tubes, 2.0 mL conical with skirting, Perfector Sci. # 6550
Tube caps with O-rings, Perfector Scientific, # 6581
Adjustable pipettors with volume range of 200-1000 µL for adding PBS-T20 to samples.
ELISA racks, Intermountain Scientific # R-7920-2
Water bath or method of heating samples to 100°C for 15 minutes

CHAPTER 7

Corroborative Testing of Bacteria by Polymerase Chain Reaction (PCR)

SECTION 1 – PCR for *Renibacterium salmoninarum*

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SECTION 2 – PCR for *Flavobacterium psychrophilum, Yersinia ruckerii*, and *Aeromonas salmonicida*

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SECTION 1 – PCR for *Renibacterium salmoninarum*

I. Introduction

The Polymerase Chain Reaction[1] (PCR) assay has been shown to be an effective method for identifying low level infections of *R.salmoninarum* in fish tissue (Brown et. al 1994). The *Renibacterium salmoninarum* Polymerase Chain Reaction (Rs-PCR) technique employs oligonucleotide primers to amplify base pair segments of the gene that codes for the 57 kDa protein of *R. salmoninarum.* DNA is extracted from fish tissues and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a primer for a smaller segment of DNA within the larger segment amplified initially. This "nested PCR" step results in extreme sensitivity in detecting the target DNA (Chase 1998, Pascho et.al 1998). The DNA products from both amplifications are then visualized by agarose gel electrophoresis. The PCR assay has been shown to be an effective and accurate method for identification of low-level infections with this bacterial pathogen in various fish tissues (Pascho et al. 2002). The technique is also used to corroborate test results from immunological assays such as Enzyme linked Immunosorbent Assay (ELISA).

The materials and methods described in the *R. salmoninarum* protocol are adapted from those developed by Ronald J. Pascho and Dorothy Chase of the Western Fisheries Research Center (USGS) in Seattle, Washington. The Service would like to thank Ron Pascho and Dorothy Chase for training and technical assistance provided to Fish Health Centers for *Renibacterium salmoninarum* testing by both ELISA and nested PCR. A special acknowledgement is in order for Dorothy Chase for the protocols and training that she has provided to numerous Fish Health Centers over the years, her continued research and validation of the Rs-PCR assay, and her overall support of Survey objectives for detection of *Renibacterium salmoninarum* in wild fish populations.

[1]The PCR Process is covered by Patents owned by Hoffman-LaRoche, Inc.

II. Nested Primer Sets for *R. salmoninarum*

Initial		
Forward		Product
P3	A GCT TCG CAA GGT GAA GGG	383 bp
Reverse		
M21	GC AAC AGG TTT ATT TGC CGG G	

Nested		
Forward		Product
P4	AT TCT TCC ACT TCA ACA GTA CAA GG	320 bp
Reverse		
M38	C ATT ATC GTT ACA CCC GAA ACC	

III. DNA Extraction using QIAGEN Kit

A. Material and Reagents

QIAGEN DNeasy Kit (QIAGEN #29304)
Ethanol (Absolute 97-100%)
Proteinase K (Sigma P2308)-(also included in QIAGEN kit)
Lysozyme Lysis Buffer
Pipettor (100-200 µL and 0.5mL)
Aerosol Barrier Tips
1.5mL centrifuge tubes
Micro centrifuge
Heat block(s) (70°C and 95°C)
Latex or nitrile gloves

Lysozyme Lysis Buffer

Lysozyme	2 g
Tris HCl Stock	2 mL
EDTA Stock	2 mL
Triton	1.2 mL

Bring to 100 mL with DI water.

Tris HCl Stock Solution
(100 mL at 1 M pH 8.0)
5.7g trizma base+8.9g Tris HCl into 85.4 mL DI water

EDTA Stock Solution
(100 mL at 0.1 M)
3.72g EDTA - qs distilled water to 100mL

B. General Quality Control Considerations

1. Wear gloves and change gloves often.
   This prevents contamination of sample DNA with degrading nucleases and acids that naturally occur on the skin. Frequent changing of gloves also prevents DNA contamination of hands and work surfaces in the laboratory. Steps in this protocol that are underlined indicate significant sources of error and/or potential for contamination should deviation from this protocol occur.

2. Utilize microcentrifuge tubes with locking caps. Heating of extraction solutions causes unlocked caps to pop open, which can cause release of aerosols that can cause cross-contamination between samples and controls.

3. Always run positive *Renibacterium salmoninarum (Rs)* control tissues as well as negative controls (water, or known negative tissue) from the start of the extraction

process through nested amplification to final electrophoresis. This is the only means of assuring validity of the assay and its results.

C. Procedure

1. Cell lysis:
   a. Kidney Tissue: Cut 25 mg of tissue into small pieces and place in a 1.5 ml microfuge tube and add 180 µl of ATL Buffer and 20 µl of Proteinase K solution. Incubate at 55°C for 1 hour or at 37°C 1-16 hours or until tissue is completely lysed. (Overnight incubation of kidney samples at 37°C is effective for complete tissue lysis.) Vortex occasionally.

   b. Ovarian fluid: Pipet 50 µl of ovarian fluid into a 1.5 ml microfuge tube and add 180 µl of ATL Buffer and 20 µl of Proteinase K solution. Incubate at 55°C until tissue is completely lysed. (usually 1 hour) Vortex occasionally.

   c. Whole blood: Pipet 10 µl of whole blood into a 1.5 ml microfuge tube and add 180 µl of ATL Buffer and 20 µl of Proteinase K solution. Incubate at 55°C until tissue is completely lysed. (usually 10 minutes) Vortex occasionally.

   d. R. salmoninarum solutions: Pellet bacteria solution by centrifugation at 7,000 x g for 15 minutes. Pour off supernatant and resuspend pellet in add 180 µl of ATL Buffer and 20 µl of Proteinase K solution. Incubate at 55°C for 1 hour. Vortex occasionally.

2. Add 180 µl of Lysozyme Lysis Buffer (LLB).

3. Incubate at 37°C for 1 hour, vortexing occasionally.

4. Add 25 µl of Proteinase K stock solution and 200 µl of buffer AL.

5. Mix by vortexing and incubate at 55°C for 30 minutes (vortex occasionally during this incubation period).

6. Incubate at 95°C for another 10 minutes.

7. Add **210µl of ethanol**, mix thoroughly by vortexing.

8. Place a QIAGEN spin column in the 2 mL collection tube provided. Pipette sample mixture onto the filter in the spin column being careful not to moisten the rim of the column. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1minute. (All centrifugation is at ambient temperature).

9. Transfer the spin column (upper section of tube with filter) into a clean 2 mL collection tube and discard lower tube containing the filtrate.

10. Carefully open the spin column and add **500 µl Buffer AW** (wash buffer). Centrifuge as above.

11. Repeat steps 9 and 10, centrifuging at 6000 x g for 1 minute, then at full speed for an additional 2 minutes.

12. At this point, place an aliquot of Buffer AE in the 70°C heat block to preheat for the next step.

13. Place spin column in clean 1.5 mL Micro centrifuge tube that has a closable cap.

14. Add **200 µl of Buffer AE** (elution buffer), which has been **heated to 70°C** (Tris, pH 9.0 or water heated to 70°C can also be used for this step as an elution buffer).

15. Incubate for 5 minutes at 70°C. Centrifuge at 6000 x g for 1 minute.

16. Repeat step 14 so that the total volume of DNA is 400 µl.

17. During the last step, the ELUTED DNA SOLUTION in the bottom microcentrifuge tube is retained and the SPIN COLUMN IS DISCARDED.

18. Store extracted DNA solution at -20 or -70°C until use.

IV. Initial Amplification of *R.salmoninarum* DNA

A. Materials and Reagents

10X Buffer
dNTP (nucleotides)
Primers (forward and reverse; dilute primers in H_2O to 100 pmole/µl if necessary)
Taq Polymerase
$MgCl_2$ Buffer (comes with some Taq products)
Mineral oil - molecular grade (Only use PCR Grade for overlaying samples. Standard mineral oil can be used for thermocycler equipment.)
Thermocycler
0.5-25µl and 20-200µl pipettors

NOTE: Use only highly accurate micro-pipettors such as positive displacement types with matching displacement tips. Use Aerosol-resistant tips for all other pipettors.

Gloves (latex or nitrile)
Bench top UV cabinet.
Cryo-rack frozen to -70°C

B. General Quality Control Considerations

One aerosol drop of amplified DNA contains approximately 24,000 thousand strands of DNA which can easily contaminate laboratory surfaces, other samples and reagents used in PCR !

1. To reduce this risk of contamination in the laboratory, it is important to establish three separate work areas for reagent preparation, sample preparation, and amplification steps, as follows:

   a. **AREA 1 - <u>REAGENT PREPARATION AREA</u>**: Mix and aliquot pre-amplification ingredients (Master Mix or "PCR cocktail") in a clean room or under a dedicated UV cabinet. Provide dedicated pipettors, preferably positive displacement type, and all supplies that are required to prepare Master Mix. **NEVER** contaminate this area with sample material or amplified DNA product.

   b. **AREA 2 - <u>SAMPLE PREPARATION / DNA EXTRACTION AREA:</u>** Extract DNA from tissue samples in an area away from reagent prep or amplification steps. Use strict containment precautions to prevent contamination of surfaces and equipment. Disinfect this area after each use with a commercial DNA-disinfectant or a strong chlorine solution.

   c. **AREA 3 - <u>AMPLIFICATION AREA</u>**: Transfer amplified DNA from Round 1 to Round 2 tubes under a Bench-top UV Hood. Gloves and all other supplies should be stored within the hood for easy access and to prevent amplified DNA from leaving this area.

2. Always wear gloves and change to a fresh pair when leaving or entering the PCR REAGENT AREA. Change gloves whenever contamination from a sample tube is even suspected in the SAMPLE PREP/EXTRACTION AREA (i.e. aerosols may have been released upon opening a tube, liquid appears on the outside edge of a tube or cap, or liquid is visible on gloves after handling an individual tube, etc.)

3. Use only aerosol barrier tips and positive-displacement pipettors for dispensing PCR reagents. Change pipette tips between all reagents and all samples. Discard dirty tips into autoclave bags and discard bags daily.

4. All sample racks and reusable equipment should be washed in DNA-away or chlorine disinfectants and autoclaved after use. Spray/wipe pipettors and working areas with disinfectant and sterilize work within the hood with at last 30 minutes of UV light (UV light denatures DNA).

5. All reagent batches should be marked and recorded for each test run so they can be checked if problems occur with the assay.

C. Procedures

1. Using Appendix 7.A – Worksheet for DNA Sample Data, record appropriate data for each sample to be tested by PCR.

2. Using Appendix 7.B – Worksheet for Initial Amplification of R. salmoninarum DNA by PCR, record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples to be processed. (Add 4 to the total number of samples to be tested - this allows enough extra reactions to run both positive and negative controls in the assay).

3. In REAGENT PREPARATION AREA (Area 1), add PCR reagents (**except for sample DNA)** to the MM tube in the order listed on Worksheet in Appendix 7.B, adding water first and Taq last. Keep all reagents cold in a cryo-rack during mixing, and return unused reagents to the freezer immediately after use.

4. Place 40 µL of MM into each 0.5 mL PCR tube, then overlay the Master Mix with one drop of PCR grade mineral oil per tube to prevent condensation within the reaction tube. Close caps tightly. Move Master Mix tubes to sample loading area (Area 2).

5. In Area 2, label the top of the MM tubes with sample identification. Load 10 ul of each sample DNA into the appropriate tube being careful to expel the sample beneath the layer of mineral oil. Close caps tightly. Change gloves and transfer the sample tubes to the thermocycler. Clean the SAMPLE PREP AREA as described above.

6. Add regular mineral oil into each reaction well of the thermocycler, and load the sample tubes into the machine (Follow manufacturer's recommendations regarding use of mineral oil and sample placement in machine).

7. Thermocycler should be programmed for 30 cycles of the following cycling regime. Record the thermocycler program on the worksheet in Appendix 7.B:

   a. Preheat or "Jumpstart" sample to 94°C for two minutes.

   b. Denaturing at 94°C for 30 seconds.

   c. Annealing at 60°C for 30 seconds.

   d. Extending at 72°C for 60 seconds.

   e. Post dwell at 4-16°C for holding samples after cycling is complete.

V. Nested PCR - Secondary Amplification of *R. salmoninarum* DNA

A. Materials, Methods, and General QA/QC Considerations of Section II also apply to the nested PCR process.

B. Procedures

1. Using Appendix 7.C - Worksheet for Nested (Second Round) Rs PCR, record assay date and perform calculations to determine the amount of each reagent to use in the second round Master Mix. This is based on the number of samples and controls to be processed (usually the same number of samples and controls that was used for initial round Master Mix).

   NOTE: The water volumes are increased in second round MM to offset the smaller volume of DNA Template used in the Nested amplification step.

2. Add PCR reagents, except the Template DNA, into the Master Mix tube. Return unused reagents to the freezer.
3. In 0.5 mL PCR tubes, place 49µL of MM and overlay samples with one drop PCR grade mineral oil. Close caps tightly. Transfer tubes to AMPLIFIED DNA AREA (Area 3).

4. Load 1µL of amplified sample DNA into the appropriately labeled PCR tubes for second round amplification. Be sure to expel PCR product beneath mineral oil layer. Gentle mixing with the pipette tip by withdrawing and expelling within the MM solution may help mix the DNA more thoroughly with the second round Master Mix.

5. Add mineral oil to each reaction well of the thermocycler. Load PCR tubes into wells.

6. Program thermocycler for 20 cycles of the following regime:

   a. Jump start thermocycler to 94°C for two minutes.

   b. Denaturing at 94°C for 30 seconds.

   c. Annealing at 60°C for 30 seconds.

   d. Extending at 72°C for 60 seconds.

   e. Post dwell at 4-16°C for holding samples after cycling is complete.

   NOTE: PCR Products can be refrigerated for one month or frozen at -70 ^{o} C for long-term storage.

VI. Visualization of PCR Product by Electrophoresis

A. Materials and Reagents

GEL ELECTROPHORESIS
SeaKem Agarose
0.5X TAE Buffer Solution
Clean glass flask (200 mL)
Hot Plate or Microwave Oven
Gel unit with Power Supply
Dedicated pipettor for Amplified DNA
Gel Loading Dye
1kbp DNA Ladder (in 100bp increments preferably)

VISUALIZATION OF BANDS
Ethidium Bromide Staining Solution
Staining dishes
Protective UV Eyewear
Transilluminator
Photo documentation Camera

B. Procedures

1. Prepare 0.5X TAE buffer for both the agarose gel and to fill the gel unit chamber (it's important to use the same buffer for both since small differences in ionic strength can affect migration of DNA). Use sterile deionized water to prepare buffer, following manufacturer's recommendations.

2. Prepare 2% Agarose Gel

   a. Assemble the gel tray and position 12 µl well volume comb in the tray according to manufacturer recommendations.

      Gel units come with various comb sizes. The final volume of sample loaded has to be adjusted to the comb size by following manufacturer recommendation.

      <u>Some guidelines follow:</u>
      4 place comb.....50 µl
      8 place comb.....20 µl
      12 place comb.....12 µl

   b. Level the chamber using a built-in bulls eye level, or with a small level. Place assembled tray into chamber.

   c. Weigh appropriate amount of agarose for 2% gel and add to proper volume 0.5X TAE buffer.

   d. Heat solution to boiling while stirring to completely dissolve the agarose.

   e. Allow solution to cool to about 65°C before pouring into gel tray (approximately 10 minutes or until still quite warm to touch).

   f. Pour agarose solution into gel tray filling to the 1cm mark. Avoid the formation of bubbles.

   g. Allow gel to cool completely, then carefully remove the comb by lifting straight up (avoid side to side motions). Additionally remove the rubber ends.

   h. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode. The mnemonic phrase "RUN TO RED" is used to remind the operator of the correct orientation of the gel tray in regard to samples running towards the red (positive) electrode.

   i. Slowly fill the chamber with the remaining 0.5 X TAE buffer solution until the top of the gel surface is submerged about 1mm.

3. Loading the gel

   a. Prepare a map of sample placement before loading the gel to ensure that samples, ladders, and controls are placed correctly.

   b. For each PCR product to be visualized, pipet 2 µl of gel loading dye onto a clean strip of parafilm. Place the dye with adequate space between each droplet to prevent cross contamination. (Clean 0.5 mL tubes can also be used).

   c. Withdraw 10 µl from the first PCR product tube and wipe the excess mineral oil from the tip (dispose of each Kimwipe® after each sample). Carefully mix the sample and the dye by repeated expulsion.

   d. When the sample and the dye are adequately mixed, carefully place the pipet tip containing the mixture over an individual well of the 2% agarose gel, and carefully load the sample into the well. Repeat steps b & c for all the samples to be tested on the gel (both initial round and second round - nested PCR products).

   e. Be sure to load the DNA ladder(s) for base pair (bp) reference.

   f. Load the positive and negative controls last.

   g. Place the safety cover on the chamber and secure shut. Connect the attached leads to the power supply, making sure the black lead is connected nearest to the samples (use the mnemonic"Run to Red" to remember the correct orientation and direction of current).

   h. Turn on the power supply and run the gel at 60-80 volts for at least one hour. Follow manufacturer's recommendations, as gel units will vary in their conductivity characteristics. Small bubbles will form in the buffer at the electrodes, and the loading dye will begin to migrate through the gel after a short period, indicating proper electrophoresis.

4. Staining the Gel

   a. Prepare a 0.5 µg/mL solution of ethidium bromide with 0.5 X TAE Buffer.

      CAUTION: Ethidium bromide is a strong mutagen. Wear gloves and follow all MSDS precautions when handling or using this chemical.

   b. Turn off power supply to chamber and remove power leads. Remove safety cover.

   c. Remove gel and tray and place in ethidium bromide solution for 15 to 20 minutes.

   d. Destain gel in water for 5 to 60 minutes.

NOTE: Ethidium Bromide solution can be reused and stored in a plastic tray container with a secure lid. Be sure to label and store this solution appropriately. Also see the Reagent list for an Ethidium Bromide extraction kit that allows filtration and proper disposal of used staining solutions.

5. Visualizing the DNA

   a. Carefully carry the gel to the transilluminator and place the gel over the light source.

   b. Be sure to wear UV protective eyewear when visualizing the gel with UV light.

   c. Carefully record locations of base pairs on positive control bands in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primer sequence used in both the first and second (nested) round PCR assays.

      Anticipated Products: 1st round primer M21 = **383bp**
      2nd round (nested) primer M38 = **320 bp**
      Bands from the second (nested) round will be brighter than the initial PCR products.

   d. Note any unusual band occurrences (bands at 750 bp can occur as a PCR artifact). Negative controls (water used as template) should not have any visible bands. If suspected contamination occurs in negative controls, or test samples, the assay should be re-run from the extracted DNA tubes.

6. Photographing the gel

   a. Assemble the Polaroid Photo documentation camera: Use an orange filter and appropriately sized snap-on camera hood. Adjust shutter speed and aperture as recommended by the manufacturer. Experimentation with aperture and shutter speed may be needed to obtain the best resolution and exposure.

   b. Position the hood over the gel on the transilluminator and take the picture using the remote shutter release button. You can photograph directly on the gel tray, however adjust the level of the hood to the top edge of the tray, otherwise the image will be slightly out of focus.

   c. **Photo document all gels (Appendix 7.D)** and attach the developed photo to the PCR Data sheet and case history information.

VII. Equipment / Reagent Source List

DNA Extraction:

Reagents/ Supplies	Source
QIAGEN Tissue Kit	QIAGEN #29304
microcentrifuge tubes (1.7mL, locking caps)	ISC #C-3251-1
Ethanol (Absolute 97-100%)	Spectrum Chem. #ET107
Proteinase K (also comes with QIAGEN kits)	Sigma # P2308
Lysozyme Lysis Buffer	Sigma # L-7651
Trizma (Tris base/Tris Hydrochloride)	Sigma # T1503/T3253
Pipettor, Oxford Benchmate (10-200µl autoclavable)	Thomas Sci. #7733F10
Oxford Benchmate (100-1000µl autocl.)	Thomas Sci. #7733F13
Aerosol barrier tips (1-300 µl)	Thomas Sci #7740-F78
Aerosol barrier tips (1-1000µl)	Thomas Sci #7740-F92
Micro centrifuge (MicroV)	Fisher Sci. #05-090-724
or Var.speed Microcentrifuge	Daigger #YX4241A
Heater - Dry bath incubator	Fisher Sci. # 11-718 series
Blocks for Fisher Dry Baths (1.5 mL mc tubes)	Fisher Sci. # 11-718-9
Gloves (nitrile : non-latex not necessary)	Fisher Sci. # 11-395-19C
Tube storage boxes (100 mc-tubes)	Daigger # YX4280C

PCR Amplification

Reagents/Equipment	Source
QIAGEN PCR Kit (includes Taq, dNTPs and buffers)	QIAGEN #201223
Or master mix ingredients can be purchased separately:	
Taq DNA Polymerase (comes with $MgCl_2$)	Sigma # D1806
PCR Buffer II Kit ($MgCl_2$ and 10X)	Sigma #PCR-II
dNTP (nucleotides)	Sigma # D7295
microcentrifuge tubes (0.65 mL)	ISC #C-3249-1

Primers (forward and reverse; dilute primers in H_2O to 100 pmole/µl if necessary)

PRIMER	DNA Sequence 5' to 3'
Forward P3	**A GCT TCG CAA GGT GAA GGG**
Reverse M21	**GC AAC AGG TTT ATT TGC CGG G**
Forward P4	**AT TCT TCC ACT TCA ACA GTA CAA GG**
Reverse M38	**C ATT ATC GTT ACA CCC GAA ACC**

Primers can be synthesized by:	Great American Gene CO. GIBCO, BRL Life Technologies
Mineral oil (PCR grade for overlay)	Sigma # M8662
PCR Grade water (1 mL quantities)	Sigma # W1754
Molecular Grade Water (Genemate gallon)	ISC #C-553-1
Finnpipette, positive displacement 0.5-25µl	Fisher Sci. # 21-377-9
Positive Displacement tips/plungers 0.5-25µl	Fisher Sci. # 21377-53
Finnpipette, positive displacement 20-200µl	Fisher Sci. # 21-377-10
Tips/Plungers (20-200µl)	Fisher Sci. # 21377-54
Cryo-rack IsoFREEZE Flipper , -holds 0.5 and 1.5 mL microcentrifuge tubes	ISC #R-2020-2
Thermal cycler ,Thermolyne Amplitron II	Fisher Sci. # DB66925 or 35

Electrophoresis:

Reagents/Equipment	Source
Tris-Acetate EDTA Buffer 10x pre-made solution	Gibco #1558-026
(Available in other forms) 25X Liter powder packs	ISC #C-5553-2
(can also be made from scratch - see Buffer Recipes)	Sigma # T4038
Agarose, Seakem	ISC # 50003
RediLoad Gel Loading Buffer (can be loaded with Master Mix) or,	Research Genetics # 750026
Agarose Gel Loading Dye 6X	ISC # C5400-5
DNA Ladder (100 bp)	GIBCO # 15628-019
Ethidium Bromide (10mg/mL solution)	ISC #C-5515-10
Extractor-Ethidium bromide waste reduction system	Sigma # Z36, 156-9
Submarine Gel Systems: A variety are available from Fisher Scientific	
Power Supply EC105	Fisher Sci. #FB-105
Photo-Documentation Camera (Snap on hoods sized according to gel size)	Fisher Sci. # FB-PDC-34
Lens Filter (Tiffen) Orange for ethidium bromide	Fisher # FB-PDF15
Film - black and white Polaroid	
Type 667 (20 exp.)	Fisher Sci. #04-441-91
or Type 107 (8 exp.)	Fisher Sci. #04-441-19
Transilluminator: Fisher Model 88, 302 nm	Fisher Sci. #FB-TI-88
UV Protection spectacles	Daigger #YX11090U

Manufacturers and Phone Numbers:

A. Daigger & Co., Inc.	**800-621-7193**
Fisher Scientific	**800-766-7000**
Gibco	**800-828-6686**
Intermountain Scientific Corp.(ISC)	**800-999-2901**
QIAGEN, Inc.	**800-426-8157**
Sigma	**800-325-3010**
Thomas Scientific	**800-345-2101**
VWR Scientific Products	**800-252-1234**

References

Brown, L. L., G. K. Iwama, T. P. T. Evelyn, W. S. Nelson, and R. P. Levine. 1994. Use of the polymerase chain reaction (PCR) to detect DNA from Renibacterium salmoninarum within individual salmon eggs. Diseases of Aquatic Organisms 18:165-171.

Chase, D. M. and R. J. Pascho. 1998. Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of Renibacterium salmoninarum that provides a highly sensitive method of detection of the bacterium in salmonid kidney. Diseases of Aquatic Organisms 34:223-229.

Pascho, R. J., D. Chase, and C. L. McKibben. 1998. Comparison of the membrane-filtration fluorescent antibody test, the enzyme-linked immunosorbent assay, and the polymerase chain reaction to detect Renibacterium salmoninarum in salmonid ovarian fluid. Journal of Aquatic Animal Health 9:99-107.

Pascho, R. J., D. G. Elliott, D. M. Chase. 2002. Comparison of traditional and molecular methods for detection of Renibacterium salmoninarum. In Cunningham, C.O. ed. Molecular Diagnosis of Salmonid Diseases. Kluwer Academic Publishers, pp.157-209.

Additional Reading

Innis, M. A., D. H. Gelfont, J. J. Sninsky, and T. J. White. Eds. 1990. PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Guide. 2nd edition. Cold Spring Harbor Laboratory Press Plainview, NY.

White, Bruce A., Ed. 1993. PCR Protocols. Current Methods and Applications in Methods in Molecular Biology Vol. 15. Humana Press, Inc. Totowa, NJ.

Appendix 7.A – Worksheet for DNA Sample Data

Case Number_____ Sample Site_____ Species_____

Tissue Sample Type*	PCR Number	Mean ELISA OD Value	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

***Record "E" for ELISA processed kidney or "F" for frozen or fresh kidney.**

Appendix 7.B - Worksheet for Initial Amplification of Rs DNA by PCR

Case Number_____ Date_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µl)	Volume for ____samples
10XBuffer		1X	___ X		
$MgCL_2$		1.5 mM	___ mM		
dNTPs		0.2 mM	___ mM		
(+)Primer		20 pM	___ pM/µl		
(-)Primer		20 pM	___ pM/µl		
TAQ		2 units	___ units/µl		
d-H_2O*		**Add to total 50µl**	**including DNA =**		
DNA		-	-	10 µl	

*Add water to Master Mix first, TAQ last. (= 50 µl)

Thermocycler Program: Number of Cycles____

Denature at _____^{0} for _____ seconds.
Anneal at _____^{0} for _____ minutes.
Extend at _____^{0} for _____ minutes.

Master Mix Formula Table for Rs-PCR

PCR Reagents	Lot #	Stock Concentration*	Final Concentration	Volume/Reaction (Total reaction volume = 50µL)
Extracted DNA		--	--	10.0 µL
10X Buffer		10X	1X	5.0 µL
dNTPs		(10*) mM/µL	0.2 mM	1.0 µL
(+) Primer	P3	*(70*)* pMole/µL	20 pMole	1.0 µL
(--) Primer	M21	*(78*)* pMole/µL	20 pMole	1.0 µL
[1]RediLoad Gel Dye™		10X	1x	5.0 µL
TAQ		*(5*)* units/µL	2 units	0.4 µL
d-H_20			(qs to 50µL)	Example only: 23.4 µL subtotal Add 26.6µL H_20 per rxn
[2]$MgCl_2$ (if not included in 10X Buffer)		25mM	1.5 mM	3.0 µL

*** NOTE: Stock concentrations will vary – those provided here are examples only. Check the specific reagents prior to use.**

[1]RediLoad Gel Dye™ - optional.
[2]$MgCl_2$ - only add not included in 10X buffer (or if concentration in buffer is insufficient for final concentration).

Appendix 7.C – Worksheet for Nested (Second Round) Rs PCR

PCR Reagent	Lot#	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µl)	Volume for ____samples
10XBuffer		1X	___X		
$MgCL_2$		1.5 mM	___mM		
dNTPs		0.2 mM	___mM		
(+)Primer		20 pM	___pM/µl		
(-)Primer		20 pM	___pM/µl		
TAQ		2 units	___units/µl		
d-H_2O*		**Add to total 50 µl**	**including DNA =**		
DNA		-	-	1 µl	-

*Add water to Master Mix first, TAQ last. (=50 µl)

Thermocycler Program: Number of Cycles____

Denature at _____° for _____ seconds.
Anneal at _____° for _____ minutes.
Extend at _____° for _____ minutes.

Appendix 7.D - Photo documentation and Report of Results

Case Number_____ Date:_____

Samples:_____

_____

_____

Affix gel photo documentation to this sheet and make notes on results:

Notes

CONFIRMED: ☐ Yes ☐ No Initial _____ Date:_____

SECTION 2 – PCR for *Flavobacterium psychrophilum, Yersinia ruckerii*, and *Aeromonas salmonicida*

I. Introduction

The polymerase chain reaction (PCR) is a rapid, nucleic-acid-based procedure that uses a pair of oligonucleotide primers and a thermostable polymerase to amplify target DNA sequences. Weisburg et al. (1991) developed a set of primers (fD2/rP2) that target highly conserved DNA sequences within the gene coding for the bacterial 16S ribosomal RNA (rRNA) and that can amplify a 1,500-base-pair (bp) PCR product from most any bacterial species. The products of these "universal" primers can be used as templates in conjunction with primers targeting species-specific 16S rRNA sequences internal to the universal primer binding sites to create an extremely sensitive "nested" PCR reaction.

Primer sets have been developed for the 16S rRNA sequences of a number of bacterial fish pathogens. Toyama et al. (1994) developed a set pf PCR primers (PSY1, PSY2) that target specific sequences within the 16S rRNA gene of *F. psychrophilum*, amplifying a 1,100bp product. Gibello et al. (1999) developed a set of 16S rRNA primers (YER8, YER10) that amplify a 575bp product of *Y. ruckeri*. Hoie et al.(1997) developed a set of 16S rRNA primers (AS1,AS2) that amplify a 270bp product from *A. salmonicida*. While all three sets of primers target species-specific sequences within the 16S rRNA gene, the three studies differed considerably in the protocols used to prepare target sequences. Taylor and Winton (2002) examined ways of optimizing the PCR reactions from tissue using the universal primers in a nested PCR, and directly using bacterial cultures using only the species specific primers in a single round PCR. For the purposes of the National Wild Fish Health Survey, only the single round PCR will be used as a confirmatory method.

II. Nested Primer Sets for F. psychrophilum, Y. ruckerii, and A. salmonicida

		Product
Forward PSY1	CGA TCC TAC TTG CGT AG	
Reverse PSY2	GTT GGC ATC AAC ACA CT	1100bp
		Product
Forward YER8	GCG AGG AGG AAG GGT TAA GTG	
Reverse YER10	GAA GGC ACC AAG GCA TCT CTG	575bp
		Product
Forward AS1	GGC CTT TCG CGA TTG GAT GA	
Reverse AS2	TCA CAG TTG ACA CGT ATT AGG CGC	271bp

III. DNA Preparation

When using a bacterial culture, it is not necessary to run through a DNA extraction kit such as Qiagen**.**

A. Add 200 µl of TE buffer to a 1.5 ml centrifuge tube.

B. Take a loop of bacteria culture of interest and add to TE buffer. Culture needs not be pure, but will work better if it is.

C. Using a spectrophotometer Spec 20, adjust O.D. value to be ≤ 0.6 at 525 nm.

Note: Culture can be diluted up to $0.6 \times 10^{-4.}$ If sample is this dilute, use 10ul in master mix.

IV. Amplification

A. Materials and Reagents

10X Buffer
dNTP (nucleotides)
Primers (forward and reverse; dilute primers in H_2O to 100 pmole/µl if necessary)
Taq Polymerase
$MgCl_2$ Buffer (comes with some Taq products)
Thermocycler
0.5-25 µl and 20-200 µl pipettors

B. Procedures

1. Using the appropriate PCR worksheet forms Appendices F-H, record appropriate data for each sample to be tested by PCR

2. Using Appendix 7.E -Worksheet for Amplification of Bact DNA for PCR (for either P.psychrofilum, Y. ruckeri, or A. salmonicida) record the date of assay and then calculate the amount of each reagent to go into the "Master mix"(MM) according to the number of samples and O.D. value of samples to be processed. (Add 4 to the total number of samples to be tested- this allows enough extra reactions to run both positive and negative controls in assay.)

3. In reagent preparation area, add PCR reagents, adding water first and Taq last.

4. Place 49 ul of MM mix into 0.5 ml tubes (depending on O.D. value of sample). Close caps tightly and move to sample loading area.

5. In Area 2, label top of MM tubes with sample identification. Load 1ul of sample into the appropriate tube.

6. Thermocycler should be programmed for the following:

    a. Initial Denaturing at 95°C for 4 minutes

    b. 30 cycles of:

        (1) Denaturing at 95°C for 45 seconds

        (2) Annealing at 55°C for 60 seconds

        (3) Extending at 72°C for 90 seconds

    c. Final extension at 72°C for 4 minutes

    d. Hold at 4°C.

V. Visualization of PCR Product by Electrophoresis

GEL ELECTROPHORESIS	VISUALIZATION OF BANDS
Low EEO Agarose	Ethidium Bromide Staining Solution
1x TBE Buffer Solution	Staining dishes
Clean glass flask (200 mL)	Protective UV Eyewear
Hot Plate or Microwave Oven	Transilluminator
Gel unit with Power Supply	Photo documentation Camera
Dedicated pipettor for Amplified DNA	
Gel Loading Dye	
1kbp DNA Ladder (in 100bp increments preferably)	

B. Procedures

1. Prepare 1 X TBE buffer for both the agarose gel and to fill the gel unit chamber (it's important to use the same buffer for both since small differences in ionic strength can affect migration of DNA). Use sterile deionized water to prepare buffer, following manufacturer's recommendations. TBE is more stable that TAE, but TAE buffer is acceptable)

2. Prepare 1.5% Agarose Gel

    a. Assemble the Gel tray and position comb in the tray according to manufacturer recommendations. Gel units come with various comb sizes. The final volume of sample loaded has to be adjusted to the comb size by following manufacturer recommendation.

b. Level the chamber using a built-in bulls eye level, or with a small level. Place assembled tray into chamber.

c. Weigh appropriate amount of agarose for 1.5% gel and add to proper volume 1x TBE buffer.

d. Microwave on high for 30 seconds. Remove flask and swirl gently. Heat on high for another 30 seconds, remove and swirl gently. Repeat process until boiling and agarose is completely dissolved.

e. Allow solution to cool to about 65°C before pouring into gel tray (should be able to touch to wrist comfortably)

f. Pour agarose solution into gel tray filling to the 1cm mark. Avoid the formation of bubbles.

g. Allow gel to cool completely, then carefully remove the comb by lifting straight up (avoid side to side motions). Remove the rubber ends also.

h. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode. The mnemonic phrase "RUN TO RED" is used to remind the operator of the correct orientation of the gel tray in regard to samples running towards the red (positive) electrode.

i. Slowly fill the chamber with 1X TBE buffer solution until the top of the gel surface is submerged about 1mm.

3. Loading the gel

a. Prepare a map of sample placement before loading the gel to ensure that samples, ladders, and controls are placed correctly.

b. For each PCR product to be visualized, pipette 2 µl of gel loading dye onto a clean strip of parafilm. Place the dye with adequate space between each droplet to prevent cross contamination. (Clean 0.5 mL tubes can also be used).

c. Withdraw 10 µl from the first PCR product tube and carefully mix the sample and the dye by repeated expulsion.

d. When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the 1.5% agarose gel, and carefully load the sample into the well. Repeat steps b & c for all the samples to be tested on the gel.

e. Be sure to load the DNA ladder(s) for base pair (bp) reference.

f. Load the negative control first and the positive control last.

g. Place the safety cover on the chamber and secure shut. Connect the attached leads to the power supply, making sure the black lead is connected nearest to the samples (use the mnemonic"Run to Red" to remember the correct orientation and direction of current).

h. Turn on the power supply and run the gel at 60-80 volts. Follow manufacturer's recommendations, as gel units will vary in their conductivity characteristics. Small bubbles will form in the buffer at the electrodes, and the loading dye will begin to migrate through the gel after a short period, indicating proper electrophoresis.

4. Staining the Gel:

a. Prepare a 0.5 μg/mL solution of ethidium bromide with 0.5 X TAE Buffer.

CAUTION: Ethidium bromide is a strong mutagen. Wear gloves and follow all MSDS precautions when handling or using this chemical..

b. Turn off power supply to chamber and remove power leads. Remove safety cover.

c. Remove gel and tray and place in ethidium bromide solution for 15 to 20 minutes.

d. Destain gel in water for 5 to 60 minutes.

NOTE: Ethidium Bromide solution can be reused and stored in a plastic tray container with a secure lid. Be sure to label and store this solution appropriately. Also see the Reagent list for an Ethidium Bromide extraction kit that allows filtration and proper disposal of used staining solutions.

5. Visualizing the DNA:

a. Carefully carry the gel to the transilluminator and place the gel over the light source.

b. Be sure to wear UV protective eyewear when visualizing the gel with UV light.

c. Carefully record locations of base pairs on positive control bands in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primer sequence used in both the first and second (nested) round PCR assays.

Anticipated Products:
F. psychrophilum = 1100bp
Y. ruckeri = 575bp
A. salmonicida = 271 bp

6. Photographing the gel:

   a. Assemble the Polaroid Photo documentation camera: Use an orange filter and appropriately sized snap-on camera hood. Adjust shutter speed and aperture as recommended by the manufacturer. Experiment with aperture and shutter speed may be needed to obtain the best resolution and exposure.

   b. Position the hood over the gel on the transilluminator and take the picture using the remote shutter release button. You can photograph directly on the gel tray, however adjust the level of the hood to the top edge of the tray, otherwise the image will be slightly out of focus.

   c. **Photo document all gels** and attach the developed photo to the PCR Data sheet (Appendix 7.D) accompanied with the appropriate PCR Worksheet from Appendices 7. F-H and the case history information.

VI. Equipment and Resource list: see previous Section 1 for R.sal

References

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Hoie, S., M. Heum, and O. F. Thoreson. 1997. Evaluation of a polymerase chain reaction-based assay for the detection of *Aeromonas salmonicida ss salmonicida* in Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 30:27-35.

Taylor, P. W., and J. R. Winton. 2002. Optimization of nested polymerase chain reaction assays for the identification of *Aeromonas salmonicida, Yersinia ruckeri*, and *Flavobacterium psychrophilum*. Journal of Aquatic Animal Health 14:216-224.

Toyama,T., K. Kita-Tsukamoto, and H. Wakabayashi. 1994. Identification of Cytophaga psychrophila by PCR targeted 16S ribosomal RNA. Fish Pathology 29:271-275.

Weisburg, W. G., S. M. Barns, D. A. Pelltier, and J. D. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173:697-703.

Additional Reading

Taylor, P. W., and J. R. Winton. 2004. PCR detection of *Flavobacterium psychrophilum* in eggs and sexual fluids of pacific salmonids:implications for vertical transmission of Bacterial Coldwater Disease. Journal of Aquatic Animal Health 16:104-108.

Appendix 7.E - Worksheet for Amplification of Bact DNA by PCR

Case Number_____ **Date_____**

PCR Reagent	**Lot#**	**Final Concentration**	**Stock Concentration**	**Volume per Reaction (to total 50 µl)**	**Volume for ____samples**
10XBuffer		1 X	___ X	5 ul	
$MgCL_2$		2.0 mM	25 mM	4 ul	
dNTPs		0.2 mM ea	__10 mM each	1 ul	
(+)Primer		100 pM	__20 pM/µl	5 ul	
(-)Primer		100 pM	20 pM/µl	5 ul	
TAQ		1.25 units	___5 units/µl	0.25 ul	
d-H_2O*		**Add to total 50 µl**	**including DNA =**	28.75	
DNA		-	-	1 µl	

*Add water to Master Mix first, TAQ last. (= 50 µl)
Note: add 1 ul of sample DNA if spec'd to ≤0.6 O.D. value. Add 10 ul if sample is diluted to 0.6 x 10-4.

Appendix 7.F – PCR Worksheet for Amplification of *Flavobacterium psychrophilum.*

Primer Sets for ***Flavobacterium psychrophilum***

	Forward	Reverse
1st round	**5'- CGA TCC TAC TTG CGT AG -3'**	**5'- GTT GGC ATC AAC ACA CT -3'**

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Appendix 7.G – PCR Worksheet for Amplification of *Yersinia ruckerii.*

Primer Sets for ***Yersinia ruckerii***

	Forward	Reverse
1st round	**5'- GCG AGG AGG AAG GGT TAA GTG -3'**	**5'- GAA GGC ACC AAG GCA TCT CTG -3'**

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Appendix 7.H – PCR Worksheet for Amplification of *Aeromonas salmonicida.*

Primer Sets for ***Aeromonas salmonicida***

	Forward	Reverse
1st round	**5'- GGC CTT TCG CGA TTG GAT GA -3'**	**5'- TCA CAG TTG ACA CGT ATT AGG CGC -3'**

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Chapter 8

PARASITOLOGY

SECTION 1 - General Parasitology

Becky Lasee
USFWS- La Crosse Fish Health Center
Onalaska, Wisconsin

SECTION 2 - Detection of Whirling Disease (*Myxobolus cerebralis*) by Pepsin-Trypsin Digest

Kimberly True
USFWS-California/Nevada Fish Health Center
Anderson, California

SECTION 3 - Diagnosis of *Bothriocephalus acheilognathi* (Asian Tapeworm) in Wild Fish Populations

Becky Lasee
USFWS-La Crosse Fish Health Center
Onalaska, Wisconsin

Appendix 7.H – PCR Worksheet for Amplification of *Aeromonas salmonicida.*

Primer Sets for ***Aeromonas salmonicida***

	Forward	Reverse
1st round	**5'- GGC CTT TCG CGA TTG GAT GA -3'**	**5'- TCA CAG TTG ACA CGT ATT AGG CGC -3'**

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Trematoda (Digenetic): flukes with flattened body; oral and ventral suckers (some exceptions); and exhibit complex life cycles involving multiple hosts.

Cestoidea (Tapeworms): worms with flattened segmented body (usually); and head (scolex) usually bears suckers, hooks or suctorial grooves, occasionally no organs of attachment.

Nematoda (Roundworms): thin, elongate worms with cylindrical body covered by a rigid cuticle; one or both ends attenuated; and no organs of attachment.

Acanthocephala (Spiny-headed worms): body cylindrical, sometimes slightly flattened; and spectacular hook-bearing eversible proboscis present.

Crustacea (parasitic): external parasites; may be louse-like (Argulus); worm-like (Lernaea); or grub-like (Salmincola, Ergasilus).

Hirudinea (Leeches): external parasites; some dorsoventrally flattened; others more cylindrical; and body segmented, with anterior and posterior suckers.

Glochidia: larval freshwater clams encapsulated in fins and gills; resemble minature clams with shells; and some armed with hooks.

II Key to Major Taxa of Adult Parasites of Fishes

(modified from Frimeth 1994 - Does not apply to larval Cestoda, Digenea or Nematoda)

1. Individual organism microscopic (except *Ichthyophthirius*) and single-celled, but may be multinuclear..... **Protozoa**
   Individual organism usually visible without a microscope and multicellular 2
2. Body worm-like..... 3
   Body not worm-like..... 8
3. Body dorsoventrally flattened, not round in cross-section 4
   Body not dorsoventrally flattened, round in cross-section 7
4. Body with segmentation or distinct external annulations or rings 5
   Body without segmentation or distinct external annulations or rings 6
5. Anterior attachment organ present which may include hooks and muscular suckers, posterior attachment organ not present; gut absent .....**Cestoda**
   Anterior and posterior attachment organs present with well defined posterior sucker, gut present .....**Hirudinea**
6. Posterior attachment organ present which usually includes hooks (hamuli) and marginal hooks; external parasites .....**Monogenea**
   Posterior attachment organ not present, possesses circumoral and ventral suckers except for blood dwelling species; internal parasites .....**Trematoda**
7. Anterior spined proboscis present ..... **Acanthocephala**
   Anterior spined proboscis not present .....**Nematoda**
8. Body in form of two hinged shells (valves); organism encysted on surface of gills or fins ..... **Glochidia**

Body not in form of two hinged valves, possesses appendages for attachment to host surface; organism not encysted, external parasite..... **Crustacea**

III. Some North American Fish Parasites Listed By Location in Host (Modified from Hoffman 1999)

A. Eggs

Fungi: Saprolegnia and relatives.

Protozoan: Carchesium (on walleye and trout eggs), Epistylis reported from catfish eggs, *Pleistophora variae* in golden shiner eggs, *P. sulci* in *Polyodon spathula* eggs, and *Thelohania baueri* in *Pungitius pungitius* eggs, former Soviet Union

B. Barbels

Protozoa: *Henneguya* sp. in *Ictalurus nebulosus*, *Ichthyophthirius* occassionally

Trematoda: *Gyrodactylus* spp.

C. Skin and Fin Surfaces

Fungi: External fungi, *Saprolegnia* and relatives, *Exophiala pisciphila*

Protozoa: Ectoparasitic protozoa (*Ambiphrya, Amphileptus, Bodomonas, Chilodonella, Colponema, Cyclochaeta, Epistylis, Ichthyoboda*, *Ichthyophthirius*, *Oodinium*, *Trichodina*, *Trichophrya*), Microsporidea, Myxosporidea, *Myxobolus squamalis* (in scales of salmonids)

Monogenea: *Gyrodactylus*, usually not other monogeneans

Trematoda: Metacercariae of many species, including *Neascus* (black spot)

Nematoda: Undescribed larval nematode in skin nodules of ictalurids (eastern half of USA)

Crustacea: *Argulus*, *Lernaea*, *Ergasilus*, *Salmincola*

D. Nares

Protozoa: *Apiosoma* sp., Europe, *Amphileptus*, *Chilodenella*, *Myxobolus*, *Tetrahymena*, *Trichodina*, *Trichodinella*

Monogenea: *Aplodiscus nasalis* in *Hypentellium etowanum*, *Cleidodiscus monticelli*, *Pellucidhaptor catostomi*, *P. nasalis*, *P. pricei*

Nematoda: *Philometra* in bluegills and largemouth black bass

Copepoda: *Ergasilus megaceros* in fallfish and catfish, *Ergasilus rhinos* in centrarchids, *Gamidactylus*, *Gaminispatulus*, *Gaminispinus*, *Lernaea*, *Paragasilus*, *Salmincola*

E. Gills

Fungi: *Dermocystidium*

Protozoa: *Ambiphrya*, *Amphileptus*, *Bodomonas*, *Chilodonella*, *Cryptobia*, *Dermocystidium*, *Epistylis*, *Ichthyoboda* (Costia), *Ichthyophthirius*, Microspora, Myxosporea, *Piscinoodinium*, *Trichodina*, *Trichophrya*

Monogenea: *Gyrodactylus*, *Dactylogyrus*, *Cleidodiscus*, and many other species

Trematoda: Many metacercariae, ova becoming miracidia of *Sanguinicola*

Copepoda: *Achtheres*, *Argulus*, *Lernaea*, *Ergasilus*, *Salmincola*, *Lepeophtheirus*

F. Mouth

Protozoa: *Apiosoma*, Europe, Myxosporea
Trematoda: *Leuceruthrus*
Nematoda: *Philometra nodulosa* in suckers and buffalo fishes
Copepoda: *Lernaea cyprinacea*, *Salmincola* (*S. lotae* in burbot)

G. Blood

Protozoa: *Trypanosoma* (*Cryptobia*), *Trypanoplasma* free, *Babesiosoma*, *Dactylosoma*, *Haemogregaerina* in red blood cells, rarely *Kudoa*, *Sphaerospora* trophozoites (Csaba bodies) free in carp and other cyprinids, Europe
Trematoda: *Sanguinicola* in blood vessels, including gill vessels
Nematoda: *Philometra sanguinea* in caudal fin blood vessels of goldfish, *P. obturans* in gill vessels of pike, former Soviet Union

H. Esophagus

Trematoda: *Azygia*, *Cotylaspis*, *Derogenes*, *Halipegus*, *Proterometra*

I. Stomach

Protozoa: *Schizamoeba* spp.
Monogenea: *Enterogyrus* spp. (freshwater, tropical), *Enterogyrus* sp. in foregut of *Pomacanthus paru*
Trematoda: *Allocreadium*, *Aponeurus*, *Azygia*, *Caecincola*, *Centrovarium*, *Derogenes*, *Genolinea*, *Hemiurus*, *Leuceruthrus*
Nematoda: *Haplonema*

J. Intestine and Pyloric Ceca

Protozoa: *Hexamita*, *Schizamoeba*, *Eimeria*
Trematoda: *Allocreadium*, *Crepidostomum*, *Lissorchis*, *Alloglossidium*, additional adults of many species
Cestoda: *Proteocephalus*, *Bothriocephalus*, *Eubothrium*, additional adults of many species
Nematoda: *Contracaecum*, *Camallanus*, *Spinitectus*, additional adults of many species
Acanthocephala: *Neoechinorhynchus*, *Echinorhynchus*, *Pomphorhynchus*, additional adults of many species

K. Swim Bladder

Trematoda: *Acetodextra* in *Ictalurus punctatus*
Nematoda: *Cystidicola* spp. in salmonids, *Huffmanella huffmanella* in *Lepomis cyanellus*

L. Body Cavity: Mesenteries, Liver, Spleen

Fungi: *Ochroconis humicola* in coho salmon; *O. tshawytscha* in chinook salmon, *Phoma herbarum* in northwest salmonids

Protozoa: Many Myxosporea, rarely Microsporea, *Goussia* spp.
Trematoda: Many metacercarial species including *Ornithodiplostomum*, white grub (*Posthodiplostomum*), adult *Paurorhynchus* (bucephalid), adult *Acetodextra* in catfish
Cestoda: Larval *Diphyllobothrium*, *Haplobothrium*, *Ligula*, *Proteocephalus*, *Schistocephalus*, *Triaenophorus*
Acanthocephala: Larvae of *Echinorhynchus salmonis*, *Leptorhynchoides thecatus*, *Pomphorhynchus bulbocolli*
Nematoda: Adult *Philonema*, many larval species
Copepoda: Rarely *Lernaea* in small fishes

M. Gall Bladder

Protozoa: Myxosporea, *Hexamita*
Trematoda: *Crepidostomum cooperi*, *C. farionis*, *Derogenes* sp., *Plagioporus sinitsini*, *Prosthenhystera* sp., *Pseudochaetosoma*, Europe
Cestoda: *Eubothrium salvelini*, larval Dilepidae (armed pleurocercoids)
Nematoda: *Rhabdochona* sp., occasionally *Capillaria catostomi*

N. Hepatic Bile Duct

Trematoda: *Phyllodistomum* spp.

O. Kidneys

Fungi: *Ichthyophonus hoferi* in many fishes, *Ochroconis* spp. in West Coast coho and chinook salmon
Protozoa: Myxosporea in tubules, sometimes interstitial, sometimes cysts
Trematoda: Metacercariae of *Nanophyetus salmincola*, *Posthodiplostomum minimum centrarchi*, probably others, adult *Phyllodistomum* in renal tubules and ureters

P. Urinary Bladder

Protozoa: Myxosporea, *Vauchomia* (trichodinid) in *Esox* spp.
Monogenea: *Acolpenteron*
Trematoda: *Phyllodistomum*

Q. Ovaries

Protozoa: *Henneguya oviperda* in *Esox lucius*, Europe, *Pleistophora ovariae* in golden shiners, *Thelohania baueri* in *Gasterosteus aculeatus* ova, former Soviet Union
Trematoda: *Acetodextra ameiuri*
Nematoda: *Philonema* spp. in salmonids
Cestoda: *Proteocephalus*

R. Testes

Protozoa: *Hexamita*
Cestoda: *Proteocephalus*

S. Eyes

Protozoa: *Henneguya episclera* in *Lepomis gibbosus*, *H. zikaweiensis* in *Carassius auratus*, China, *Myxobolus corneus* in cornea of *Lepomis macrochirus*, *M. hoffmani* in sclera of *Pimephales promelas*

Digenea: *Diplostomum spathaceum* in lens, *Diplostomulum scheuringi* in vitreous humor and other species of *Diplostomulum*

Nematoda: *Philometroides* sp. in eye orbit of southeastern centrarchids

T. Cartilage

Protozoa: *Henneguya brachyura* in fin ray of *Notropis* spp., *H. schizura* in sclera of *Esox lucius*, *Henneguya* sp. in branchial arch of *Pomoxis* spp., *M. cartilaginis* in centrarchids, *Myxobolus cerebralis* in salmonids, *M. hoffmani* in sclera of eye of *Pimephales promelas*, *M. scleropercae* in cartilaginous sclera of eye of perch and additional species

U. Nervous System

Fungi: *Ichthyophonus hoferi* in brain

Protozoa: *Mesencephalicus* in brain of *Cyprinus carpio* (Europe), *Myxobolus cerebralis* affects CNS (although parasite is in cartilage), *M. hendricksoni* in brain of *Pimephales promelas*, *M. arcticus*, *M. kisutchi*, *M. neurobius* in CNS of salmonids

Monogenea: Some species in lateral line pits

Trematoda: *Diplostomulum*, *Euhaplorchis*, *Ornithodiplostomulum*, *Parastictodora*, metacercariae on brain, *Psilostomum* metacercariae in lateral line canal.

V. Muscle and Connective Tissue

Protozoa: *Myxobolus insidiosus* in muscle of cutthroat trout, chinook and coho salmon, *Heterosporis* in muscle of yellow perch, and many Myxosporea and Microsporea

Trematoda: Many metacercarial species including yellow grub (*Clinostomum*), and black spot (*Neascus* spp.)

Cestoda: Larval *Diphyllobothrium*, *Triaenophorus*

Acanthocephala: Larval forms

Nematoda: Larval *Eustrongylides*

IV. General Methods

(Brown 1980; Frimeth 1994; Dailey 1996; Hoffman 1999)

Following collection, fish should be held in well-aerated containers at the appropriate temperature. Care should be taken to handle fish humanely and euthanize with approved anesthetic prior to examination. After euthanasia, fish can be examined in the field (if microscopes are available) or transported, on ice, to the laboratory.

A. Necropsy Procedures

See Appendix 8.B for pictures and diagrams of some parasites described in Section 1 of this chapter.

1. Examine skin, fin and gills for larger parasites that can be seen with the naked eye (e.g., *Ichthyophthirius*, larger monogeneans, leeches, etc.). If the fish is small, it can be placed into a petri dish with water or normal physiological saline and examined using a dissecting microscope. The fins and gills can be removed from larger fish and examined similarly. If a microscope is not available, a hand held lens can be used for external examinations

2. Prepare a mucus wet mount by scraping the dorsolateral surface of the fish with the dull side of a scalpel blade. Transfer the mucus to a clean microscope slide, add a drop of saline and cover with a cover slip. Remove several fins and prepare a wetmount. Examine preparations for smaller external protozoa using 100 and 450X magnification of a compound microscope.

3. Remove the operculum of the fish with a scissor. If the fish is small, remove the entire gill arch and transfer to a slide. Add saline and cover slip. With larger fish, it is necessary to remove the bony arch before preparing the gill wetmount or use a few filament tips removed with a scissor. Examine the gills with 100X of a compound microscope looking for external protozoans, cysts (which may be *Ichthyophthirius*, trematode metacercariae, Microsporea or Myxosporea) and monogenetic flukes.

4. Open the fish. Examine the body cavity for encysted parasites. Remove a small amount of blood from the heart. Dilute 1:1 with saline and examine at 100X for *Trypanoplasma* and *Trypansoma*. Blood smears can also be prepared at this time and later stained for blood sporozoa.

5. Remove the viscera *in toto* from fish. For small fish this should be done in saline with the use of a dissecting microscope. Tease apart the organs with fine forceps or dissecting needles. Remove a drop or two of fluids and mucus from the intestinal tract and transfer to a slide. Add a coverslip and examine for protozoa (*Hexamita*, *Schizamoeba* and *Eimeria*). Open the intestinal tract the entire length and examine with a dissecting microscope for helminths. Examination of the intestinal tract may also be facilitated by compressing a longer section of the intestine between two glass slides and examining with a compound microscope.

Individual organs of larger fish can be removed, transferred to saline in a Petri dish and examined for larval parasites. Squashes of kidney, liver, spleen and gonads can be prepared. Remove the swim bladder, being careful not to deflate it. Examine with a dissecting microscope for nematodes and trematodes. Stretch out the entire intestinal tract and cut open longitudinally with a small fine point dissecting scissors (subdivide the tract if it is too large).

The tract may be flushed with saline and gut contents examined separately from the tract wall. Keep in mind, that most parasites will be small and transparent.

6. Carefully remove the gall bladder with fine forceps, and examine for trematodes. Prepare a wetmount and examine for sporozoans and ciliates. Likewise, remove the urinary bladder and examine for *Phyllodistomum* and sporozoans.

7. Remove each eye using a forceps and scissor. Place into a petri dish with saline. Use low power of a dissecting microscope to observe any movement of digenetic unencysted larval flukes (*Diplostomulum*). Cut open the eye and examine the lens for the eye fluke *Diplostomum spathaceum*.

8. Cut the head lengthwise and remove the brain. Squash some of the brain onto a slide, add a drop of saline and coverslip. Examine for Myxosporea and trematode metacercariae (e.g., *Ornithodisplostomum*).

9. The musculature can be examined by slicing the epaxial musculature at regular intervals and looking for larval worms, sometime encysted, and Microsporea and Myxosporidea cysts. Some can only be seen with higher magnification.

10. Concentration methods for myxosporidans are sometimes required, such as for detection of *Myxobolus cerebralis --see* Section 2: Detection of Whirling Disease (*Myxobolus cerebralis*) by Pepsin-Trypsin Digest (PTD) (Modified from Dr. Rich Holt, Oregon Department of Fish and Wildlife (1987) and excerpted from the Alaska Department of Fish and Game, Fish Pathology Section Laboratory Manual, Meyers, ed. 1997.).

    See also Whirling Disease Foundation-AFS Standard Protocols for Whirling Disease Research (version 1.0 - May 2001) and Markiw and Wolf (1974) for digestion techniques, and O'Grodnick (1975) for the plankton centrifuge method.

B. Fixation of Parasites

The most commonly used fixative for preserving and storing parasites include alcohol-formol-acetic (AFA or Davidson's Fixative), Bouin's, formalin and glycerine alcohol (Humason 1979). Formalin is probably the most commonly used and preferred fixative. Preservation in cold fixatives is not recommended because most parasites will contract and make identifications difficult or impossible. For a list of reagents used in the following procedures for parasites fixing and examination see Appendix 8.A.

Prior to fixation, worms should be thoroughly washed in saline and cleansed of mucus.

1. Protozoa

   a. For Myxosporea: cut out the cyst with enough adjacent tissue and place in 10% formalin.

   b. For trophozoites of motile forms: place as many protozoa as possible on a clean microscope slide, add one drop of Low Viscosity-Polyvinyl Alcohol (LV-PVA) or Zinc Sulfite-Polyvinyl Alcohol (Zn-PVA) fixative adhesive, mix, spread over slide and allow to dry. Also, protozoans can be transferred to a vial of 10% formalin (keep in mind they will usually shrink).

2. Monogeneans/Trematodes
   Trematodes should be transferred to a small glass petri or stender dish. Remove excess saline or water. Heat 10% formalin or AFA to 85-90°C (begins to steam but not boil) in a fume hood. Add hot fixative to dish containing trematodes. For thicker worms, flatten under a coverslip and flood with warm fixative. For monogenea, drop infected gills into 10% formalin. Larger monogenes can be removed and fixed under light coverslip pressure.

3. Cestodes
   Procedures are similar to that of trematodes; kill in 80°C water or formalin and store in buffered 10% formalin.

4. Nematodes
   Kill in warm (80°C) glycerine alcohol (1 part glycerine:3 parts 95% ethanol) and transfer to cold glycerine alcohol for storage.

5. Acanthocephala
   For acanthocephalans it is necessary to evert the proboscis prior to fixation. Place worms in distilled water and refrigerate overnight. Transfer to warm 10% formalin or AFA.

6. Leeches
   Fix in warm 10% formalin, or if very thick, flatten between two slides and flood with 10% formalin.

7. Copepods
   Remove copepods, if possible, and drop into glycerin alcohol or 70% ethanol. If not easily detached, cut out a small piece of tissue containing parasite and place in 10% formalin or 70% ethanol.

C. Preparation of Parasite Whole Mounts

Only two whole mount techniques will be presented here: Semichon's Acetocarmine Technique for monogoneans, trematodes, cestodes and acanthocephalans and Glycerin Jelly Technique for whole mounts of small nematodes and microcrustacea. More comprehensive staining and whole mount procedures can be found in parasitology and histology laboratory manuals (Luna 1968; Cable 1977; Humason 1979; Dailey 1996).

Staining should be done using glass dishes with ground covers (stender preferred). Screw-cap vials may also be used. Always use at least four times the volume of reagent to the bulk of specimen. Containers should always be covered, except when changing solutions or observing specimens. Care must be taken when transferring specimens from one liquid to another. Several methods can be used: 1) pour off the first liquid and then add second liquid immediately (never allow the specimens to dry out); 2) if the specimens are small and delicate, transfer with a bulbed pipette using a dissecting microscope or 3) most specimens can be successfully transferred to a second liquid by lifting them gently with a camel hair brush. A small slip of paper labeled in pencil can be used to identify specimens through staining process.

The fixative should be thoroughly removed from specimens prior to staining. Wash out formalin in distilled water and AFA in 70% alcohol.

1. Semichon's Acetocarmine Staining Technique (AFA Fixative preferred) To stain, cover specimens with Semichon's aceto-carmine stain and allow to stand overnight.

    a. Remove stain and save (it can be reused). Wash off excess stain in two changes of 70% ethanol (5-10 minutes for each change).

    b. Destain using 70% acid alcohol. Observe specimens using a dissecting microscope and destain until parenchyma and muscles are nearly free from stain (light pink) while internal organs are well stained (red).

    c. Stop destaining by replacing or pouring off acid alcohol with two changes of basic alcohol (5-10 minutes each change).

    d. Dehydrate in 95% ethanol for 15 minutes.

    e. Counterstain with dilute fast green in 95% ethanol. Observe specimens with dissecting microscope using care not to over stain (until very light bluish green).

    f. Dehydrate in two changes of 100% ethanol for 10-15 minutes each.

    g. Clear in methyl salicylate (or xylene replacement) for at least 10 minutes. Specimens are ready for mounting when they sink.

    h. After clearing is complete, transfer specimen to a microscope slide using a clean

camel hair brush. Center specimen and orient correctly (ventral side up and anterior end towards bottom of slide).

i. Add a coverslip and add sufficient mounting medium (Permount™) to fill space under the coverslip (try to avoid introducing any bubbles). As the mount dries, additional Permount may need to be added.

j. When completely dry, excess Permount may be cleaned from the slide using a razor blade.

k. Label slides and store in a horizontal position.

2. Glycerin Jelly Technique (Double coverslip method, Figure 1)

a. Transfer specimens to glycerin alcohol if they were not fixed in hot glycerin alcohol.

b. Place specimens in stender staining dishes containing glycerin alcohol. Cover dish with filter paper or a Kimwipe to allow slow evaporation of the alcohol in the glycerin alcohol. The specimens are ready to mount when all the alcohol has evaporated and there is only glycerin jelly remaining in the dish. This may take one to several days.

c. Heat glycerin jelly mounting medium in a water bath until it liquefies. Do not allow jelly to exceed 60°C as it will caramelize and become brown. Glycerin jelly will solidify at room temperature, so it will be necessary to use a warmed pipette to transfer the molten glycerin jelly to your coverslip.

d. Add a small drop of liquefied glycerin jelly to the center of a large coverslip (22 or 25 mm diameter). Use a camel-hair brush to transfer specimen to the drop of jelly. Place the coverslip on a slide warming table to prevent the jelly from hardening.

e. Add a smaller diameter coverslip (18 mm) to cover the specimen. The glycerin jelly should completely fill the space covered by the smaller coverslip. If not, add a small drop of glycerin jelly.

f. Allow the glycerin jelly mount to harden by removing preparation from warming table.

g. Clean off any excess glycerin jelly from around the rim of the larger coverslip using a razor blade and a cotton applicator stick dipped in 70% ethanol.

h. Place a drop of Permount onto the center of a clean microscope slide. Center the mount with the larger coverslip up on top of the drop of Permount. Add sufficient Permount to the slide to fill the remaining space created by the overhanging larger

coverslip. Allow Permount to harden.

i. Label slide.

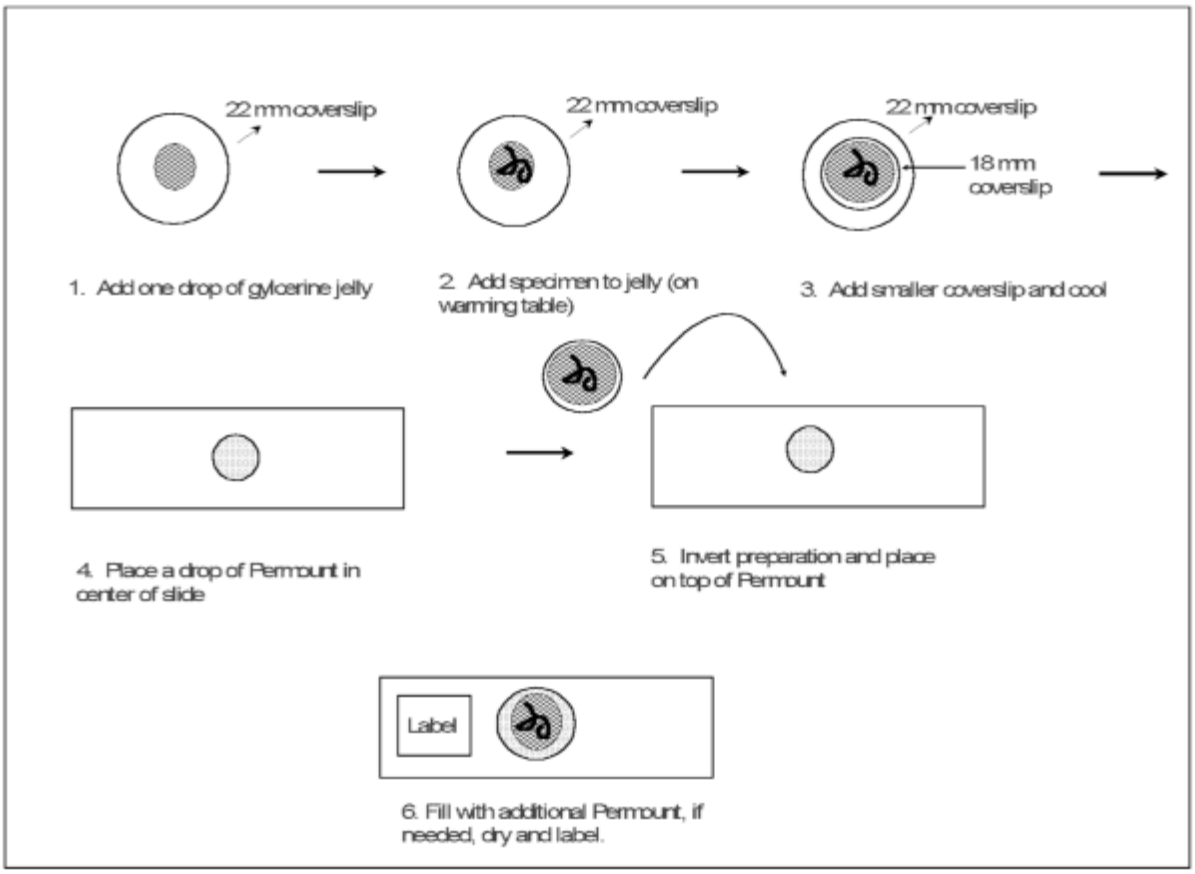

Figure 1. **Double coverslip method for Glycerine Jelly Mounts.**

V. Keys for Identification of Parasites

Taxonomic keys for presumptive identifications of parasites can be found in Hoffman (1999). In most cases, definitive identifications will require more extensive review of primary and secondary literature. When in doubt, samples (slides or preserved) can be sent to a parasitologist for confirmatory identification.

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Appendix 8.A – Reagents and Solutions

Acid Alcohol

Ethanol, 70%	98 mL
Hydrochloric acid	2 mL

AFA (Alcohol-formol-acetic) Fixative

Ethanol, 85%	85 mL
Formalin	10 mL
Acetic acid, glacial	5 mL

Glycerine Alcohol

Ethanol, 70%	95 mL
Glycerine	5 mL

Alkaline (Basic) Alcohol

Ethanol, 70%	500 mL
Ammonium hydroxide, concentrated	0.5 mL

Bouin's (picro-formol-acetic) Fixative

Picric acid, saturated aqueous solution	75 mL
Formalin, commercial	25 mL
Acetic acid, glacial	5 mL

Specimens are usually left in it for about 24 hours before being transferred to 70% ethanol.

Fast Green, Stock

Fast green, powdered	0.2 gm
Ethanol, 95%	100 mL

Formalin

Commercial formalin is treated as 100% (although it is a 40% solution of formaldehyde gas in water). Thus 5% formalin, which is 2% formaldehyde, contains

Water, distilled	95 mL
Formalin	5 mL

Glycerine Jelly

Soak 7 gm of granulated gelatin in 40 mL of distilled water for 30 minutes. Then melt in a warm water bath and filter through several layers of cheesecloth previously moistened with hot water. Dissolve 1 gm phenol in 50 mL of glycerin and add to the gelatin. Stir until the mixture is homogeneous.

Low Viscosity-Polyvinyl Alcohol (LV-PVA)

Fixative adhesive that is commercially available.

Saline Solution

Sodium chloride	7 gm
Water, distilled	1000 mL

Semichon's Acetocarmine

Acetic acid, glacial	100 mL
Water, distilled	100 mL
Carmine "in excess"	1.5 gm (about)

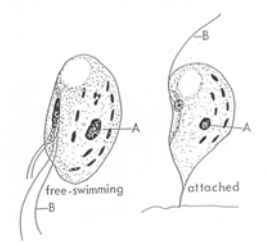
Mix distilled water and acetic acid in an Erlenmeyer flask and add carmine. The objective is to prepare a saturated solution of carmine; however, do not add more than will go into solution. Heat in boiling water bath for 15 minutes, then cool flask in cold water and filter the contents. This stock stain should be diluted with approximately two parts of 70% ethanol before use.

Zinc Sulfite-Polyvinyl Alcohol (Zn-PVA)

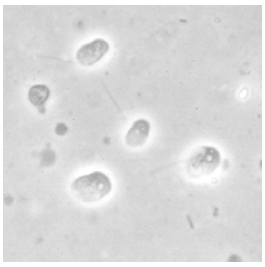
Fixative adhesive that is commercially available.

Appendix 8.B

EXTERNAL PROTOZOANS



Free-swimming and attached form of *Ichthyoboda.* (from Rogers 1994)



Ichthyoboda in skin wetmount. (from GL Hoffman)

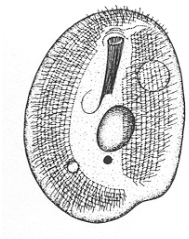
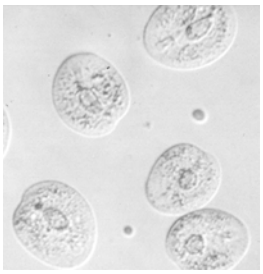


Diagram of *Chilodonella*. (from Lom 1995)



Chilodonella from skin. (from GL Hoffman)

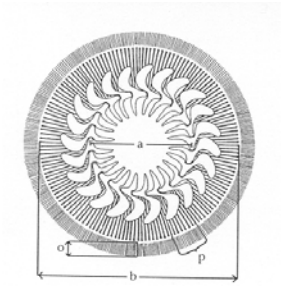
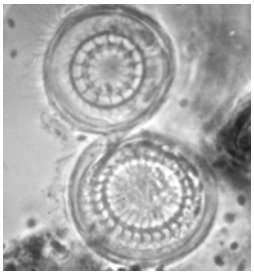
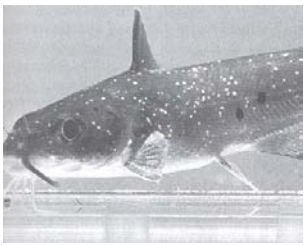


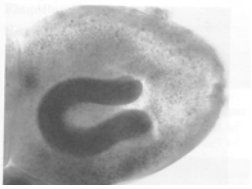
Diagram of *Trichodina*. (from Lom 1995)



Trichodina from the skin.



Channel catfish infected with *Ichthyopthirius.* (from Lom 1995)



Stained *Ichthyopthirius* trophont showing horse-shaped macronucleus.

MYXOSPOREANS

Spores of *Henneguya*.

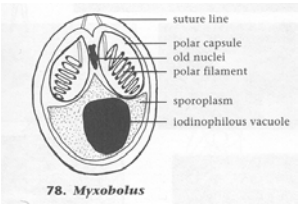
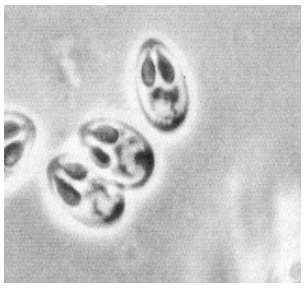
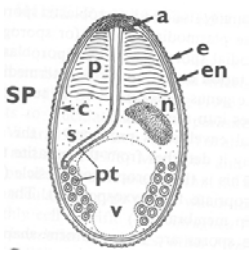


Diagram of *Myxobolus* spore. (from Hoffman 1999)

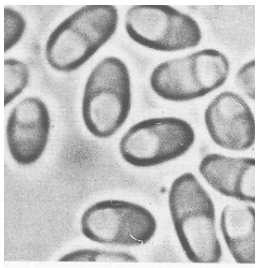


Top and bottom photos: two different *Myxobolus* spores.

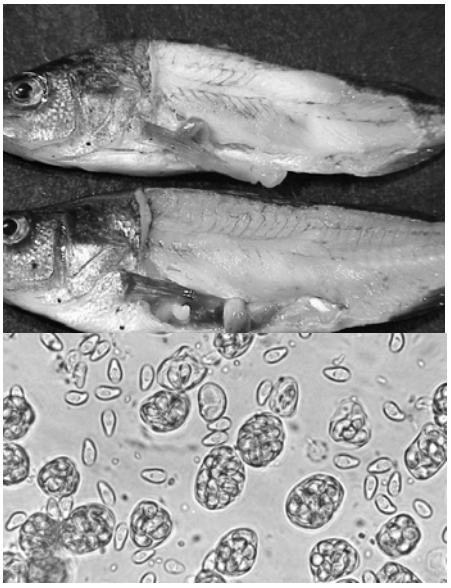
MICROSPORANS



Microspora spore. (from Dykova 1995)

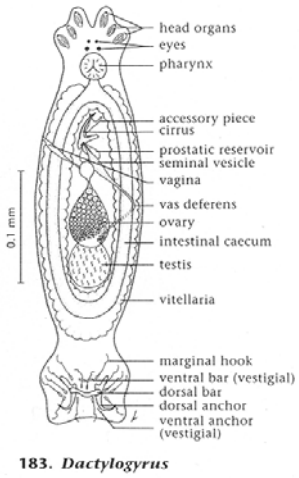


Spores of *Glugea*. (from Woo 1995)

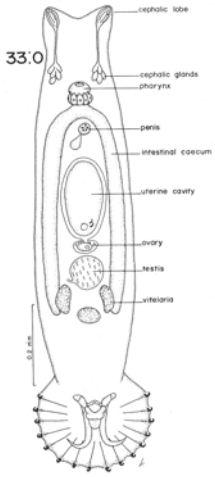


Heterosporis. Top photo: large white cysts in musculature o f yellow perch (infected fish above, uninfected below). Bottom photo: spores and vesicles. (from D.R. Sutherland)

MONOGENES



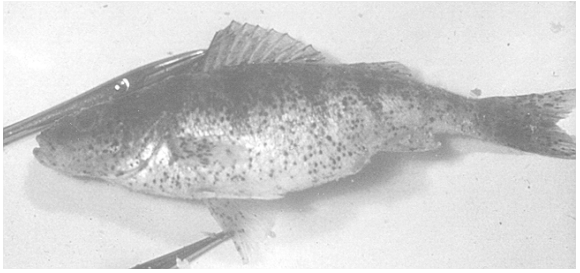
Line drawing of *Dactylogyrus*. (from Hoffman 1999)



Line drawing of *Gyrodactylus*. (from Hoffman 1999)

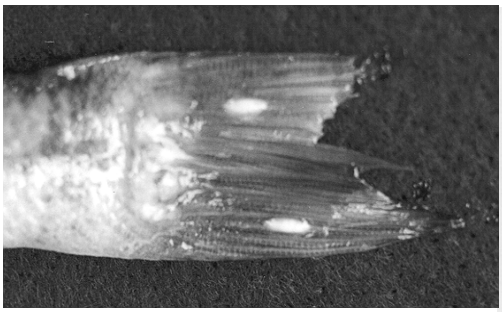
Gyrodactylus in skin wetmount. (from A.J. Mitchell)

DIGENETIC TREMATODES



Yellow perch infected with metacercariae of black grubs (*Neascus*). (from Michigan DNR)

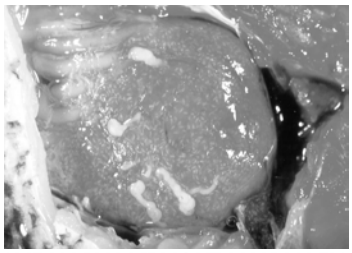
Metacercarial cysts of white grub (*Posthodiplostomum*).

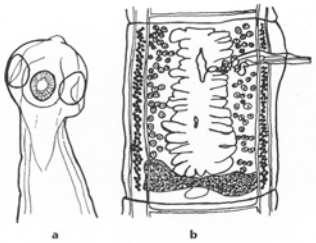


Yellow grub (*Clinostomum*). Top photo: metacercarial cysts on caudal fin. Photo on right: acetocarmine stained wholemount (w.m.).

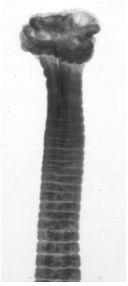
Adult *Allocreadium* (stained w.m.).

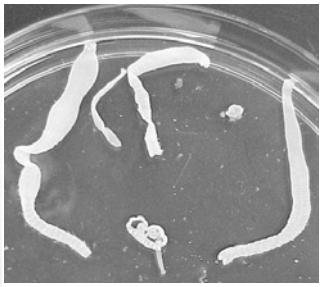
Adult *Alloglossium* (stained w.m.).



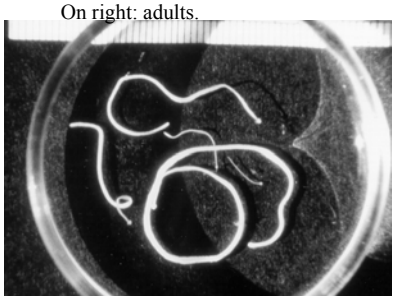


Proteocephalus or Bass tapeworm. Top photo: larval or metacestodes on the liver of bass. Bottom: diagram of the adult, scolex and proglottid. (from Hoffman 1999)





Corallobothrium (Catfish tapeworm) adults. On left: stained wholemount (note fleshy appendages surrounding suckers). On right: adults.



Bothriocephalus acheilognathi or Asian Tapeworm. Note arrow-shaped scolices.

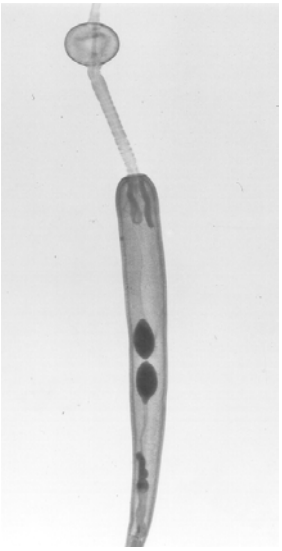
NEMATODES

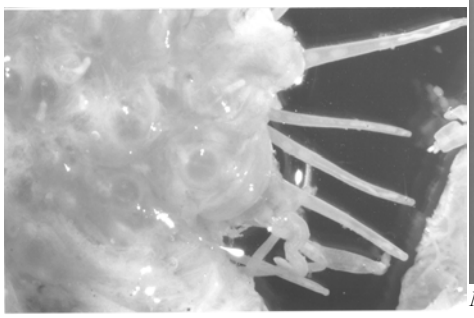
Cystidicola (swim bladder nematode) from a bloater chub.

Capillaria from intestine of lake trout.

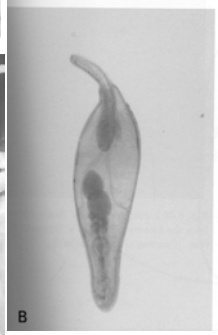
Philometra in fascia of cheeks of a white sucker. (from Ribelin and Migaki 1975)

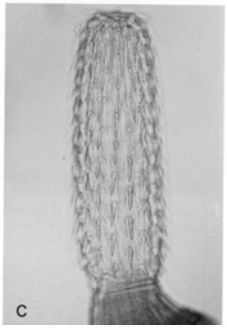
ACANTHOCEPHALANS





Pomphorhynchus bulbocolli. Left: wholemount, acetocarmine stained (note large bulb below proboscis). Top: Live worms, *in situ*. Bottom: Attached worms with bulb protruding through intestinal wall.

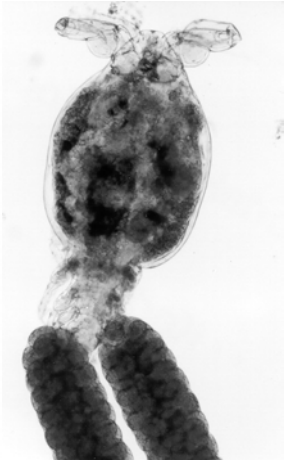




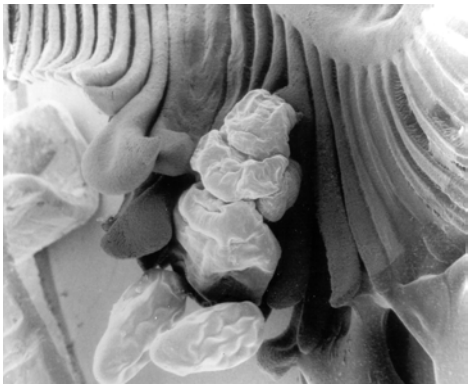
Metechinorhynchus salmonis. A: adults, *in situ*. B: wholemount, acetocarmine stained. C: proboscis armed with spines.

CRUSTACEANS

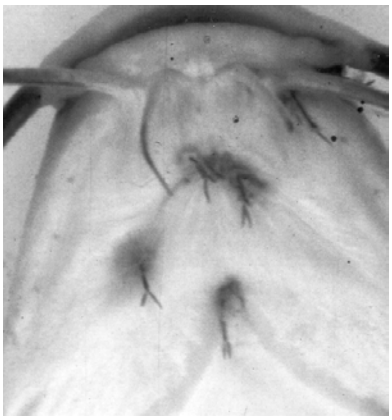
The fish louse, *Argulus* (acetocarmine stained).



Ergasilus from the gills of a deepwater sculpin.



Scanning electron micrograph of *Salmincola*, the gill maggot.



The anchor worm, *Lernaea*. Top: adults attached to ventral surface of a channel catfish. Note egg sacs of copepods. Right: Stained wholemount preparation.

Section 2 – Detection of *Myxobolus cerebralis* (The Causative Agent of Whirling Disease) by Pepsin-Trypsin Digest (PTD)

I. Sample Collection

Samples are collected for preliminary testing of *Myxobolus cerebralis* by the Pepsin-Trypsin Digest method. If samples test positive for Myxobolus spores, additional corroborative testing will be performed on the digested spore preparation by Mc-PCR or the archived half of the head, either by histology or Mc-PCR. The first step is to divide individual fish tissues for Pepsin-Trypsin Digest (PTD) and Archive (ARC) sample sets. Caution should be used in sample collection with forethought to the corroborative method to be used and to prevent cross-contamination of Mc-DNA. Disinfect tools with 10% chlorine (followed by a di$H_2$0 rinse) between sample pools as necessary.

A. For Small Fish – Less Than 6 Inches

1. Remove head including gill arches, using a sterile scalpel for each set of 5 heads. Cut the heads longitudinally from the snout to the operculum.

2. Place one half of the 5 heads in a whirl-pak™ bag labeled with fish ID and "PTD".

3. Place the second set of 5 halved heads in another whirl-pak bag labeled with fish ID and "ARC" for corroborative testing by histology or Mc-PCR.

4. Keep samples cold or on ice during collection. Place both sets of samples in a –20 or -70°C freezer upon return to the laboratory.

B. For Larger Fish – Greater Than 6 Inches

It may be necessary to excise the target tissue and then halve the material into the separate bags labeled for PTD and ARC.

1. Use a large biopsy punch (12-18mm for fish 6-12 inches) or a coring tool for adult fish.

2. Remove the tissue punch, or core, and cut it in half using a scalpel.

3. Place one half of tissue from 5 heads in a whirl-pak bag labeled with fish ID and "PTD".

4. Place the second set of 5 halved tissues in another whirl-pak bag labeled with fish ID and "ARC" for corroborative testing by histology or Mc-PCR.

5. Keep the PTD samples cold and the Archive samples frozen. Place the archive in a –70C freezer upon return to the laboratory.

C. Histology

If histology will be used for corroborative testing, follow the above procedure except place the second set of halved head tissue in Davidson's or Bouin's fixative and label as Archive. Tissues are transferred to alcohol at 24 hours, however heads do not need to be processed unless a positive PTD test occurs.

II. Preliminary Detection Procedure - Pepsin-Trypsin Digest (PTD)

SOLUTIONS: **0.5% Pepsin**: To 1 L water add 5.0 g pepsin and 5 mL concentrated hydrochloric acid.

0.5% Trypsin (in Rinaldini's Solution):
Make a 1-L solution of distilled water containing

NaCl (sodium chloride)	8.0 g
KCl (potassium chloride)	0.2 g
$HOC(COONa)(CH_2\ COONa)_2 \bullet 2H_2O$ (sodium citrate)	1.0 g
NaH_2PO_4 (sodium phosphate)	0.05 g
$NaHCO_3$ (sodium bicarbonate)	1.0 g
Glucose	1.0 g
Phenol Red (0.5%)	1.0 mL
Trypsin	5.0 g

A. Thaw the frozen samples overnight in the refrigerator. Frozen samples can also be thawed the day of the assay, but will require more time to process in step B.

B. To deflesh the soft tissue, soak the heads in 60°C water for 35 minutes (smaller fish) and up to 60 minutes (larger fish).

C. Remove soft tissue from bone and cartilage by rinsing heads in a fine mesh strainer, using water pressure and a rubber spatula to dislodge all soft tissue (skin, eyes, lower jaw, muscle). Discard all wash solution and soft tissues into 10% chlorine solution.

Note: For Larger heads, it may be easier to dissect bone and cartilage directly from the soft tissue using forceps.

D. Collect all bone, cartilage and gill elements in tarred aluminum foil, a tarred electric blender, or a tarred beaker. Record the weight of the sample on the datasheet.

Note: This is a good stopping point for the assay if unable to complete in one day. Store the tissue preparations in the refrigerator overnight, or frozen for long-term storage. Continue the assay the next day by allowing the samples to reach room temperature before proceeding with next step.

E. Add 20 mL of pepsin solution per gram of tissue. Blend in electric blender for 2-3 minutes. (If the blender cannot hold the entire volume of pepsin needed, add a small

volume, blend the sample, then add the remainder of pepsin to the sample after it is transferred to a beaker in step F below).

F. Transfer the tissue and pepsin into a beaker and stir for at least 30 minutes at 37°C.

G. Pour the digest into a pre-weighed 50 mL conical tube. Centrifuge at 1200 x g for 10 minutes. Discard supernatant into 10% concentrated bleach.

H. Weigh the 50 mL tube and tissue, subtract tube weight and record tissue weight on the datasheet.

Optional: Check digest material for spores using a wet mount. If negative, proceed to next step.

I. Add 20 mL trypsin solution per gram of tissue in the centrifuge tube. Shake tube, and then pour back into original beaker used in step F.

J. Adjust pH to 8.5 with 1 N NaOH. Digest at room temperature for minimum of 30 minutes. (Be sure to sanitize pH probe after each use).

K. Pour digested sample through cheesecloth or non-adsorbent disposable filter (mesh size approximately 200um). Retain filtered fluid. Discard cheesecloth or filters in biobag and autoclave.

L. Centrifuge fluid in original tube (step G) at 1200 x g for 10 minutes.

M. Re-suspend pellet in 0.5-1mL of sterile di$H_2$0, or PBS. If samples cannot be examined immediately, freeze the preparations to prevent growth of bacteria and/or fungi.

Note: Formalin inhibits the detection of Mc by PCR. Therefore DO NOT USE FORMALIN as a preservative if PCR will be utilized for corroborative testing of the digest material.

Researchers have also found better extraction of DNA when PTD spore preparations are stored in water, or frozen when compared to samples stored in ethanol (John Wood, Pisces Molecular, LLC., personal communication).

Therefore, if corroborative testing of digest samples will be performed with Mc-PCR, samples can be frozen (-20°C or -70°C). Thaw samples and process the digest material through the DNA extraction step for Mc-PCR (see Chapter 9 – Section IV for DNA Extraction from PTD preparations). Freezing will not affect the quality or quantity of DNA provided samples are not frozen and thawed repeatedly.

N. Thoroughly mix the sample and load a hemacytometer using a pasteur pipet. Examine the pellet suspension for spores, using the 40x objective.

O. A minimum of 150 fields should be examined at 200x magnification. Observe for spores with appropriate morphology (slightly ovoid, lenticular in lateral view, 2 polar capsules with a sporoplasm, a clearly visible suture line with no sutural ridge, no valve extensions or processes, 8-12µm in size).

P. Optional: Calculate spores per mL using the following equations:

Cells per mL = the average count per each square **x** dilution factor **x** 10^4

Total cell number = cells per mL x the original volume of fluid from which cell sample was removed.

III. Identification of *Myxobolus cerebralis*

Myxobolus cerebralis can easily be confused with its congeners in the genus *Myxobolus*; therefore, the following should facilitate identification. In wet mount and, in some cases, stained preparations from digested specimens, it is difficult to distinguish these species. A combination of several species may be detected in one sample.

Myxobolus cerebralis: ovoidal front, lenticular profile; 2 pyriform polar capsules at anterior end. Sporoplasm without iodinophilous vacuole; therefore, no stain is taken up by the sporoplasm and the entire spore stains the same color using Lugols iodine. Vacuole staining is not a very accurate method due to variation within the species. Some spores have unusual processes but generally are 2/3 the size of *Myxobolus kisutchi*. *M. cerebralis* is about 8-10 µm and is histozoic in cartilage/bone tissue, primarily in the head, but also in the spinal column. Capsules are about 2/5 of spore length. Common hosts of *M. cerebralis* include *Oncorhynchus nerka*, *O. clarki*, *O. mykiss*, *O. aquabonita, Salmo salar, Salvelinus fontinalis*, and *O. tshawytscha*. Refractory hosts include *O. kisutch* and *S. trutta, Salvelinus namaycush* and splake.

Myxobolus kisutchi: ovoidal with 2 polar capsules at anterior end. Sporoplasm with iodinophilous vacuole. Sometimes with posterior prolongation of shell. It is about 7-8.5 µm and is histozoic in or adjacent to nervous tissues. Hosts include *O. kisutch*, *O. tshawytscha*, and *O. mykiss*. *Myxobolus insidiosus* spores are pyriform or tear-drop shaped and the long axis is longer than *M. kisutchi*. The iodinophilous vacuole stains dark orange if the spore is young and the storage area has not been used up. Giemsa stains vacuoles very well. Skeletal muscle is infected and white patches on the skin have been associated with heavy infections.

Other species of Myxosporidia that may confuse diagnosis of *M. cerebralis* in salmonids include:

Myxosoma dermatobia: in *O. kisutch*: spores 8-10 µm in size. In skin under epithelium or scales. Narrow ends of polar capsules widely apart. Produces ulcers.

Myxosoma squamalis: in *O. kisutch, O. mykiss*. Found in scale pockets. Scales are raised, giving the appearance of warts. Fixed spores are 8-9

µm in diameter, uniform and have equal polar capsules with a narrow ridge paralleling either side of suture ridge.

Myxobolus neurobius: in *O. kisutch*, *O. nerka, Thymallus arcticus, S. trutta*, *S. alpinus, Salmo salar*. In spinal cord, brain, and nerves. Fixed spores pyriform 8 x 10-12 µm. Polar capsules occupy less than half of the spore length. Fresh spores are larger (8-14 µm).

Myxobolus arcticus: in central nervous system of *O. kisutch, O. nerka, S. malma, S. neiva* (Russian char), *T. arcticus, S. alpinis,* and *Coregonus clupeaformis.* Fresh spores are large, 7.5 x 14-16 µm, with elongated polar capsules.

M. insidiosus: in muscle of *O. clarki, O. tshawytscha,* and *O. kisutch* of Western U. S. Fresh spores are similar in size and shape to *M. arcticus* (9-11 x 12-17 µm).

IV. Corroborative Testing for *Myxobolus cerebralis*

PCR technique may be superior to other detection and corroborative methods due to the specificity and extreme sensitivity of this molecular tool. The Survey offers an excellent opportunity to utilize the PCR method and evaluate this tool alongside the standard detection methods, such as PTD, and confirmatory methods such as histology.

A. Polymerase Chain Reaction (PCR) Testing of Archive Tissue

To utilize PCR as a corroborative method, heads are halved during the sample collection process. One set of halved heads is used for preliminary testing for Mc spores. The second set of halved heads is archived for corroborative testing by PCR (frozen) or histology (preserved in fixative). If the Digest sample set tests positive for Myxobolus spores, the corresponding samples from the frozen archive set can be used to confirm the identification of the spores as *Myxobolus cerebralis* by PCR. Drs. Ron Hedrick and Karl Andree recommend using the archived head tissue for PCR corroborative testing rather than the digested spore material.

B. PCR of Pepsin-Trypsin Digest (PTD) Spore Preparations

An alternative method for corroborative testing is to utilize the spore preparation obtained from the Pepsin Trypsin Digest method (Baldwin 2002). The PTD spore preparation must be centrifuged to concentrate the spore material, then heated in a microwave oven to disrupt the spores and release the Mc DNA. See Chapter 9 - Corroborative Testing of Parasites by PCR, for procedures for each method.

C. Confirmatory Identification by Histology

Confirmatory diagnosis can be accomplished by histology: Fresh or frozen heads are fixed in Bouin's or Davidson's. These fixatives are preferred over 10% neutral buffered

formalin because the acetic acid assists in decalcification. Decalcify as specified below, then paraffin embed, section and stain (May-Gruenwald Giemsa or Toluidine blue). Scan cartilage tissues at 40-400x magnification. The presence of spores in cartilage confirms diagnosis.

D. Decalcification Procedure for Detection of Whirling Disease

1. Purpose

   Tissue sectioning of large fish heads for the presence of *Myxobolus cerebralis* spores can be facilitated by chemical decalcification of fixed bone/cartilage and of frozen samples which are later placed into a fixative. Only heads or wedges from fish over 8" should be decalcified. Wedges from fish 6-8" in length are adequately decalcified in Bouin's or Davidson's fixative.

2. Equipment/supplies

   Lerner D-Calcifier solution (Hydrochloric acid, polyvinylpyrrolidone)
   Dissecting needles
   pH paper
   Forceps
   Beakers
   Pipets
   Magnetic stirring rods
   Stirring plate
   Graduated cylinders
   Watch glasses
   0.1 N NaOH
   Scalpels
   5% Ammonium Oxalate solution $C_2H_2O_4 \bullet (NH_3)$

3. Procedure

   a. A 2½ - x 1½- x ¼ cm section of the skull (Bouin's fixed) is removed with scalpel and forceps from an area encompassing the otolith/auditory canal. The section is rinsed, weighed and placed in a cassette. The size of the section may vary according to the size of cassettes, molds, etc., that are available in the lab.

   b. The cassette is placed in a beaker with a magnetic stirring rod and sufficient D-calcifier to provide a ratio of 20 mL D-calcifier to 1 g of tissue sample. Cassettes from the same case number or lot of fish may be pooled into one beaker. Care must be taken not to overload the beaker with cassettes, or the stirring rod will not function. Stirring assists in decreasing the decalcification time by increasing the permeation of the specimen with the solution. Cassettes should be labeled with a marker that will withstand acidic solutions, or identity of the specimens will be lost. The beakers should be covered to reduce evaporation and for safety reasons.

c. The covered beakers are placed onto a stirring plate for 4-16 hours at room temperature. The time depends on the thickness of the specimen and the amount of bone/cartilage present. If laboratory personnel cannot be present to monitor this process during these hours, then the cassettes must be removed from the solution, rinsed with distilled water, and submerged in a beaker of distilled water until the next day when the digestion procedure can be resumed with fresh decalcifier.

d. The degree of remaining calcification is tested by a combination of physical and chemical testing during the decalcification process.

4. Physical test
A dissecting needle is pressed against the bone/cartilage area to test its softness. Puncturing is not suggested since this alters the integrity of the specimen. This physical test will give some indication of the progress of the digestion and should be performed approximately 3 hours after the initiation of digestion and periodically thereafter.

5. Chemical test
Decalcification is complete when the chemical test cannot detect any calcium in the decalcification solution.

a. This test requires removal of 5 mL of the decalcifying solution from the beaker of specimens after 3 hours of decalcification, and also every hour thereafter; and placing that amount into a small beaker with a piece of pH paper.

b. The solution is neutralized (pH 7.0) with 0.1 N sodium hydroxide as indicated by the pH paper which is immediately removed with forceps.

c. 1 mL of ammonium oxalate solution is added to the neutralized solution and mixed. This solution is allowed to stand for at least 15 minutes to determine if a precipitate forms. The precipitate is calcium oxalate and indicates that the decalcification is incomplete. Decalcification should be continued until no calcium oxalate precipitate can be detected.

d. After decalcification is complete, the specimens are rinsed in distilled water and loaded into a tissue processor.

6. Discussion
This procedure was developed for the histological examination of large heads that were fixed or frozen/fixed. A 10% nitric acid solution was found to be essentially equal to the D-calcifier in decalcification time and effectiveness, but required a larger ratio of solution per gram of specimen (50:1). The D-calcifier was selected as the agent of choice due to its commercial availability. Other commercial products,

especially those that do not require overnight monitoring of the decalcification process, and formic acid may also be suitable but have not been tested.

Giemsa stains of decalcified tissue sections are not as intense as normally fixed samples, but *Myxobolus cerebralis* spores are still evident. Specimens have also been "partially" decalcified before loading them into the tissue processor and these specimens, although a little more difficult to section, appeared to retain better staining properties.

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Section 3 -Diagnosis of *Bothriocephalus acheilognathi* (Asian Tapeworm) in Wild Fish Populations

I. Introduction

Various tapeworms can be found in the gastrointestinal tract of fish. Of particular concern is the segmented Asian tapeworm, *Bothriocephalus acheilognathi* (Yamaguti 1934) which was introduced into the United States from Asian fish imports. This tapeworm's preferred infection site is the stomach and can exceed 50 cm, but most infections are made up of large numbers (more than 100) smaller worms. A review of procedures to evaluate fish for this species can be found in the AFS-FHS Blue Book (Mitchell 1994). Mitchell reported the tapeworm in cyprinids, poecilids, silurids, percides, and centrarchids. Since that publication, confirmed infections have been documented in other Cyprinidae and Poeciliidae: humpback chub, *Gila cypha*; bonytail chub, *Gila elegans*; and Yaqui topminnow, *Poeciliopsis occidentalis yaqui* (Landye and McCasland 1997). It has been found commonly in young of the year common carp, *Cyprinus carpio* and adult western mosquito fish, *Gambusia affinis*. The genus *Bothriocephalus* found parasitic in American freshwater fish was revised by Scholz (1997). Dove et al. (1997) reported the tapeworm in common carp, mosquito fish, *Gambusia holbrooki* and western carp gudgeon, *Hypseleotris klunzingeri* in Australia.

II. Sampling Methods

If a stereomicroscope is available, gastrointestinal tracts can be examined in the field after viral, bacterial, and other parasitic samples are obtained. It is best to remove the gastrointestinal tract and place in physiological saline in a plastic bag. Transport samples on ice to the laboratory. The examination for tapeworms should be within 48 hours of euthanasia, but viable Asian tapeworms have been seen after five days of refrigeration. In heavily infected fish, a few Asian tapeworms will dislodge from the stomach during sample collection.

A. Fish Less Than ~ 15 cm (with relatively thin walled internal tissue)

1. Multiple gastrointestinal tracts (GI) can be laid on a piece of plexiglass or glass and then another piece of the same material can be laid on top of the tissues (Mitchell 1994).

2. Using binding clips, the pieces of glass/plastic can be compressed and viewed utilizing a stereo microscope. If possible Asian tapeworms are found, they can be extracted from the preparation for further viewing under the stereomicroscope.

B. Fish larger than 15 cm

1. Remove the entire GI tract and place in saline solution contained in a large Petri dish. In extremely large fish the tract can be subdivided into several dishes, but the stomach should be examined first.

2. Stretch out the GI tract in the dish and cut longitudinally and examine the contents with a stereo microscope.

3. Most Asian tapeworms will be found in the gastric section of the tract, but examination of the remaining sections and pyloric caecae might reveal segments of the tapeworm. Usually, this condition is found in fish species that are accidental hosts. Obviously with this type of examination other types of tapeworms, nematodes, and trematodes might be encountered.

C. Quick Freezing Method of Preservation

An alternate method for maintaining samples for a later examination is to utilize the quick –freezing method of preservation. Samples can be stored at -20°C until examined. To "super cool" alcohol, place 95-100% Ethyl alcohol in 0.5 L to 1 L polyethylene bottles and place in a cooler containing dry ice. Alcohol can be also cooled by placing alcohol bottles in-80°C ultra cold freezer. Chill at least 2-3 hours before use.

1. Fish less than approximately 15 cm:

   a. Make a small incision in the abdominal cavity of the fish, being careful not to cut the GI tract.
   b. Place the fish into a deep dissecting pan.
   c. Pour super-cooled alcohol over the fish (wear protective gloves, safety glasses and apron). Alcohol can be reused by using a large funnel to pour alcohol from the pan back to bottle. Chill before using on another fish.
   d. Store fish in a plastic bag on dry ice or in -20°C freezer.

2. Fish larger than 15 cm:

   a. Remove the GI tract from fish and place into a deep dissecting pan.
   b. Pour super-cooled alcohol over the tract (wear protective gloves, safety glasses, and apron).
   c. Store frozen GI tracts in a plastic bag on dry ice or in -20°C freezers.

III. Presumptive Diagnosis

Live Asian tapeworms are segmented and can be recognized by their arrowhead or pit viper shape of the scolex. This shape appears due to the posterior part of the scolex being wider than the first few proglottids of the tapeworm. If only dead or preserved samples of the worm are available, this shape can be hard to determine, but usually the pit viper shape is present in a few of the specimens. The tapeworm, which is found in the stomach or upper gastrointestinal tract, has two bothria that are deep, elongated sucking grooves on the scolex and has no neck. The scolex is wider than the first few proglottids of the worm.

IV. Corroborative Diagnosis

Several other genera of tapeworms have similar scolices to Asian tapeworms (Mitchell 1994). After eliminating the nonsegmented tapeworms and those with suckers on the scolex, *Eubothrium*, *Bathybothrium,* and *Marsipometra* species remain. If a neck (a nonsegmented area which is at least twice as long than the first segment) is present, these latter three species can be eliminated. Not all *Eubothrium* sp. have a neck, but they do have a dorsal or ventral furrow. *Bothriocephalus acheilognathi* can be distinguished from other bothriocephalids by the presence of incision-like openings in the bothria (Scholz 1977). Thus an Asian tapeworm, *B. acheilognathi* will have no neck, no furrow, no suckers, but will have bothria. An excellent review of the Asian tapeworm is presented in Hoffman (1999). For a complete understanding of the systematics of *Bothriocephalus* in American fishes, Scholz (1997) should be reviewed. When in doubt, samples of the tapeworm preserved in alcohol can be sent to a parasitologist for corroborative identification.

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CHAPTER 9

Corroborative Testing of Parasites by Polymerase Chain Reaction (PCR)

SECTION 1 - Corroborative Testing for *Myxobolus cerebralis* by PCR

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SECTION 2 - Corroborative Testing for *Ceratomyxa shasta* by PCR

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I. Introduction

This chapter provides protocols for corroborative testing of *Myxobolus cerebralis* and *Ceratomyxa shasta* by Polymerase Chain Reaction[1] (PCR). PCR is a relatively new molecular tool to the field of fish health and is utilized in the Survey alongside standard detection methods for these two parasites. PCR will increase our knowledge of the presence of Whirling Disease and Ceratomyxosis in natural populations and allow the Service to fully evaluate the potential of this molecular tool for use in fish health diagnostics. As additional DNA sequences are elucidated for other pathogens, PCR may become an invaluable tool for detecting fish pathogens in both hatchery and wild populations.

[1]The PCR Process is covered by Patents owned by Hoffman-LaRoche, Inc.

II. General Guidelines for PCR

The specificity of oligonucleotide primers and the shear quantity of DNA that is produced during amplification makes PCR an extremely powerful detection method. These same characteristics, however, can lead to problems with sample and/or laboratory contamination with escaped DNA if protocols are not strictly followed. All extracted and amplified DNA must be confined to the sample tubes to ensure laboratory workspace is free of excess target and non-target DNA. The protocols in this chapter are intentionally very detailed to ensure quality control in using PCR. The following guidelines will be followed for all work with PCR:

A Always place fresh bench coat paper over work areas before starting to do DNA extractions, PCR set-up, and dispensing or allocating PCR reagents. (All three of these activities will be done in physically separated areas.) Dispose of the bench paper when your work is complete to ensure that trace amounts of DNA can be disposed of before they get dispersed around the lab.

B. Work surfaces should be decontaminated by washing down the work area with 10% chlorine to hydrolyze possible DNA contaminants. Tube racks will be soaked in 10% bleach for 30 minutes.

C. All PCR reagents are stored separately from DNA samples.

D. Always use aerosol resistant pipette tips when you are working with PCR reagents and amplified DNA samples.

E. Use <u>only</u> pipettors dedicated for pipetting PCR reagents (Master Mix "cocktail") and <u>never</u> use these pipettors for DNA or tissue of any kind.

F. Primers and other reagents are to be aliquoted in a clean area separate from the diagnostic area where the PCR is taking place. DNA samples never enter this room. After the PCR "cocktail" has been prepared in this room it can be taken to the diagnostic room for dispensing the DNA template into the reaction tubes.

G. Dispense the DNA samples into the reaction tubes in a laminar flow hood to avoid contaminating the lab (the blower need not be running). When you are done clean the work area in the hood and turn the germicidal UV lamp on for several hours or overnight. This will "UV crosslink" any contaminants making them unable to be amplified.

H. Dispense your positive control sample last to minimize handling this sample before diagnostic samples are handled.

I. Change gloves frequently especially after handling DNA samples of any kind. Dispose of gloves and bench liner immediately if you suspect any amplified DNA has been spilled on them.

J. Dispose of trash containing amplified DNA or contaminated materials frequently to prevent accidental spills that could contaminate the lab.

K. When opening and closing tubes containing DNA samples of any kind avoid touching the inside of the cap and the rim of the tube to prevent contamination of the samples.

L. Wear gloves that fit snugly to prevent contamination from baggy fingertips dragging inside the sample tubes or across the inside of the caps when opening and closing them. Especially when transferring material from the round 1 reaction to round II.

M. Use a heated lid Thermocycler when possible to prevent vapors generated during the PCR cycling from condensing near the lid of the tube where they are more likely to become airborne when the tube is opened.

N. Use a mineral oil overly for your PCR reaction to prevent condensation of vapors near the lid during the cycling or after prolonged refrigeration of the PCR sample.

O. Have a separate pipettor to use only for loading amplified DNA samples into the agarose gels. This will be contaminated with very high doses of amplified DNA and should never be used for DNA extraction or PCR "cocktail" preparation. The pipettor should be cleaned with chlorine (followed by di$H_2$0 rinse) or a UV lamp on a regular basis.

P. Dispose of the agarose gels and running buffer in an appropriate manner keeping in mind they contain amplified target DNA that can get spread about the lab.

Q. In general treat any amplified DNA as if it were radioactive! Don't be careless with it and keep it contained at all times.

SECTION 1 - Corroborative Testing for *Myxobolus cerebralis* by PCR

I. Introduction

This nested PCR protocol for *Myxobolus cerebralis* (Mc) was developed by Dr. Karl Andree and Dr. Ron Hedrick of University of California, Davis. This highly sensitive and specific detection method utilizes two rounds of PCR amplification and specific primer sequences that target the ribosomal DNA of this fish parasite.

For the Wild Fish Survey, the standard method for detecting this pathogen is the Pepsin-Trypsin Digest Method (PTD) described in Chapter 8 (Parasitology).

Mc-PCR is used as the corroborative technique to positively identify *Myxobolus cerebralis* when spores are detected in the initial PTD method. It may not be necessary to use a nested PCR (two rounds of PCR amplification using two primer sets) for corroborative testing when sufficient numbers of spores (a moderate to heavy infection) are found in the initial detection method (Dr. Karl Andree, University of California at Davis (UCD), personal communication,). Single round PCR (Baldwin and Myklebust 2002) has been validated for detection of spores in the digested tissue obtained with the Pepsin Trypsin Digest method.

As previously noted in the PTD protocol, formalin should not be used to preserve the digest material if PCR will be used for corroborative testing. Also an additional heat and microwave step is recommended to disrupt spores and release Mc-DNA prior to PCR testing.

This section is divided into the following major steps for the Mc-PCR assay:

I. Sample Processing
II. DNA Extraction from Archived Cranial Tissue
III. DNA Extraction Method for PTD Spore Preparations
IV. Preparation of Master Mix (MM) ("Cocktail" for PCR amplification)
V. Amplification - Round 1 (R1)
VI. Nested PCR - Amplification of Round 2 (R2)
VII. Gel Electrophoresis
VIII. Interpretation of Gel and Photo Documentation
IX. Reporting Results
X. References
Appendix 9.A - Equipment, Supplies and Reagents
Appendix 9.B - Mc-PCR Data Sheet
Appendix 9.C - Analysis of Extracted DNA using a UV Spectrophotometer.

II. Sample Processing

Tissue dissection can be performed on the bench top in a designated area of the laboratory, or in a bench top hood with a UV lamp for disinfection. Proper disinfection of work surface and tools is necessary to prevent laboratory contamination with specific DNA. Use 10% chlorine solution, a commercial DNA detergent, or 60-minute disinfection with UV light to decontaminate all work surfaces and supplies. All supplies, instruments and sample tubes should be sterile prior to use. All excess tissue should be autoclaved prior to disposal.

If the archive tissue was correctly labeled with fish ID per 5-pool sample (as described in the PTD protocol), only the single sample that corresponds with the digest sample has to be tested by Mc-PCR to confirm the identification of *Myxobolus cerebralis* spores. If samples were not labeled and tracked with corresponding digest samples, then entire archive sample set for that case history will have to be tested to confirm the presence and identification of *M. cerebralis*.

A. Archived Heads - Dissection Methods (Necropsy or Miltex 6 mm Biopsy Punch)

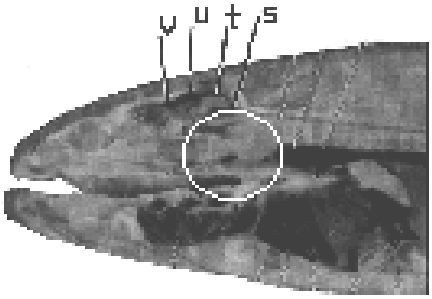
1. Sample Size – 6 mm biopsy punches work well with fish smaller than 6 inches.

2. For larger fish, use a scalpel to excise the tissue aiming for tissue size of ~ 5-6 mm^2.

B. Wear clean gloves and use individual sterilized instruments for each fish tissue

C. Target Tissue – Dissect the cartilage and bone tissue from the caudal ventral portion of the skull; posterior to the optic lobe and include the majority of the auditory capsule.

D. Keep the tissue cold during collection to prevent degradation of DNA.
(Store fish tissue frozen if DNA extraction will be done at a later time. Avoid repeated freeze thaw cycles to prevent shearing of DNA).



s. Medulla oblongata
t. Cerebellum
u. Optic Lobe
v. Cerebral hemisphere

Figure 1 – Target tissue site detection of Myxobolus cerebralis by PCR

III. DNA Extraction for Archived Cranial Tissue

(QIAGEN DNeasy Extraction Kit using the Rodent-tail Protocol)

A. Follow the DNA Extraction method in the QIAGEN DNeasy Kit Handbook - using the Rodent Tail Protocol . Follow all manufacturer's instruction for initial preparation of kit solutions.

B. Quantifying extracted DNA for use in PCR:

Quantification of DNA by spectrophotometry requires that the preparation of DNA be pure, as evidenced by a 260/280 reading of 1.8 or higher by UV spectrophotometry. If the DNA preparations are not pure enough, then the relationship upon which spectrophotometric quantification of DNA is based (1 A_{260} =50ug/mL) is not valid.

Furthermore, the quantities of DNA template recommended in this protocol are based on DNA preparations with a 260/280 ratio of at least 1.8. If DNA extracted from tissue or spore samples do not meet this criterion, additional steps will be required to further purify the preparations, or to utilize alternate purification methods.

All samples should be "spected" prior to testing by PCR to minimize the risk of false negative reactions due to poor quality, or inadequate DNA. **See Appendix 9.C for measuring extracted DNA using a UV Spectrophotometer.** Follow the manufacturer's instructions for setting the correct measuring parameters for the UV spectrophotometer being used. The recommended quantity of DNA for Mc-PCR is approximately 300 ng per reaction, with a 260/280 ratio of 1.8 or higher (Andree, 1998).

Using a spectrophotometer to quantify the DNA ensures that the quality and quantity of DNA is sufficient for PCR testing, an important step in quality assurance for the extraction and amplification process.

IV. DNA Extraction Method for PTD Spore Preparations

A. Pellet the spores collected from PTD by centrifugation in a microtube at 14,000 rpm for 2 minutes.

B. Aspirate the supernatant and dry the pellet for 30 minutes at 50°C.

C. After drying, microwave the spores in the same microfuge tube for 1 minute (standard microwave set on high power).

D. Add 180µL of the ATL (Lysis Buffer) from the QIAGEN Kit and follow the Mouse-tail protocol through completion.

V. Preparation of Master Mix (MM) "Cocktail" for PCR amplification

Preparation of the PCR cocktail, or Master Mix (MM), is done in a designated clean area of the laboratory, preferably in a UV hood. NO TISSUE SAMPLES, EXTRACTED DNA, OR AMPLIFIED PRODUCTS ARE BROUGHT INTO THIS AREA. Dedicated pipettors, racks, tubes, and all supplies should be located in this area to prevent cross-contamination from other areas of the laboratory.

A. **Preparing the MM "Cocktail"** - always WEAR CLEAN GLOVES when handling MM reagents.

1. Thaw the frozen MM REAGENTS - Taq (in glycerol) should be kept on ice, thaw the other reagents. Keep the MM tubes (R1 & R2) on ice during preparation.

2. Determine amount of MM to prepare as follows:

   a. Number of samples to run.

   b. Enough additional cocktail for 2 reactions (for pipetting loss).

   c. Prepare cocktail for all positive and negative controls.

   d. **The total number of reactions will be prepared twice** -
   one set for each of the R1 and R2 tubes (Specific primers for each round of amplification will be added to the corresponding tube last - all other components and volumes are the same for each round of MM).

   EXAMPLE: You have 6 samples and 2 controls (1 positive and 1 negative control). You'll prepare one extra reaction to cover any lost volume during pipetting.

   You prepare the volumes indicated for 10 reactions by rounding up to the nearest value that allows easy calculations. You'll prepare MM for 10 reactions, but you will REPEAT all dispensing steps (except primers) into the second tube designated as R2 MM. (You simply prepare 20 reactions by repeating each dispensing step, except primers, into the second R2 tube).

3. Set up a clean rack with TWO, 1.5mL microcentrifuge tubes labeled R1 and R2 MM (Prepare MM for both R1 and R2 amplification steps, however store the tubes for R2 in the freezer for later use). Keep MM solution on ice during preparation.

4. Place labeled, sterile PCR sample tubes in the rack including tubes for the controls. Use clear tubes for R1 samples and color-coded tubes for R2 (or label each tube with both the ROUND NUMBER as well as the Sample ID).

5. Using dedicated pipettes (labeled MM ONLY) carefully dispense all reagents in order into the R1 & R2 tube, EXCEPT THE PRIMERS and the TAQ.

Table 9.1(section 1) - Master Mix Formulation and Primer Sequences

STOCK Concentration	**FINAL Concentration**	**1 rxn**	**10 rxn**	**25 rxn**	**50 rxn**
DNase-free		Water add to 50µL total Rx volume considering µL of extracted DNA or PCR product used.			
Taq Buffer 10X	**1X**	5	50	125	250
$MgCl_2$*	**25mM**	5	50	125	250
dNTPs 10mM	**400µM**	2	20	50	100
TMAC 50uM	**5uM**	5	50	125	250

***Recommend using Taq Buffer without MgCl2 and adding this as a separate component to achieve the final concentration of 2.5mM per reaction.**

ADD TAQ LAST – AFTER SPECIFIC PRIMERS

Taq Polymerase 5U/µL	**2U/rxn**	0.4	4	10	20

ROUND 1 Primer Sequences:

Tr3-16 5' - GAATCGCCGAAACAATCATCGAGCTA - 3'

Tr5-16 5' - GCATTGGTTTACGCTGATGTAGCGA - 3'

ROUND 1 PRIMERS are added to the R1 TUBE ONLY. Add in numeric order to prevent errors (i.e., Tr3-16, then Tr5-16).

Primer #1 (Tr3-16) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-16) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100

ROUND 2 Primer Sequences:

Tr3-17 5' - GGCACACTACTCCAACACTGAATTTG - 3'

Tr5-17 5' - GCCCTAT TAACTAGTTGGTAGTATAGAAGC - 3'

ROUND 2 PRIMERS are added to the R2 TUBE ONLY. Add in numeric order to prevent errors (i.e., Tr3-17, then Tr5-17).

Primer #1 (Tr3-17) 20 pmole/µL	**40 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-17) 20 pmole/µL	**40 pmole/rxn**	2	20	50	100

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6. Carefully add the appropriate primer sets to their corresponding tube, i.e., R1 tube receives primers Tr3-16 and Tr5-16 and R2 tube receives primers Tr3-17 and Tr5-17.

7. Vortex all tubes gently. ADD THE TAQ LAST.

8. Dispense 47 µL of R1-MM to each labeled tube for R1 amplification.

9. Close the caps, vortex gently by hand.

10. Repeat this process for the **R2-MM tubes if they will be used the same day*.** Freeze the R2 tubes (-20°C) for use following R1 amplification.

    ***NOTE on Adding TAQ**:
    It is recommended that the Taq not be added to R2 MM tubes unless they will be used within 3-4 hours, i.e. immediately following completion of R1.

    If R2 will not be completed until the next day, do not add Taq to R2 MM tubes. In this case, it is recommended that the Taq be added to the R2 MM just prior to aliquoting into PCR tubes and as soon as possible prior to beginning R2 amplification.

11. Take the R1 tubes to the appropriate workspace designated for TEMPLATE DNA (extracted DNA from test samples).

VI. Amplification - Round 1 (R1)

All transfers of DNA material, both extracted DNA from tissues, and amplified products following amplification should only be done in an UV HOOD to prevent cross-contamination of samples and laboratory space. Again, dedicated pipettors should be used for this purpose only

and labeled clearly for their specific use. Always disinfect the hood area with UV (30-60 minutes) between transfers and overnight at the end of the day.

A. Adding DNA Template (extracted DNA from samples) - WEAR CLEAN GLOVES.

1. Without touching the rim or upper edges of the tubes, carefully add DNA Template (150-300 ng/rxn based on DNA quantification) to each corresponding sample tube containing the MM.

2. Add 1-2 drops of sterile mineral oil (molecular grade only) to each tube. Use caution in controlling how mineral oil is applied to prevent cross-contamination of samples (drop from above the tube, avoid aerosols, close caps carefully).

B. Adding the Negative Control:
Sterile, DNase-free water is used as the negative control by using the same volume as the DNA template in each amplification round (3 µL for R1 and 1 µL for R2).

C. Adding the Positive Control:
The positive control is plasmid DNA p18Tr29, or another known positive tissue for Mc. The plasmid contains a 1934 bp insert of 18s rDNA from *M.cerebralis*. The stock concentration of this positive control is 15ng/µL and is approximately equal to 2.9×10^9 plasmid molecules/µL. You can dilute a control of this concentrate at least ten-fold and still get a good strong signal.

For dilution of the positive control plasmid, use Tris-EDTA (TE) Buffer (10 mM Tris, 1mM EDTA final concentration). You can prepare a stock 10X TE Buffer for more accurate measure of the small weights used in this formula, then perform an additional 1:10 dilution with DNase-free water for the working concentration. Alternatively, DNase-free water can be used as a positive control diluent, but the control will be less stable for long-term storage (approximately 2-3 months). The Positive Control, diluted in TE Buffer, can be stored in the refrigerator (4°C) for 1 year.

USE EXTREME CARE IN HANDLING THE POSITIVE CONTROL, especially when transferring amplified product from R1 to R2 tubes. Risk of laboratory and sample contamination is highest with this reagent.

1. Carefully add 1 µL of positive control plasmid DNA to the appropriate MM tube labeled for positive control (1 µL is adequate volume for both R1 reaction tube).

2. Add 1 drop of sterile, PCR-grade mineral oil (NOTE: A separate tube of mineral oil can be designated for use with positive controls only).

3. Close the cap immediately.

4. Change gloves immediately after handling the positive control tube.

5. Disinfect the work area with the UV lamp for 30 minutes.

D. Amplification using a Thermocycler:

1. Most thermocycler units must run ~ 30 minutes prior to use (follow the manufacturer's recommendation for machine warm up).

2. Place one drop of standard grade mineral oil in each well of the machine block (if recommended by manufacturer). Oil aids in transfer and uniformity of heat within the sample tube during the processing cycles.

3. Add sample tubes to the block.

4. If the thermocycler includes a heated lid, place it over the tubes and close the chamber.

5. Select a program for Mc-PCR (pre-programmed) or set the thermocycler program as follows:

STEP	TEMPERATURE (°C)	TIME (minutes)	
Preamplication	95	5	
Denaturing	94	1	**35 CYCLES**
Annealing	65	2.5	
Extension	72	1.5	
Post-amplication	72	10	
Hold	15	(hold temp at 15°C until unit can be turned off. Colder hold temperatures can be used but are not necessary and cause un-due wear on the unit.)	

This program takes approximately 3.5 hours to run; it can be initiated in the afternoon and left overnight - the amplification will stop after 35 cycles, the machine will run through the post-amplification step and hold at the designated temperature.

VII. Nested PCR - Amplification of Round 2 (R2)

After R1 amplification is complete, DNA is transferred from R1 tubes to R2 tubes for the nested PCR. Again, all transfers are done in the UV hood with dedicated pipettors, followed by complete disinfection of the work surface.

A. Transfer R1 PCR products to R2-MM tubes:

1. Thaw the R2 MM tubes that were prepared earlier and frozen.

2. Wear clean gloves, remove tubes from thermocycler and wipe excess oil from tube bottom with Kim-wipes. Place sample tubes in a rack and place in the UV hood.

3. Use a dedicated pipettor for amplified DNA only.

4. Carefully place pipette tip under the mineral oil interface and mix the solution by gently pipetting up and down 1-2 times. Withdraw 1 µL of the R1 PCR PRODUCT.

5. Without touching the rim or upper edges of the R2-MM tubes, transfer each PCR product from R1 to the appropriate sample tubes and negative control tube containing the R2-MM.

6. Add 1 drop of mineral oil to each tube without cross-contaminating samples or the mineral oil.

B. Add the positive control R1 PCR product to the R2 MM labeled "Mc+":

1. Pipette the positive control product last and immediately CHANGE GLOVES.

2. Add 2 drops of mineral oil to the tube, being extremely careful (use the dedicated source of mineral oil for this purpose).

3. Turn on UV lamp for 30 minutes to disinfect the hood area.

C. The same thermocycler program is used in R2 amplification of the nested primer sequence:

1. Place one drop of mineral oil in each well and use heated lid if available.

2. Add sample tubes to the block and close the chamber.

3. Select the program for **Mc PCR**.

Program takes approximately 3.5 hours to run, can be left overnight - machine will stop and hold at the programmed temperature.

VIII. Gel Electrophoresis

The Anticipated Product (AP) from R2 of the nested Mc-PCR is 410 base pairs. The R1 AP is 1300 bp, but normally will NOT be present in diagnostic PCR because insufficient copies are produced in R1 amplification. However for corroborative testing of PTD-positive samples with moderate to heavy infection levels with Mc spores, this band should be present. As noted in the introduction, a single amplification PCR, rather than the nested PCR, may be more appropriate for corroborative testing of known-positive samples.

If nested PCR is used, amplified products from both R1 and R2 are run on the gel as this may help with interpretation of positives and troubleshooting if anticipated products are not resolved.

TABLE 9.2(section 1) - Size of DNA fragments (kb) optimally resolved
(modified from Sambrook, et al. (1987))

AGAROSE CONCENTRATION	Size of DNA fragments (kb)
0.7%	0.8 to 12
1.0%	0.5 to 10
1.2%	**0.4 to 3**
1.5%	**0.2 to 3**
2.0%	0.01 to 1

A. **Determine the Gel Size** needed by the number of samples to be run. Select the appropriate gel apparatus. The following instructions and tables are provided as a guide only for the equipment specified in this protocol. Consult the manufacturer of the gel unit to determine the optimum conditions for gel electrophoresis.

1. Determine the number of samples for both rounds of PCR products and DNA LADDERS and select the gel unit and comb (Fischer Mini-EC370 unit has combs with 8-12 wells; the larger unit, Midi-EC350 has combs with 22-40 wells).

2. Determine the quantity of agarose gel needed by following the recommended gel volumes for each unit. A consistent gel thickness of 1/2 to 2/3 cm is recommended for all Mc-PCR gels.

TABLE 9.3(sec. 1) - AGAROSE VOLUMES needed for gel apparatus (and buffer volumes)
Tris Acetate-EDTA (TAE Buffer) is used in this protocol. See Appendix 9.A for more information on buffers.

GEL UNIT	GEL THICKNESS	Volume OF 0.5X-TAE Buffer to mix with Agarose	Agarose (grams)	0.5X- TAE Buffer in Gel Chamber
MIDI-EC350	1.0 cm	150 mL	2.25 g	1200 mL
(10x20cm)	**0.7 cm**	**110 mL**	**1.65 g**	**1050 mL**
8x4"	**0**.5 cm	85 mL	1.28 g	1000 mL
Mini- EC370	1.0 cm	50 mL	0.75 g	425 mL
(6.5x9cm)	**0.7 cm**	**35 mL**	**0.53 g**	**350 mL**
2 3/4 x 3"	0.5 cm	25 mL	0.38 g	300 mL

B. Set up the Gel Apparatus

As per manufactures recommendations. See below and Table 9.3(sec. 1) above for examples.

1. Level the gel apparatus using leveling screws (clockwise lowers corner, counter-clockwise raises corner).

2. Place gel tray with rubber dams on each end in chamber.

3. Place COMB near the cathode end (NEGATIVE ELECTRODE) of gel chamber. The mnemonic phrase "RUN TO RED" is used to remind the operator of the correct orientation of the gel tray in regard to samples running towards the red (positive) electrode.

4. Use the COMB SPACER placed under the COMB to set the height of the wells and ensure uniform depth of wells across the entire gel. The 0.75 mm spacer is used (1.5mm used for very fragile gels with low agarose content).

5. Tighten the COMB HOLDER SCREW when the comb is in place and adjusted correctly.

C. Prepare a 1.5% Agarose Gel

1. Weigh out agarose and add to an Erlenmeyer flask (oversized for volume needed) – see Table 9.2(section 1) for more information of selection of a 1.5% agarose gel.

2. Add appropriate volume of buffer and swirl to dissolve.

3. Microwave on HIGH heat watching carefully to prevent boiling over or burning of unmelted agarose (approximately 2 minutes). Stop and swirl contents periodically to

distribute heat evenly and dissolve completely. CAUTION: USE A HEAT MIT AND EXTREME CARE AS FLASK AND AGAROSE ARE VERY HOT.

4. Once agarose boils, gently heat an additional 30-45 seconds to dissolve completely.

   Agarose should appear completely dissolved and clear when held up to light; no clumps or semi-dissolved agarose should be present.

5. COOL GEL to approximately 55°C (usually 10-15 minutes at RT). The gel can also be cooled by swirling in cool water, or placing in a water bath at 55°C for several minutes (If the gel sets up prior to pouring, it can be melted again).

6. Pour gel in gel tray carefully avoiding bubbles.

7. Let gel cool completely in tray (approximately 30 minutes). Remove the comb by gently pulling it directly up (avoid side to side or uneven motions that will distort the well chambers).

D. Test the Gel Unit

Test unit prior to committing samples to the gel. Follow manufacturer's directions.

1. Add appropriate buffer volume to unit chamber. Buffer should cover the gel with ~ 1-2 mm of buffer over the surface of the gel.

2. The gel tray should be oriented so that the combs are closest to the black/negative electrode, and running towards the red/positive electrode. The mnemonic phrase "RUN TO RED" is helpful to ensure proper orientation of the gel in the electrophoresis unit.

3. Carefully slide the interlocking safety cover onto the chamber and make sure that both banana plugs are securely attached.

4. Connect the attached leads to the Power Supply making sure that the polarity of the leads agrees with the polarity of the output.

5. Refer to Table 9.4(section 1) to select appropriate voltage, switch the Power Supply "ON" and adjust the output to the desired level.

6. Check that the system is functioning properly - the voltage and current levels should correspond to the expected levels and should not fluctuate. Bubbles should rise from the electrodes in the chamber when current is passing through the unit.

7. After verifying proper operation, switch the Power Supply "OFF" and disconnect the leads from the Power Supply. Remove the cover from chamber.

TABLE 9.4(section 1) (Minutes Required by Marker Size (for fragments to move 6.2 cm))

(Conditions given for a 1% agarose gel at 0.5cm thickness in 1X TAE buffer)

Marker/Fragment Size	50V	100V	150V
100 bp	102	49	30
500 bp	121	60	36
1000 bp	145	72	42

NOTE: The Mc-PCR recommended gel is 1.5%, 0.5cm thick agarose gel, therefore this table is a guideline only. Refer to the section RUNNING THE GEL for recommended voltage and time.

E. Load Samples on Gel

1. The Gibco 100bp DNA Ladder has bands at 100-1500 bp, with a brighter band at 600bp. - See Sambrook, et al. (1987) or other reference for preparing loading buffer.

2. Prepare the LOADING BUFFER/DYE for the samples:
   Loading buffer has two purposes; it adds color to samples for placement into wells and monitoring of band electrophoretic movement across the gel. Secondly, it adds weight to the sample so the sample solution is heavier than the TAE buffer. Generally the sample is mixed with buffer at a ratio of 5:1, hence the designation of loading buffer at "6X" concentration.

3. Pipette 3µL of 6X LOADING BUFFER for each sample you will load; place in rows on parafilm in same order as samples (use spacers to help keep track of different samples or wells you plan to skip, for example if leaving empty wells around the positive control).

4. Add 15 µL of the sample, mix by pipetting up and down 1-2 times in the dye solution. Load 10-15 µL in the correct well. Loading volumes are dependant on the size of the wells prepared in the gel.

5. When loading sample, hold pipette tip just above well, this prevents tearing the well (dye is heavy and sample will sink into the well as it is loaded).

6. Load the POSITIVE CONTROL last, leave wells on either side if extra space is available on the gel.

7. Place the chamber cover on in the correct direction (RUN TO RED).

8. Plug in corresponding leads into the Power Supply and turn power “ON”.

9. Run at corresponding voltage for gel unit, and gel size as follows:

F. Run the gel - Power Supply Settings:

The voltage used for the gel is determined by the size and resistance of the gel unit, the thickness of the gel, and the desired resolution of the bands - generally the slower the electrophoresis rate, the "cleaner" the resolution of DNA on the gel (faster rates can create smears and/or band artifacts).

Each unit has a maximum voltage that should not be exceeded per manufacturer recommendation. The following are recommendations by EC (manufacturer) and a "rule of thumb" based on the LINEAR DISTANCE (LD) of the gel apparatus (LD = Distance between electrodes on the gel chamber).

EC Recommends	"Rule of Thumb" is DO NOT EXCEED	(LD)	3.5 - 4.0 Volts per LD (cm)
Mini-EC370	15 WATTS	16.5 cm	60 - 70 VOLTS
MIDI-EC350	30 WATTS	21.5 cm	75 - 85 VOLTS

NOTE : Watts = volts x amps {1 amp = 1000mA}.

Migration Rate: A 410bp band migrates at a distance of 4 cm (1.5 inch) in a gel system with the following parameters:

- **1.5% Agarose gel, at 1/2 to 2/3 cm thickness**
- **Gel apparatus - linear distance between electrodes is 15 cm**
- **Run at 4 v/cm = 60 VOLTS for 45 minutes**

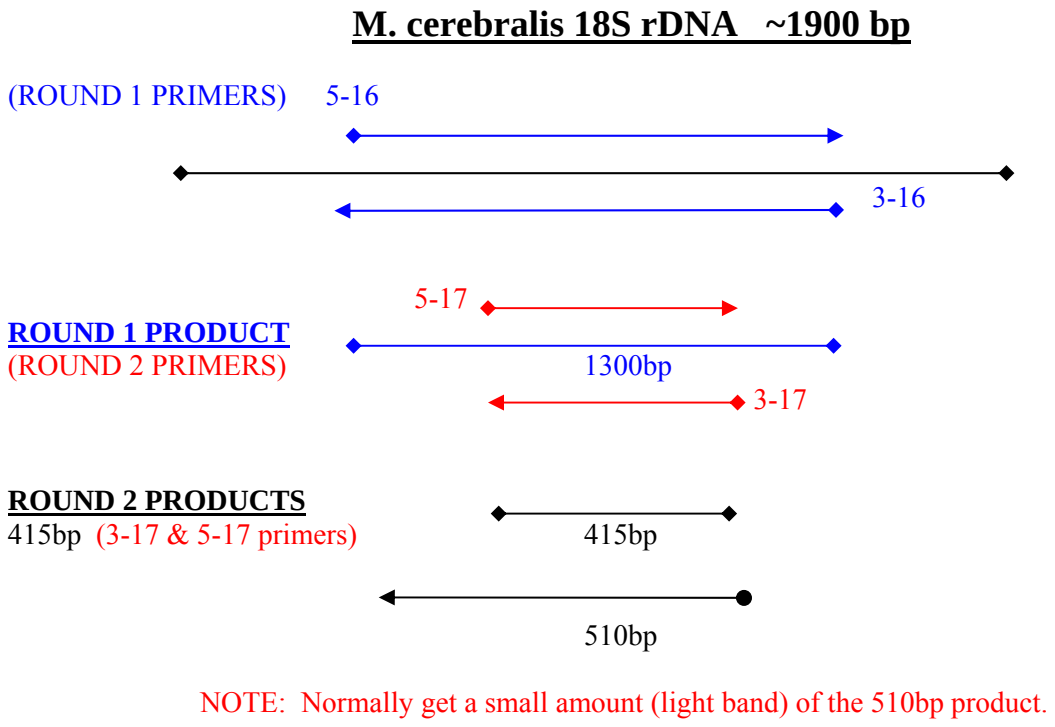
IX. Interpretation of Gel and Photo Documentation

A. Stain the gel with Ethidium Bromide for 10 minutes. Destaining for 20-40 minutes is an optional step; longer destaining can produce clearer photographs of the gel.

B. Anticipated product from nested PCR is 410 bp. The 415bp product is diagnostic and must appear in the positive control and any test samples if they are to be rated positive. A light band at 510 bp may also be present.

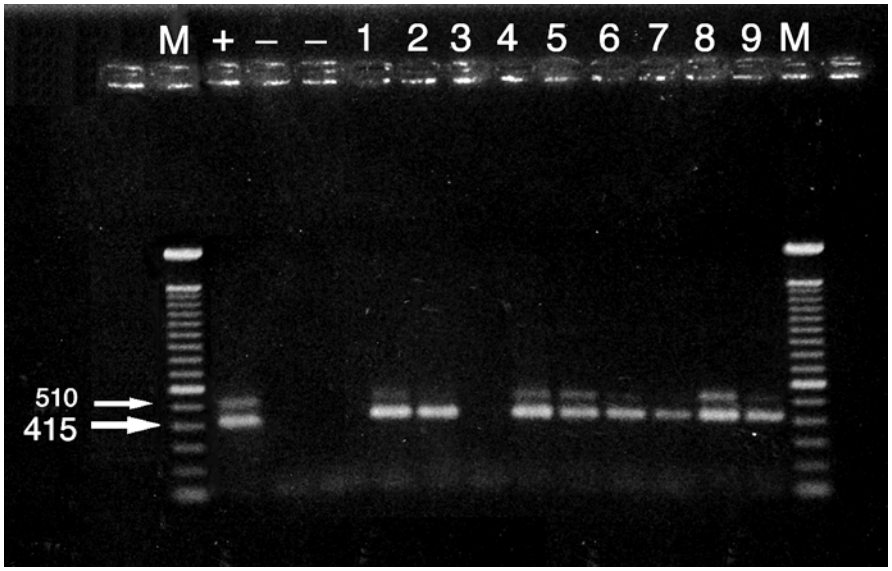
The larger 1300bp product should be present when a single amplification using the Round 1 primers is performed on samples with significant quantities of Mc DNA (as recommended for corroborative testing of spores detected by PTD).

Figure 2 – Schematic of Anticipated Products based on primer sequence.



NOTE: Normally get a small amount (light band) of the 510bp product.

Figure 3. Anticipated Products for Mc Nested PCR
Photo courtesy of Dr. Karl Andree, University of California at Davis (UCD).



X. Reporting Results

A. Document all procedures for each assay using the MC PCR DATA SHEET (Appendix 9.B)

B. Report positive bands (415bp for nested and 1300bp for single round PCR) as positive for *Myxobolus cerebralis.* All positive and negative controls must also support the findings or the results of the assay/gel are equivocal.

C. Photograph the gel and keep for a permanent laboratory record.

D. Positive PCR products can be archived as a laboratory stock or for future reference.

References

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Additional Reading

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Appendix 9.A - Equipment, Supplies and Reagents

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service and/ or the United States government. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

EQUIPMENT:

EC Mini-submarine Gel	Fischer
EC MIDI-submarine Gel	Fischer
Hoefer Power Supply, model	Hoefer
Thermocycler (suggest MJ Research, Perkin-Elmer, or Thermolyne)	
Finnpipettes - digital, various volumes	VWR
(Dedicated to Tissue DNA, Amplified DNA, PCR-MM ONLY, and Gel-Loading).	
Aerosol-resistant tips, 200 & 1000µL sizes.	VWR
Positive-displacement tips, 1-25µL & 200-1000µL sizes	VWR
Micro-7 Minicentrifuge	Fischer
Standard Heatblock (55C incubation)	VWR
Polaroid DS34 Camera	Fischer
Polaroid 667 B&W Film	Fischer
Camera Hood -	Fischer

SUPPLIES / REAGENTS:

Description	Catalog #	Source
PCR Marker with darkened 400/500 bands	15628-019	Gibco
PCR MARKER (DNA bp Ladder)	P-9577	Sigma
Taq Polymerase	D-1806	Sigma
DNase-free Water	W-4502	Sigma
dNTPs	D-7295	Sigma
Tetramethyl Ammonium Chloride (TMAC)	T-3411	Sigma
DNA/RNA-free mineral oil	M-8662	Sigma
Tris-Acetate-EDTA Buffer (TAE 10X)	T-4038	Sigma
Ethidium Bromide (EtBr)*	E-7637	Sigma
S&S Extractor (EtBr Decontamination Kit)	448031	Intermountain Scientific
	(Manufacture is Schleicher & Schuell)	
SeaKem GTG (Genetic Technology Grade)	50070	Intermountain Scientific
	(Manufacturer is FMC Bioproducts)	
Miltex Biopsy Punches (6mm)	21909-144	VWR
(other sizes are available)		

***CAUTION:** Strong mutagen, wear gloves and use hazardous chemical precautions.

FORMULAS FOR PREPARING REAGENTS USED:

REAGENT:	REFERENCE:	DIRECTIONS TO PREPARE:
TAE BUFFER 10X	Sigma T-4035	Comes in prepared packets, add Gibco DI water & qs to 1L Label as 10X- STOCK (store at RT). Also can prepare a 50X stock (Sambrook et al. 1987)
TAE BUFFER 1X WORKING SLN	TAE-1X	Diluted 1:10 from 10X stock Label as TAE - 1X (store at RT)
PCR LOADING BUFFER -OR- LOADING DYE	Sigma P-7206 LOADING DYE 6X	Pre-made 6X concentrate, ready to use (store -20˚C) Prepare in-house (Sambrook et al. 1987)-store at 4˚C): Bromophenol blue 0.25% Xylene cyanol 0.25% Glycerol 30.0%

USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH "CHEMICAL CARGINOGEN".

ETHIDIUM BROMIDE - Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

EtBr STOCK SOLN - 10mg/mL	Ethidium Bromide 100 mg Gibco DI water 10 mL Label as EtBr STOCK (10mg/mL) Protect from light (store at RT)
EtBr-WORKING SOLN - 4.0ug/mL* WORKING STAIN SLN	Add 200µL STOCK SLN to 500mL TAE-1X Buffer Label EtBr - Working Sln (store at RT)

*References may suggest weaker working solutions (0.5ug/mL) and staining periods of 45-60 minutes. Most researchers prefer to use at a stronger working concentration (8 X stronger in this example) and stain gels for 10 minutes. Karl Andree recommends the 4.0ug/mL concentration and staining for 10 minutes.

OTHER BUFFER FORMULAS:
An excellent reference for PCR is Sambrook et al. (1987). This reference describes how the electrophoretic mobility of DNA is affected by the composition and ionic strength of the

electrophoresis buffer. For example, in the absence of ions (e.g., if electrophoresis buffer is omitted from the gel by mistake), electrical conductance is minimal and DNA migrates very slowly. In buffers of high ionic strength (e.g., if 10X electrophoresis buffer is used by mistake), electrical conductance is very efficient and significant amounts of heat are generated. In the worst case, the gel melts and the DNA denatures.

According to Sambrook et al. (1987), several different buffers are available for electrophoresis of double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50mM (pH 7.5-7.8). For historical reasons, TAE is the most commonly used buffer. However, its buffering capacity is rather low, and it tends to become exhausted during extended electrophoresis. Both TPE and TBE are slightly more expensive that TAE, but they have significantly higher buffering capacity. Double-stranded linear DNA fragments migrate approximately 10% faster through TAE than through TBE or TPE, but the resolving powers of these systems are almost identical, with the exception that the resolution of supercoiled DNAs is better in TAE than in TBE. (See Sambrook, et al. (1987) for more information on the various types and uses of electrophoresis buffers).

NOTE: The use of 0.5X TAE in this protocol is a non-standard and more dilute concentration of this buffer than that which is normally used for electrophoresis. TAE is normally used at a concentration of 1.0X or 50 mM as noted above. The 0.5X TAE works well for Mc-PCR in this researchers hands, however this concentration is only a recommendation and individual researchers may prefer to use the standard concentration of 1.0X, or 50 mM.

Appendix 9.B **Mc-PCR DATA SHEET:** **ASSAY DATE:_____**

I. Sample Information

Case Number	Collection Date	Sample ID	NOTES

II. Control Information

POSITIVE CONTROLS		NEGATIVE CONTROLS	
Extraction Positive Control		Extraction Negative Control	
Plasmid + Control		R1-MM	
		R2-MM	

III. Primer Sets

Initial Amplification		Nested Amplification	
Forward	Reverse	Forward	Reverse

IV. Master Mix Preperation

Total number of reactions (R1&2):_____

STOCK CONCN.	FINAL CONCN.	1 rxn	10 rxn	25 rxn	50 rxn
DNase-free Water --		Add to 50µL total Rx volume considering µL of extracted DNA or PCR product used.			
Taq Buffer 10X	**1X**	5	50	125	250
$MgCl_2$	**2.5mM**	5	50	125	250
dNTPs 10mM	**400uM**	2	20	50	100
TMAC 50uM	**5uM**	5	50	125	250
Taq Polymerase 5U/µL	**2U/rxn**	0.4	4	10	20

ROUND 1 PRIMERS are added to the R1 TUBE ONLY.
Add in numeric order (Tr3-16, then Tr5-16).

Primer #1 (Tr3-16) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-16) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100

ROUND 2 PRIMERS are added to the R2 TUBE ONLY.
Add in numeric order (Tr3-17, then Tr5-17).

Primer #1 (Tr3-17) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-17) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100

TEMPLATE DNA (Round 1)	**150-300ng/rxn**	Adjust to meet target quantity
***PCR PRODUCT DNA (Round 2)**		1µL

***product of first round

V. Amplification (Thermocycler Process)

Round Number (Date & time)	Program #	NOTES

VI. Gel Preperation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (mL)

VII. Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

INITIAL PCR PRODUCTS (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

NESTED PCR PRODUCTS (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
23	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**

VIII. Photo Documentation

CAMERA SETTINGS:

F-stop setting _____ Aperture ____ Exp time _____

Attach photo

RESULTS SUMMARY:

Appendix 9.C - Analysis of Extracted DNA using a UV Spectrophotometer.

The GeneQuant II (Pharmacia Biotech) is a spectrophotometer specific for obtaining concentrations of either Double Stranded DNA (dsDNA); Single Stranded DNA (ssDNA) or RNA in units of weight, molar fraction, moles of phosphate and total molecules. The instrument is capable of measuring the RNA or DNA using UV cells at 230nm, 260 nm, 280 nm, and 320 nm simultaneously. The 230, 260 and 280 wavelengths provide the readings for quantification and purity and the 320 wavelength provides a reading for background compensation.

1. Instrument Set Up
   The instrument has a set of default parameters that can be altered to meet the needs of the molecular biologist. For >spec readings= of dsDNA the standard settings on the instrument are as follows:

Using the >setup= and >enter= key enter the following parameters:

Cell path length (mm) ----------5(OD_{260} range---5-0.2;ug/mL range---250-10)
Printer----------off (on if using a printer)-press >enter=
Sample Number----------enter specific sample number-press >enter=
Date----------key in date-press >enter=
Month----------key in month-press >enter=
Year----------key in year-press >enter=
Use 320 background compensation----No (unless suspect background interference)
Dilution Factor----------1 (unless samples have a dilution factor)
Factor----------press select to key in dsDNABpress >enter=
Molecular Weight----------this displays default calculations of instrument

The remaining parameters are basic default parameters that are entered specifically for the instrument when dsDNA was keyed in.

2. Reference Measurement
   A reference measurement must be taken prior to obtaining any sample readings. This is the base reading and will be stored for all samples measured until a new reference is read. DNase free water can be used for setting the reference. If the DNA to be Aspect=d@ was extracted and purified using a commercial kit (such as QIAGEN) the reference background can be set using the final elution reagent from the kit, since that is the reagent solution containing the purified DNA.

To set the reference background;

Add 7 µL of reference reagent or DNase free water to the Ultra micro-volume cell
Press >set ref=---------->Please wait= is displayed
When the instrument beeps-------->Insert reference= is displayed
Insert the Ultra micro-volume cell
Wait for the beep----------Remove reference is displayed
Remove the cell and the absorbance will display: 260 nm----------0.000AU

3. Sample Measurement

   After all the above parameters are set and the reference background has been measured, samples can be quantitated. Add 7 µL of purified DNA to a clean (rinsed with DNase free water) ultra micro-volume cell.

   Press sample----------->please wait= will be displayed
   At the beep----------->insert sample= will be displayed
   Insert the sample cell and wait for the beep and >remove sample display=
   After the cell is removed in a few seconds the absorbance will be displayed
   press >conc= and select to obtain the reading in units of choice:
   Conc 1-------ug/mL (range 1C4000 ug/mL)
   Conc 2-------ug/µL (range 0.001B0.2 ug/µL)
   Conc 3-------pmol/µL (range 0.001-200 pmol/µL)
   Conc 4-------phosphate concentration

4. For measurement of oligos (if "spec-ing" primers) follow the same instructions listed in instrument set up except at the setup factor, key in either oligo RNA or oligo DNA (depending on the primers used).

5. Maintenance:
   The instrument can be kept clean with a soft damp cloth. A drain hole in the sample compartment allows for drainage of any spillage during sample measurement. This also can be cleaned with a soft damp cloth and should be immediately cleaned with a spillage occurrence.

   Fuses can be replaced as recommended by the manufacturer in the Instrument User Manual.

6. Troubleshooting:
   For instrument problems it is recommended to contact the manufacturer's technical support.

Section 2 - Corroborative Testing for *Ceratomyxa shasta* by PCR[1]

I. Introduction

Review of the known geographical range of *Ceratomyxa shasta*, susceptible host species, epizootiology, clinical signs of disease, and diagnostic procedures can be found in Fish Disease Leaflet 80 (Bartholomew 1989) and in the American Fisheries Society Bluebook (Bartholomew 1994).

[1]The PCR Process is covered by Patents owned by Hoffman-LaRoche, Inc.

II. Sampling Methods

Wet mount and Sample Collection for PCR - *C. shasta* has an affinity for the lower gastrointestinal tract, however, spores may be found in the pyloric caecae, gall bladder, kidney, liver, spleen, and in ascites of severely infected fish. Samples should be taken from living, moribund, or freshly killed fish. Samples intended for visual examination should be held at low temperatures or on ice until assayed. Freezing (-20°C) intestine samples for the PCR procedure is acceptable and will not affect the extraction of *C. shasta* DNA.

A. Expose viscera of fish by making a lateral incision along the length of the abdominal cavity.

B. Excise a 1 - 2 cm long section of the lower intestine using sterile forceps and scalpel.

C. Place sample on a suitable sterile surface such as a section of Parafilm® or disposable petri dish.

D. For PCR, aseptically section a 25 - 50 mg sub-sample of intestine (approximately 1 - 3 mm in length) and place in a sterile 1.5 or 2.0 mL screw cap microtube. Samples from 5 fish may be pooled. The use of a separate blade for each fish or each 5 fish pool is necessary to prevent cross contamination. Inexpensive, non-sterile razor blades (Fisher # 12-640) can be used instead of expensive scalpel blades. Products such as DNA-AWAY can be used to remove DNA if the same blade must be used between samples.

E. If processing several fish at once, place remaining sample for wet mount in a sterile tube or bag and hold at low temperature (<10°C) or fix tissue in 10% neutral buffered formalin.

F. To prepare wet mount, cut along length of intestine to expose inner wall. Using the scalpel blade, scrape intestinal wall and smear onto a sterile glass slide. Dilute smear with 1 - 2 drops PBS, add coverslip, and examine with phase contrast or bright field microscopy at 400X.

Intestinal Lavage - A lavage technique (Coley et al. 1983) can be used to sample the posterior intestine for *C. shasta* without sacrificing the fish. This procedure may be

desirable when sampling fish that are listed as threatened, endangered, or species of special concern. The diameter of tubing, syringe size, and volume of PBS needed to perform intestinal lavage successfully will need to be determined for each particular size class of fish. The technique of Coley et al. (1983) was developed for adult salmon. A separate lavage apparatus (syringe and tubing) would be necessary for each fish or sample site to prevent cross contamination. The sample taken with lavage is examined in a wet mount as above. Lavage samples may also be suitable for the PCR although this has not been tested.

III. Presumptive Diagnosis

Presumptive diagnosis of *C. shasta* is accomplished by examining wet mounts of tissue scrapings and or fluids collected from the posterior intestine of suspect fish. Wet mounts are examined using a systematic search pattern with phase contrast or bright field microscopy at 400X magnification. Presumptive diagnosis requires the observation of multicellular myxosporean spores consistent with the size and shape of *C. shasta*.

IV. Corroboration Diagnosis and Detection of Sub clinical *C. shasta* Infections Using Polymerase Chain Reaction Assay

Amplification of *C. shasta* DNA by means of the polymerase chain reaction (PCR) were developed by Bartholomew et al. (1997a and 1997b), Oregon State University (OSU), Corvallis, Oregon.

A. Preparation of Extraction Buffer

Table 9.1(section 2) - Reagents for DNA extraction buffer.

Stock Reagent	Final Concentration*	Recipe for 100 mL (mL)
NaCl, 5M	100 mM	2.0
Tris-HCl pH 7.8, 1M	10 mM	1.0
EDTA, pH 8, 0.5M	25 mM	5.0
SDS, 10%	1.0%	10.0
H_2O, tissue culture grade		82.0

*NOTE: The buffer recipe is based on molar concentration which is dependent on the concentrations of the stock solution purchased. To calculate the quantities needed, follow the formula C1V1=C2V2, solving for V1=C2V2/C1 as given in the examples below:

Stock Reagents	Catalog Number	Volume for 100 mL	Molar calculation
NaCl 5M	Sigma: S5150	2.0 mL	(100mM) (100 mL)/ 5000mM = 2.0 mL
Tris-HCl pH 7.8 1M	Sigma: T2913	1.0 mL	(10mM) (100 mL) / 1000mM = 1.0 mL
EDTA pH 8.0 0.5M	Sigma: E7889	5.0 mL	(25mM) (100 mL) / 500mM = 5.0 mL
SDS 10X	Gibco: 15553-035	10.0 mL	(0.01) (100 mL) / 0.10 = 10 mL
		qs w/ d-H2O	

1. Prepare DNA Extraction buffer using sterile disposable pipettes, combine reagents in a sterile container and aliquot to 25 mL volumes. The extraction buffer is stable at room temperature so a working stock can be stored at the bench.

2. Store extra buffer at -20°C until needed. Stock reagents (molecular biology grade) can be purchased ready to use from commercial suppliers such as Sigma and Life Technologies-Gibco.

3. **Proteinase K**. This enzyme can be purchased from commercial suppliers such as Boehringer Mannheim (800-262-1640; Cat# 1-373-196) as a solution (14-22 mg/mL) which is stable for > 1 year when stored at 4°C.

4. **RNAse A**. This enzyme can be purchased from commercial suppliers such as 5' - 3' (800-533-5703; Cat# 5-888777) at a concentration of 10 mg/mL. The enzyme is available in a 50% glycerol solution that remains liquid at -20°C.

B. DNA Extraction

1. Collect intestine samples according to methods above. Use all necessary procedures to prevent carry-over and cross contamination.

2. Add 500 µL of DNA extraction buffer to microtube with sample. Add Proteinase K solution to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL).

3. Incubate microtubes at 37°C in a horizontal position on a slow platform rocker or with frequent inversion by hand. Digestion of sample will require about 4-5 hours, but overnight incubation does not affect the quality of the DNA and is recommended.

4. After samples are completely digested, add 5 µL of RNAse A (10 mg/mL stock) to each microtube and digest for 1 hour at 37°C with rocking.

   NOTE: Use care when opening microtubes. Digested sample material may be stuck to the walls and cap of the tube. To minimize the possibility of cross contamination, either by aerosol or contact, centrifuge tubes briefly before opening. Do not touch edge or inside of microtube cap.

5. Place tubes with digested samples in a floating rack and then place in a water bath at 100°C for 5 minutes.

6. Remove rack and cool at room temperature. Upon cooling samples are ready for dilution and PCR. Samples at this stage may be stored at -20°C until needed.

C. Preparation of Master Mix

1. Dilute the DNA template (sample) 1:100 with ultrapure sterile water. Less diluted samples are not recommended and will likely produce variable results because the "crude" DNA preparation contains PCR inhibitors. At OSU, very low level infections were still positive by PCR when samples were diluted 1:1000.

2. Calculate the amount of master mix (MM) required for sample assays (Table 9.2(section 2). Prepare MM without sample in a sterile area separate from any DNA/RNA preparations. Add Taq to MM last. Keep enzymes and reagents on ice during MM preparation.

Table 9.2 (section 2). Reagents for PCR Master Mix for 20 μL reactions (19 μL MM + 1 μL sample).

Reagent	Stock concentration	Final concentration	Vol/reaction (μL)
Sterile d-H_2O			12.8
10X PCR buffer (supplied with Taq)	10X	1X	2.0
$MgCl_2$, 25mM (supplied with Taq)	25 mM	2.0 mM	1.6
dNTPs (Sigma D7295, diluted 1:5)	2.0 mM	0.2 mM	2.0
CS1 primer	50 pmol/μL	0.5 pmol/μL	0.2
CS3 primer	50 pmol/μL	0.5 pmol/μL	0.2
Taq polymerase (Perkin Elmer N808-0161)	5 units/μL	1 unit/reaction	0.2
DNA template (sample diluted 1:100)			1.0
Total			20.0

D. Adding Template DNA

1. In an area separate from MM preparation, assemble 20 μL reactions, 19 μL MM + 1μL diluted template DNA. Keep reactions on ice during assembly.

Note: Relatively large volumes of MM (5.0 mL = 250 reactions), without Taq or sample, can be made-up in advance, aliquoted to convenient volumes (500 µL = 25 reactions), and stored frozen (-20°C) until needed. For each reaction combine 18.7 µL MM + 0.2 µL Taq + 1 µL sample following steps 1 - 3 above. Repeated freeze/thaw of MM is not recommended, but 1-2 times is acceptable.

2. Overlay samples with approximately 25 µL (1 drop) sterile mineral oil. The use of mineral oil to prevent condensation is critical, even with heated lid Thermocycler, because of the small reaction volume.

E. Amplification

Load thermocycler and run the following program:

95°C	3 min	initial denaturation	
94°C	60 sec	denature	35 cycles
58°C	30 sec	annealing	
72°C	60 sec	extension	
72°C	10 min	final extension	
4°C	hold PCR products chilled		

F. Analysis of PCR product

1. Prepare a 1% agarose gel solution and cool to casting temperature. Just before casting, add 1 µL ethidium bromide (EtBr, 10 µg/mL stock) for each 10 mL of agarose gel solution. (Note: staining gels with EtBr solution after electrophoresis is also acceptable).

2. Remove tubes from thermocycler and add 2 µL gel loading solution (Sigma, G-7654) to each reaction. Centrifuge tubes briefly allowing gel loading solution to migrate below the oil overlay.

3. Prepare 100 bp DNA ladder.

4. Load gel with 10 µL of each product and run electrophoresis at 70 - 100 V until products and DNA ladder have sufficient separation.

5. Visualize DNA with UV light. *C. shasta* positive samples will have DNA amplicons of 638 bp from the CS1-CS3 primer set (Table 9.3(section 2)-below).

Table 9.3(section 2) Primers used in the *C. shasta* PCR assay.

Primer	Sequence (5' - 3')	Amplicon size with (reverse primer)
CS1	GGGCCTTAAAACCCAGTAG	(CS3) 638 bp
CS3	CCGTTTCAGGTTAGTTACTTG	

References

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Additional Reading

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CHAPTER 10

Cell Culture of Fish Cell Lines

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I. Introduction

The purpose of this protocol is to provide fish health personnel guidelines for effective poikilothermic cell culture techniques. Cell culture techniques are an important aspect of the quality control program for the National Wild Fish Health Survey. Healthy, sensitive and mycoplasma-free cells are essential for detection of fish viruses in free-ranging fish populations. This chapter outlines specific cell lines to utilize for various fish species, the steps necessary for optimal growth of cell lines, standardization among laboratories, and quality assurance procedures for cell culture.

Redundancy is the cornerstone of a successful cell culture program. Every cell line cultured must be "backed-up" by cells in frozen storage. Cell culture systems are biological entities with specific physiological needs, much like any other laboratory animals. They require ongoing care, adequate nutrition, a proper environment, and regular checkups. The fish health biologist must provide the cultures with an optimum environment for survival. If this environment is not provided, the cells can be unacceptable for viral testing of free-ranging fish populations.

II. Recommended Cell Lines for Various Fish Species

Refer to Table 11.1 (in Chapter 11) - Virology, for a listing of recommended cell lines and incubation temperatures to detect target viruses.

III. Maintenance of Stock Cell Lines and Passage of Cell Monolayers

A. Materials

Fish cell line of choice
L-glutamine - 200 mM (100x)
Minimum Essential Medium (Eagle) with 10% Fetal bovine serum
Trypsin - EDTA
Culture flask (75 cm^2 or 25 cm^2)
Pipettes, sterile, cotton plugged, 1 mL, 5 mL, and 10 mL
70% isopropanol
Graduated cylinder, 100 mL
Glass bottle 100 mL
Beaker, 500 mL
Bleach

B. Selecting and Examining Cell Cultures

1. Work with only one cell line or one kind of primary culture at a time and decontaminate work surfaces between lines with 70% isopropanol. In this way, a single incident of contamination will not affect the entire stock.

2. Make certain that culture medium over cells is optically clear and free of evidence of microbial or fungal contamination.

3. Only mycoplasma-negative cell lines should be used. The presence of infected lines in the laboratory provides an ongoing threat to the health of all cell lines.

4. Examine cell sheet with an inverted microscope and determine that cell morphology agrees with that of the description for that cell line. The cell culture must be confluent, have no foci or areas of necrosis or other factors that indicate culture may be infected or contaminated.

5. Use cells that are relatively young in the stationary phase. Cells allowed to remain too long in the stationary phase do not survive trypsinization and transfer.

C. Preparing a Sterile Work Area

1. Thaw trypsin-EDTA and keep solution cool during use.

2. Wipe down all interior surfaces of the laminar flow hood with 70% isopropanol.

3. Disinfect all items introduced into the hood with 70% isopropanol, e.g., media containers and pipettors.

4. Load work area with appropriate pipettes, beaker, graduated cylinder, glass, bottles, and 75 cm^2 or 25 cm^2 culture flasks.

5. Wash hands and wrists thoroughly with an antibacterial soap prior to working with cell lines. Additional disinfection of the hands and wrists can be done with 70% isopropanol before and after each operation. Disposable latex gloves can also be worn to prevent the drying effects of alcohol to the skin.

D. Preparing a Cell Suspension

1. Aseptically decant medium from the flask of cells into a beaker containing diluted bleach or other disinfectant taking care to prevent backsplash of chlorine or disturbance of the cell layer.

2. Rinse cell monolayer twice with about 5 mL per 75 cm^2 flask or about 2 mL per 25 cm^2 flask of dispersing solution (trypsin-versene (TV) or trypsin – EDTA), wash all internal surfaces of the culture vessel and then decant solution into a beaker containing bleach.

3. Add about 2-3 mL per 75 cm^2 flask or 1-2 mL per 25 cm^2 flask, of fresh dispersing solution using pipettor and make certain that it contacts the entire cell sheet. Rock the culture vessel during the next 3-5 min to ensure contact between dispersing solution and cell sheet and expedite cell separation.

Note: Cells will appear rounded when examined with an inverted light microscope and the monolayer will become opaque and grossly visible.

4. When cells are sufficiently loosened by the dispersing solution, strike the flask lightly on the palm of your hand to dislodge cells. Triturate with a sterile pipette until cells are single or in aggregates of two or three cells. A sterile cell culture scrapper may be used to dislodge a stubborn monolayer.

5. Immediately add at least 10 mL of fresh growth media (MEM-10) to the flask and pipette several times to break up any aggregates of cells.

6. Considerations when working with either ASK or SHK-1 cell lines.

   a. Cell dispersion:

      i. Wash cells twice with sterile PBS without Ca^2.

      ii. Add 1.5 mL trypsin solution to flask (75 cm^2). Watch cells in microscope and pour off excess trypsin before the cells have completely rounded-up. Strike the flask with the palm of your hand to loosen cells from the plate surface. A sterile cell scraper may also be used to remove cells from the flask.

   b. Split ratio:

      i. 1:2 is adequate, but if cells are growing well, 1:3 will also work.

      ii. Split every 7-10 d at 20°C.

   c. Use Leibovitz L-15 cell culture medium (Appendix 11.B-H).

E. Determine the Appropriate Medium Volume and Split Ratio.

Determine the total volume of medium needed per flask to maintain the cell lines until the next propagation. Table 10.1 provides general guidelines of split ratios for various cell types. The culture flasks require minimum volumes as follows:

Medium volume	Flask size
5 mL	25 cm^2
20 mL	75 cm^2
50 mL	150 cm^2

Table 10.1 - Seeding Guidelines for Subculture of Fish Cell Lines.

Cell Lines		Nominal split ratio	Suggested seeding rate (cells/mL)	For 24-well plates (cells/mL)	Incubation temperature	
ATCC[a] designation	Common name[b]				Suggested (°C)	Range (°C)
CRL-1681	CHSE-214	1:4 – 8	200,000	300,000	15 - 20	4 – 27
CCL-55	RTG-2	1:3 – 6	200,000	300,000	15 - 20	4 – 26
	EPC	1:4 – 8	500,000	500,000	20 – 25	15 - 30
CCL-42	FHM	1:4 – 6	500,000	500,000	25 – 30	0 – 36
CCL-59	BB	1:3 – 4	200,000	300,000	25 – 30	20 - 30
CCL-91	BF-2	1:2 – 3	200,000	300,000	25 – 30	20 - 30
	WSSK-1	1:4 – 8	300,000	300,000	15 - 25	15 - 30
	WSS-2	1:4 – 8	300,000	300,000	15 - 20	15 - 25
	CCO	1:2 – 4	200,000	300,000	25 – 30	20 - 30
CRL-2747	ASK	1:2 – 3	200,000	300,000	15 - 20	15 - 20
	SHK-1	1:2 - 3	200,000	300,000	15 - 20	15 - 20

[a]American Type Culture Collection, Rockville, MD designation for Certified Cell line.
[b]CHSE-214,Chinook Salmon Embryo; RTG-2, Rainbow Trout Gonad; EPC, Epithelioma Papulosum Cyprini; FHM, Fat Head Minnow; BB, Brown Bullhead; BF-2, Bluegill Fry; WSSK-1, White Sturgeon Skin; WSS-2, White Sturgeon Spleen; CCO, Channel Catfish Ovary; ASK, Atlantic Salmon Kidney; SHK-1, Salmon Head Kidney.

1. EPC and CHSE-214 cells lines can be split 1:8 provided cells are a confluent monolayer and were recently passed but are older than 10 d. Other cell lines can be split depending on prior performance and the flask size following the guidelines above. Cells should not be allowed to become too thick or old before splitting or they will clump and generally not perform as well as regularly passed cell cultures. Changing the medium in older flasks the day before splitting will help revive inactive cells and reduce clumping and cell debris.

2. If cell counts are done using a hemocytometer they should be approximately 2×10^5 to 5×10^6 cells/mL. Decant or pipette the correct aliquots of cell suspension into each flask; i.e., when diluting a 75 cm^2 flask of EPC cells to make four daughter flasks at a 1:8 split ratio. You must place 84 mL MEM-10/Hepes into a 100 mL glass bottle. After cell sheet is lifted, withdraw 8 mL MEM-10 from glass bottle and place in flask of cells. This will give you the 1:8 split ratio. Triturate to break up clumps. Remove 4 mL of MEM-10 w/ cells from flask and place in 100 mL bottle containing 76 mL MEM-10. The 80 mL of cell suspension is then decanted equally into four 75 cm^2 flasks. Label the flasks with cell line, passage number, date, splitting ratio, operator initials and lot number of medium used as follows:

   EPC Pass #99 08-14-97 1:8 TJO lot 105B (NaHCO3)

3. Incubate cells at the optimum growth temperature (Table 10.1) and allow them to form a cell monolayer without changing the medium.

4. After a salmonid cell layer is confluent, the flask can be transferred to a 10 - 15°C incubator for holding until use. If they are to be held for extended periods of time (up to 3 months) the medium should be replaced with MEM-5 and the cells incubated at 4°C. With a change of growth medium and return to appropriate incubation temperatures the cells resume normal replication.

IV. Cell Counting Using the Hemocytometer

A. Introduction

Rarely are cells counted during routine propagation of cell lines, however the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0 mm^2 squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 $mm^2 \times 0.1$ mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 mL, the cell concentration/mL is the average count per square $\times 10^4$. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 min at 500 rpm) and resuspended in protein-free medium or HBSS prior to counting.

B. Materials

Hemocytometer chamber
75 cm^2 flask of cells
Trypan Blue (0.1% in PBS)
Microscope
Dilution tubes (12 x 75 mm)
Pasteur pipette
HBSS, or MEM-0 (Appendix 11.B: Media for Cell Culture and Virology)
Trypsin - EDTA
Pipettes 1 mL, sterile, cotton plugged

22 x 22 mm cover-slips

C. Procedure

1. Select a healthy (log phase) 75 cm^2 flask of cells and remove cells from flask surface following methods described in "Maintenance of stock cell lines and passage of cell monolayers".

2. Re-suspend cells in MEM-0. For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20 - 50 cells/mm^2 (1×10^5 to 2×10^5 cells/mL). Dilutions vary depending on age of the cells, cell density and cell aggregation.

3. Aseptically transfer 0.5 mL of the cell suspension into a dilution tube.

4. Add 0.5 mL Trypan Blue stain (0.1%).

   Note: if cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take-up dye.

5. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipette to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or under-fill the chambers.

6. Using a microscope with a 10X ocular and a 10X objective, count 10 squares (5 from each chamber) as outlined above.

7. Calculate the # of cells/mL and the total # of cells as follows:

   Cells/mL = x (mean) count per square $\times 10^4 \times$ Trypan Blue dilution factor

   Total cells in flask = cells/mL $\times$ total volume of cell suspension

   e.g., total # cells counted in 10 squares = 300 cells

   x count/square = 300 cells/10 squares = 30 cells

   cells/mL = $30 \times 10^4 \times 2$ (dilution factor)
   cells/mL = 60×10^4 cells/mL
   cells/mL = 6.0×10^5 cells/mL

   Total cells = $6.0\ 10^5$ cells/mL $\times$ 8 mL (original volume cell suspension)
   Total cells = 48.0×10^5 cells
   Total cells = 4.80×10^6 cells

8. If the cells/mL calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

   mL medium needed = (actual cells/mL) (vol. of cell suspension)/ desired cells/mL

   e.g., actual count = 6×10^6 cells/mL
   desired count = 1×10^6 cells/mL
   volume of cell suspension = 8 mL
   mL medium needed = x

   x = mL medium needed = 6×10^6 cells/mL × 8 mL / 1×10^6 cells/mL

   mL medium needed = 48×10^6 mL / 1×10^6

   = 48 mL

   Since you have 8 mL already in the flask, you would need to add 40 mL of medium to the flask before splitting to get the recommended seeding cell density for each new culture.

V. Cryopreservation of Fish Cell Lines

A. Materials

Fish cell line of choice (75 cm^2 flask)
Trypsin-versene solution
MEM-10
Freezing medium (Origen or similar product)
Sterile (internal thread) cryovial tubes
Pipettes, 5 mL, sterile, cotton plugged
12x75 mm test tube, sterile
75 cm^2 flasks

Note: protective safety glasses and gloves are required when handling vials stored in liquid nitrogen.

B. Freezing Cells

1. Change medium in a flask of rapidly growing (log phase) cells. Incubate at appropriate temperature for cell line of choice for 24 h.

2. Pour off medium and harvest cells according to method in Section III. Seed two 75 cm^2 flasks and incubate overnight at appropriate temperature.

3. Remove cell monolayers from the flasks using method described in Section III. The cells should be rapidly growing however the flasks do not need to be 100% confluent.

4. Transfer the cell suspension to a sterile 12x75 mm test tube and centrifuge at 1000 rpm for 15 min to form a cell pellet.

5. Pour off supernatant and re-suspend cell pellet with 3 mL of Origen Freezing Medium™, or other suitable solution such as 7% DMSO.

6. Aliquot 1.0 mL of cell suspension into each of 3 cryovials. Seal and label with cell line, passage number, and date.

   Note: before beginning the freezing process, the cells should equilibrate with the freeze medium for 5 min but no longer than 10 min, including the time it takes to resuspend and transfer the cells to the cryovials.

7. Place the cryovials in a freezing container containing isopropanol and freeze at -70°C for 24 h.

8. After 24 h, remove one cryovial to test for cell viability:

   a. Rapidly defrost the contents of the cryovial in 30°C water bath.
   b. Disinfect the outside of the cryovial with isopropanol.
   c. Resuspend the cell pellet in 5 mL of MEM-10 and plate in a 25 cm^2 flask.
   d. Incubate the cells for 24 h an appropriate temperature.
   e. After 24 h, observe flask for the attachment of cells and cell growth.

9. If the test culture has survived the freezing process, the remaining cryovials can be transferred into liquid nitrogen or -70°C ultra-low freezer for long term storage.

C. Thawing Cryopreserved Cells

1. Before thawing the cells, prepare and warm the MEM-10 to 15°C.

2. Pipette warmed growth medium (10 x the cell suspension) into a sterile 12x75 mm test tube. Repeat for the number of cryovials being thawed.

3. Thaw the cryovials of cells quickly in a 37°C water bath. As soon as the ice has melted, remove the cryovial from the water bath and decontaminate the outside of the cryovial with 70% isopropanol.

4. Aseptically transfer the cryovial contents to a sterile 25 cm^2 culture flask containing 5 mL of MEM-10. Incubate 24 h at 20°C to determine cell viability. Exchange the growth medium after 24 h.

VI. Quality Control in Cell Culture

A. Precepts of Cell Culture

The following list provides guidelines in maintaining fish cell lines and avoiding cell culture contaminants:

1. Maintain stock cultures separate from working cultures.

2. Employ a redundant approach for maintaining stock cultures.

3. Employ a testing program for mycoplasma for each serum supply (Section VII).

4. Use high-purity or cell culture quality water for all solutions.

5. Test the sterility of all locally prepared solutions before use. Discard any reagent of medium in which contamination is observed.

6. Avoid the use of any antibiotics in maintaining stock cultures.

7. Never mouth pipette samples, cell lines, or media.

8. Work with only one cell line at a time to prevent cross contamination by cells and/or potential contaminants.

9. Never handle all cultures of a cell line in one day. This prevents the risk of contaminating an entire active stock of a cell line if contamination is accidentally introduced.

10. Prepare and keep multiple daughter cultures.

11. Subculture stock cultures infrequently and keep salmonid cell lines at low temperature (4°C).

12. On an annual basis, discard working cultures of the previous year and replace with new working cultures from active or frozen stock cultures that have been tested for viral sensitivity and are mycoplasma free.

13. Test cell lines for mycoplasma contamination at 6 month intervals (Section VII).

14. Keep detailed records of solution preparation and cell histories.

15. Have laminar flow hoods certified by a reputable company on an annual basis.

B. Standardization of Cell Lines for the National Wild Fish Health Survey

Standardization of initial viral testing and corroborative methods is an important component of the Wild Fish Health Survey. All viral testing will utilize cell lines traceable to cell lines from the American Type Culture Collection (ATCC) when available. At the minimum, cell lines will be tested annually for mycoplasma infection and every three years for viral sensitivity. See Chapter 11, Table 11.1, for a list of primary and complimentary cell lines for the detection and isolation of specific viral pathogens of fish.

VII. Mycoplasma Screening of Continuous Cell Lines

Mycoplasma screening should be an integral part of the quality control program within all fish health diagnostic laboratories utilizing cell culture systems. Mycoplasmas are microbes that lack cell walls and are related to, but distinct from, bacteria. With a minimum size of 0.2 to 0.3 µm, mycoplasmas are among the smallest free living organisms known.

Mycoplasmal contamination affects virtually every measurable cell culture parameter. Various enzymes, cytokines, and differentiated cellular functions can be suppressed and/or induced. Mycoplasmas may alter cell surface antigenic characteristics. Mycoplasmal contamination may induce chromosomal breakage, deplete nutrients from growth medium, and interfere with viral expression and/or viral isolation. Plaque and cellular morphology may be altered.

Mycoplasmas may be introduced by contaminated sera or reagents of a bovine or piscine origin (serum and trypsin), or contamination during handling of cell lines. However, the majority of contamination can be attributed to cross-contamination from other infected cell lines. Critical to preventing the majority of mycoplasma contamination is the establishment of effective aseptic techniques and routine testing. Effective aseptic techniques such as clean disinfected work surfaces, clean hands, and workers abstaining from talking during culture manipulations will prevent most sources of mycoplasma infection.

A. Frequency of mycoplasma testing.

1. Routine testing should include semiannual screening of stock cell lines. Sera and trypsin should be tested on a lot number basis.

2. All cell line stocks to be stored under cryogenic conditions should be tested prior to freezing. In addition, all cell lines received from outside sources, including commercial cell banks, should be quarantined and tested prior to use.

B. Screening Methods.

There are a number of direct and indirect procedures for the detection of mycoplasma contaminants in cell cultures and in cell culture reagents and media. No single method may be entirely adequate and each should be evaluated for overall sensitivity and performance. Several options are included here in no particular order These include biochemical analysis, DNA staining, DNA probes, PCR primer kits, and standard and real-time QPCR kits.

1. Fluorescence microscopic assay. The MycoFluor™ Mycoplasma Detection Kit may be purchased from Molecular Probes, Invitrogen Detection Technologies (Cat. no. M-7006). (800) 438-2209 or on the web at: http://www.probes.com/servlets/product?item=7006

2. Enzyme immunoassay for the detection of mycoplasma/acholeplasma in cell culture (*M. arginini*, *M. hyorhinis*, *A. laidlawii*, *M. orale*). This mycoplasma detection kit may be purchased from Roche Applied Science. (800) 428-5433 or on the web at: http://www.roche-applied-science.com/cfm/country_id_a.jsp

3. Photometric enzyme immunoassay for the detection of PCR-amplified DNA of mycoplasma/acholeplasma/ureaplasma in cell culture. This mycoplasma detection kit may be purchased from Roche Applied Science. (800) 428-5433 or on the web at: http://www.roche-applied-science.com/cfm/country_id_a.jsp

4. MycoTect™ Kit. A mycoplasma-infected culture can be detected by incubation with 6-MPDR and subsequent monitoring for mammalian cell toxicity. Kit may be purchased from Invitrogen™ (Gibco), (888) 584-8929 or on the web at https://catalog.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&productDescription=171

6. MycoAlert™ Mycoplasma Detection Assay, a selective biochemical test that exploits the activity of mycoplasmal enzymes. May be purchased from Cambrex Bio Science Rockland, Inc. On the web at: http://www.cambrex.com/CatNav.asp?oid=887&prodoid=Mycoalert

5. Mycoplasma Detection Kit, 16S rRNA Gene (Mixture of 18 Different Primers), 50 Rxns. This PCR kit may be purchased from Maxim Biotech, Inc., (800) 989-6296 or on the web at: http://www.maximbio.com/

7. MycoProbe™ Mycoplasma Detection Kit from. Probes will detect *M. hyorhinis* , *M. arginini, M. fermentans, M. orale, M. pirum, M. hominis, M. salivarium,* and *A. laidlawii*. Kit may be purchased from R&D Systems, (800) 343-7475 or on the web at: http://www.rndsystems.com/asp/g_sitebuilder.asp?bodyId=648

8. Mycoplasma Plus™ PCR Primer Set. Primer design permits higher annealing temperatures, reducing spurious bands from contaminants, Contains: Mycoplasma Plus PCR primers, Positive control, Internal control, StrataClean™ resin. May be purchased from Stratagene, (512) 321-3321 or on the web at: http://www.stratagene.com/products/

9. MycoSensor™ QPCR Assay Kit. Detects mycoplasma infection of cell cultures by real-time quantitative PCR (QPCR), using SYBR® Green dye detection, in less than 2 h. Convenient master mix format provides all the reagents needed for amplification and fluorescence detection, and the kit detects the eight most common cell culture contaminating species of mycoplasma. Can detect as few as 50 copies of contaminant

genomic DNA from 100 µl of cell culture supernatant, or test for weak Mycoplasma infection in extracts made from cell pellets. May be purchased from Stratagene, (512) 321-3321 or on the web at: http://www.stratagene.com/products/

10. DNA Fluorochrome Staining
DNA fluorochrome procedure has been shown to be 96-98% effective. It is a good, reliable indirect assay, however it requires some expertise in diagnosing low-level contamination.

a. Materials

Cell culture cells grown in antibiotic-free medium for at least 3 passages.
Trypsin-EDTA solution
Petri dish (use sterile non-toxic, "cell culture clean")
Sterile coverslips 22x22 mm (heat or flamed sterilized)
MEM-10
Pipettes 5 mL, 10 mL, sterile, cotton plugged
20 mL cold , fresh fixative
Forceps
Bisbenzimide staining solution
Microscope slides
Mounting medium
Pasteur pipettes
Positive and Negative controls
Coverslips

b. Reagents
Refer to MSDS and use personal protective equipment when working with Bisbenzamide which known to be a strong mutagen.

i. Bisbenzamide Fluorochrome Stain

<u>Stock Solution</u>

Bisbenzamide fluorochrome stain	5.0 mg
HBSS 1x, w/o NaHCO3 and phenol red	100 mL
Thimersol (final conc., 1:10,000)	0.01gm

Mix thoroughly at 22-25°C w/ magnetic stirrer for 30 min. Wrap bottle in aluminum foil and store in dark at 2-8°C.

<u>Working Solution (100 mL, 0.05 µg/mL)</u>

Bisbenzamide fluorochrome stock solution	0.1 mL
HBSS 1X	100 mL

(Optimal fluorescence may range from 0.05 to 0.5 ug/l)

Store in a dark bottle wrapped in aluminum foil at 2-8°C. Mix thoroughly with magnetic stirrer at 22-25°C for 30 min prior to use. Examine periodically for microbial contamination. Millipore filtration diminishes fluorescence.

ii Glacial Acetic Acid - Methanol (1:3) Fixative

c. Procedures:

i. Plating cells:

1) Examine the cell sheet under inverted microscope.

2) Decant growth medium from flask.

3) Remove cell monolayer from flask using method described in "Maintenance of stock cell lines: passage of confluent cell monolayers".

4) Place 10 mL MEM-10 in petri dish containing sterile coverslips.

5) Using the same pipette, add 4 to 5 mL MEM-10 to the flask of cells and triturate the cell suspension to disrupt clumps.

6) Place 5 mL of the cell suspension into the petri dish and discard the remainder.

7) Label the petri dish with your initials, and date. Incubate at the same temperature used for propagation of the cell culture being tested.

ii. Staining cells:

1) Carefully examine coverslips in petri dish using inverted microscope.

2) If the cells are healthy and confluent, draw off the medium with a 10 mL pipette.

3) Immediately add 10 mL cold, fresh fixative to the petri dish. Fix for 5 to 10 min.

4) Pipette off the fixative and repeat the fixative step.

5) Remove the second fixative application and thoroughly drain the coverslips.

6) Flood the coverslips with several drops of bisbenzimide stain, and allow them to incubate for 30 minutes at ambient temperature. Remember to mix stain prior to use.

7) Remove the stain with a Pasteur pipette.

8) Rinse the coverslips 3 times with sterile distilled water.

9) Drain one coverslip on a paper towel.

iii. Mounting and Examining Cells:

1) Label a slide with the position of the coverslips; i.e., (+), (-), and test.

2) Place 3 drops of mounting medium on the slide.

3) Mount positive and negative controls and test coverslips cell side up on the microscope slide.

4) Add an additional drop of mounting medium to the top surface of each coverslip.

5) Place a 22x50 mm coverslip over all.

6) Examine the slide using oil immersion with a fluorescence microscope for the presence of yellow-green fluorescent mycoplasma on the surface of individual cells.

7) Compare the test samples to the negative control to assess the amount of non-specific background staining or fluorochrome debris that may occur.

8) Compare any suspect samples to the positive control slide for size, location and staining intensity of any suspect bacteria.

Additional Reading

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CHAPTER 11

Virology

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I. Introduction

Detection or isolation of aquatic animal viruses historically has been by inoculation of tissue homogenates onto fish cell or tissue cultures appropriately researched and chosen for the propagation of target viruses. A review of many of the important viruses affecting fish and tissue culture techniques for their isolation can be found in Wolf (1988). For this Survey, standardized cell culture techniques will be used for initial screening of appropriate fish tissues for listed viral pathogens (Table 11.1). When CPE is detected in cell culture, suspect tissue culture fluids (TCF) will be tested with appropriate corroborative tests (Chapter 12 - Corroborative Testing of Viral Isolates).

Currently, eight viral pathogens of fish are included in the Survey (Table 11.1). These are divided into primary viral pathogens and pathogens of regional importance (PRI). Screening for primary viral pathogens is performed on all appropriate fish (taxonomic family) at all Service Fish Health Centers (Table 11.2). Screening for viral PRI is performed at the discretion of the testing laboratory.

Definitions: Several terms are used routinely in virology and throughout this chapter. A glossary of terms is provided in Appendix 11.A.

Media Formulations: See Appendix 11.B: Media Used in Tissue Culture and Virology.

Table 11.1.– List of fish viruses approved for the Survey and recommended cell lines for their detection and isolation. Corroborative tests given here are explained in detail in Chapter 12.

Fish virus[a]	Primary cell line	Complimentary cell line	Incubation temperature (°C)	Corroborative test(s)[c]
IHNV	CHSE-214	EPC	15	PCR, SNA, IFAT, BDNAP
IPNV	CHSE-214	EPC, FHM	15	PCR, SNA
ISAV[b]	ASK, SHK-1	CHSE-214	15	PCR
LMBV	FHM, BF-2	EPC	20-25	PCR
OMV	CHSE-214	EPC	15	PCR
SVCV[b]	EPC, FHM	CHSE-214	20-25	PCR
VHSV	EPC	CHSE-214	15	PCR, SNA, BDNAP
WSHV[b]	WSS-2	WSSK	20	PCR

[a] Viruses: IPNV- Infectious Pancreatic Necrosis Virus and other related aquatic birnaviruses; WSHV - White Sturgeon Herpesvirus; IHNV- Infectious Hematopoietic Necrosis Virus; VHSV - Viral Hemorrhagic Septicemia Virus; OMV - *Oncorhynchus masou* Virus; LMBV – Largemouth Bass Virus; SVCV, Spring Viremia of Carp Virus.
[b] Viral Pathogen of Regional Importance (PRI).
[c] PCR- Polymerase Chain Reaction; SNA- Serum Neutralization Assay, IFAT- Indirect Fluorescent Antibody Test; BDNAP- Biotinylated DNA Probe.

II. Selection of Appropriate Cell Lines

All viral testing will utilize cell lines traceable to cell lines from the American Type Culture Collection (ATCC) when available. At the minimum, cell lines will be tested annually for viral sensitivity and semiannually for mycoplasma infection (Chapter 10, Sections VI and VII).

Two cell lines, primary and complimentary, will be used for each tissue sample set. Cell lines in addition to the primary and complimentary lines may be used at the discretion of the testing laboratory. In some cases, wild fish surveys may target specific viral pathogens based on local or regional interests. Refer to Table 11.1 for recommended cell lines based on the list of fish viruses included in the Survey and refer to Table 11.2 for recommended cell lines based on the taxonomic family of fish being tested.

Table 11.2.– Recommended cell lines to detect target viruses in various fish families. Abbreviations of fish viruses are explained in Table 11.1.

Fish family	Primary cell lines	Incubation temperature (°C)	Complimentary cell line	Incubation temperature (°C)	Fish virus
Acipenseridae (sturgeon)	WSS-2, WSSK-1	15-20	CHSE-214	15	WSHV
Polyodontidae (paddlefish)	CHSE-214	20	WSS-2, WSSK-1	15	IPNV
Percichthyidae (temperate bass)	FHM	15	CHSE-214	25	IPNV, LMBV
Cyprinidae (minnows / carp)	EPC, FHM	15	CHSE-214	25	IPNV, SVCV
Catostomidae (suckers)	FHM	15	CHSE-214	25	IPNV
Centrarchidae (sunfishes)	FHM, BP-2	15	CHSE-214	25	IPNV. LMBV
Percidae (perch)	FHM	15	CHSE-214	25	IPNV. LMBV
Salmonidae (trout / salmon)	CHSE-214	15	EPC	15	IPNV, IHNV, VHSV, OMV
Clupeidae (herring)	CHSE-214	15	EPC	15	IPNV, IHNV, VHSV, OMV
Ictaluridae (Catfish)	BB, FHM	15	CHSE-214	25	IPNV
Other	FHM	15	CHSE-214	25	IPNV

[a] Viruses: IPNV- Infectious Pancreatic Necrosis Virus and other related aquatic birnaviruses; WSHV - White Sturgeon Herpesvirus; IHNV- Infectious Hematopoietic Necrosis Virus; VHSV - Viral Hemorrhagic Septicemia Virus; OMV - *Oncorhynchus masou* Virus; LMBV – Largemouth Bass Virus; SVCV, Spring Viremia of Carp Virus.

III. Target Tissues

For purposes of the Survey, most tissue collection will focus on tissue samples from sub-adult animals. If sexually mature adults are available, the additional testing of coelomic fluid (ovarian fluid) from gravid females or seminal fluid from gravid males may also be performed. Individual samples are encouraged, but samples may be pooled if necessary. No more than 5 individuals may be pooled into a single sample and samples from individuals must be approximately the same size. The following outline summarizes suggested sampling but the individual situation will dictate the best sample for viral testing.

A. Juvenile Fish ≤ 7.0 cm in Length.

1. Fish 2.5 cm or less in length - use whole, cut off and discard any visible yolk sac.
2. Fish 2.5 to 4.0 cm - cut off and discard heads and tails.
3. Fish 4.0 to 7.0 cm - use viscera.

B. Juvenile Fish > 7.0 cm in Length

1. Kidney and spleen.

C. Adult Fish (sexually mature)

1. Females: kidney and spleen, ovarian fluid from spawning or post-spawning fish.
2. Males: kidney and spleen, seminal fluid can be useful if organs are unavailable.

IV. Tissue Collection Procedures

Care is taken during sample collections to protect samples from UV light, freezing, or high temperatures that are lethal to the viruses of interest. Aseptic technique is required.

A. Whole Alevin or Juvenile Fishes < 7 cm in Length

1. Place 1 to 5 fish samples as described above into each Whirl-Pak® bag or sterile snap cap tube per pooled sample. Keep all samples cool during the collection procedure.

B. Tissue Samples from Fish > 7 cm in Length

1. Aseptically, remove a piece of the kidney and spleen from each fish with forceps, scissors, scalpel and/or tongue depressor. For pooled samples, combine tissue samples from each fish into a single Whirl-Pak® bag or snap cap tube. The total sample for each bag or tube should be at least 0.5 g of tissue.

2. Seal Whirl-Pak® bag or tube. Keep samples cool while collecting remainder of samples.

3. Between each fish or pooled sample, clean instruments of any tissue with gauze sponges dipped in 70% ETOH, alcohol rinse, or wipe with iodophor followed by sterile water rinse.

C. Procedures for Collecting Coelomic (Ovarian) Fluid Samples

1. Disinfect the abdomen of the fish with iodophor and wipe with a clean paper towel to remove any disinfectant or mucous which could drip into the sample.

2. Partially strip ovarian fluid from one female fish into a clean paper cup. If possible, avoid extrusion of blood, fecal material, and nematodes.

3. Crimp edge of paper cup to "strain out" any eggs present and pour 2-5 mL ovarian fluid from each fish into one tube per fish if samples are individual. Pour 1-2 mL per per fish if samples are pooled. Do not fill tube completely full. Do not palm or warm fluid, which could inactivate low levels of virus if present.

4. Tightly cap and place in tube rack. Keep all filled tubes in cooler chest on blue or wet ice while collecting remainder of samples.

D. Alternative Method for Collecting Coelomic (Ovarian) Fluid Samples

Method for drawing ovarian fluid using an automatic pipettor if *in situ* contamination is a problem.

1. Install sterile tip on pipettor. Insert tip into the uro-genital opening of the fish while applying light pressure to the body.

2. Draw up a sample of ovarian fluid and place in one sterile tube per fish or pooled sample. Discard tips between samples.

E. Procedures for Collecting Seminal Fluid

1. Express seminal fluid from gravid male into clean paper cup.

2. Pour 2-5 mL into tube for individual samples. Pour 1-2 mL into tube for pooled samples.

3. Tightly cap and place in tube rack.

4. Keep all filled tubes in cooler chest on blue or wet ice while collecting remainder of samples.

V. Transport of Tissue Samples

A. Packing and Shipping Samples

1. Check seals on bags or tubes to ensure closure.

2. Place Whirl-Pak® bags into large plastic bag. Label bag with number of samples, location sample taken, sample type, date, life stage, species of fish and enclose a completed sample Submission Form (Appendix 2.B).

3. Place tubes in proper rack, enclose in large plastic bag and label with appropriate information according to submission form.

4. Keep samples cool by transporting them in a suitable container with blue ice or bagged wet ice. Do not allow samples to freeze or expose them to UV light.

5. Check with receiving laboratory on special shipping instructions if samples are not delivered in person.

VI. Processing Tissue, Coelomic and Seminal Fluid Samples

A. Processing Tissue Samples

All samples for viral testing should be processed within 48 h and inoculated onto cell lines within 72 h of collection. As during sampling and transport, care is taken to protect samples from UV light, freezing, or high temperatures that are lethal to the viruses of interest. Aseptic technique is required.

1. If transport medium (Appendix11.B-B.2) is used, it is poured off and disinfected before discarding.

2. Tare balance with an empty tube or bag and weigh sample to the nearest 0.1 g.

3. Add sample dilution medium (Appendix 11.B-A) to equal a 1:10 dilution (w/v). If toxicity is likely or suspected, additional tissue dilutions from 1:20 to 1:100 can be made. Final dilution prior to inoculation of tissue samples onto cell cultures must not exceed 1:100 (v/v).

4. Homogenize samples using a Stomacher® (Virtis® or Contorque® grinders require considerable disinfection of containers between samples). Pour or pipet ~ 3-4 mL into a 12x75mm snap-cap tube.

5. Centrifuge tissue samples at 2000-3000 X g for 20 min.

6. Without disturbing the pellet, aseptically pipette 1 mL supernatant from each sample into its respective tube containing 1 mL of antibiotic incubation medium (Appendix

11.B-B or C) and vortex. Sample dilution is now 1:20 v/v. Depending on tissue type, fatty material may accumulate near the surface of the tube. Be sure to collect supernatant from below the floating material. Label tubes.

7. Incubate for 2 h at 15°C or 12-24 h at 4°C.

8. Samples are re-centrifuged at 2000-3000 X g for 15 min and supernatant is inoculated onto tissue cultures as described in inoculation procedures (Section VIII).

B. Processing Coelomic (Ovarian) and Seminal Fluids

1. Centrifuge ovarian or seminal fluid samples at 2000X g for 20 min if using polypropylene tubes. It is optimal to process in a refrigerated centrifuge at 4°C to prevent warming of the sample.

2. Undiluted ovarian fluid may be used to inoculate cell cultures or up to a 1:5 dilution (1 part ovarian fluid to 4 parts antibiotic incubation medium; Appendix 11.B-B) may be used. If a dilution is made, aseptically pipette supernatant without disturbing pellet from each sample into its respective tube containing antibiotic incubation medium and vortex. Label tubes.

3. Incubate for 2 h at 15°C or 12-24 h at 4°C.

4. Samples are re-centrifuged at 2000-3000 X g for 15 min and supernatant is inoculated onto tissue cultures as described in inoculation procedures (Section VIII).

VII. Preparing Viral Test Plates

The quantal assay (also referred to as endpoint dilution) is used to examine fish when only the presence or absence of a virus needs to be verified which is the purpose of the Survey. Flat-bottomed 24-well plates are usually used for this assay but other cell culture plates may be used if applicable.

For determination of the Tissue Culture Infective Dose - 50% endpoint ($TCID_{50}$) of a virus sample or isolate, replicate samples are necessary and 96-well plates become more useful. The $TCID_{50}$ assay is not routinely used in the Survey because the numbers of replicate dilutions required are often not practical. Thus, no methods will be included in this manual. Reed and Muench (1938) and Rovozzo and Burke (1973) describe the procedures for the $TCID_{50}$ assay. The plaque assay is another quantification method that determines plaque forming units (PFU) or infectious particles (I.P.) of a sample. Flat-bottomed 24-well plates may be used for this, but several dilutions are necessary to accurately assess the titer of PFUs. The Survey has determined not to use this test for screening. Burke and Mulcahy (1980) describe the procedure for the plaque assay.

A. Seeding Flat-Bottomed 24 (16mm) Well Plates

1. Determine number of plates needed for the assay. Remember that all samples will be inoculated onto at least two cell lines. Samples will be inoculated in duplicate on each cell line (typically 12 samples per 24-well plate). Additionally, both negative (sham and monolayer controls) and positive (when appropriate) controls should be performed each day plates are inoculated with test samples.

2. Remove confluent cell monolayer from a tissue flask using methods described in Chapter 10, Section III, Maintenance of Stock Cell Lines and Passage of Cell Monolayers.

3. Pipette 0.5–1.0 mL cell suspension into each well of the plate(s). Mix the cell suspension frequently to keep the cells homogeneously suspended.

4. Add a few extra drops of MEM-10 to all corner wells to compensate for evaporation.

5. Any liquid spilled between wells may be aspirated off or dried by use of sterile gauze. Cover each plate with the accompanying lid.

6. Label each plate with the date, cell line initials, passage number, and operator initials. Seal lid to base with tape and place plate(s) in a plastic bag or into an airtight plastic container.

7. Incubate at a temperature appropriate for the particular cell line (Table 11.1) until at least 80% confluent without changing the medium. Following seeding guidelines from Chapter 10 (Table 10.1), monolayers should be confluent within 24 h. If necessary, plates can be made the same day as inoculation but they will have to be seeded with more cells. However, same day inoculation does not necessarily result in any earlier detection of virus.

VIII. Inoculating Plates with Samples

A. Materials.

1. Appropriate number of cell monolayers to be inoculated which are at least 80% confluent, approximately 24 h old (do not use plates in which cells have been confluent more than 3 d), and are visually healthy. EPC cell monolayers may need to be 100% confluent to avoid retraction of cell margins when incubated at 15°C.

2. Ovarian fluid and/or tissue samples.

3. Dilution blanks with HBSS or MEM (Appendix 11.B).

4. Pipettor and sterile pipette tips.

5. Plate seal, plastic bags, or airtight plastic containers.

B. Inoculation Cells with Samples.

1. Label each plate with the inoculation date and case number. The assay is usually done with the plate aligned with 6 columns across the top and 4 rows down. Label wells with sample numbers and identify controls.

2. Decant medium from wells by inverting the plates over a bleach bucket or autoclave bag. A small amount of medium may remain on the cells to prevent drying in the center.

3. Inoculate with replication a minimum of 50 µL of sample per 1.0 cm^2 of cell sheet. Wells of most 24- well plates are 2 cm^2, therefore each well is inoculated with a minimum of 100µL of sample.

4. Inoculate negative control wells with 100 µL of antibiotic incubation medium diluted to the same concentration as the test samples.

5. To allow for viral adsorption, incubate plates for 1 h with gentle rocking at least every 15 min or continuously on a laboratory rocker at temperatures as follows:

   15°C for IPNV, IHNV, VHSV, ISAV, and OMV.

   20-25°C for LMBV, SVCV, and WSHV.

6. Dispense an adequate volume of appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (Appendix 11.B-F) works well in an open system for all cell lines listed in Table 11.1 except ASK and SHK-1 which respond better to Leibovitz L-15 (Appendix 11.B-H). When using 24-well plates, 0.5 mL of medium per well is generally adequate.

7. Seal each plate with plate film, or place in an airtight container.

8. Following inoculation of monolayers, remaining tissue or ovarian fluid products are kept at 4°C until completion of all assays. Subsequent to the completion of all assays, all material is decontaminated and discarded.

C. Minimum Levels of Detection (assuming replicate wells)

1. For tissues, it is 50 infectious particles (I.P.)/g pooled sample or 250 I.P/mL/fish.

2. For ovarian fluid, not pooled, it is 10 I.P./mL.

3. For ovarian fluids, pooled, it is 10 I.P./mL pooled sample or 50 I.P./mL/fish for a 5 fish pool.

IX. Viral Plate Observation

Following inoculation of plates, all wells will be monitored on the following day and every other day for the next two weeks for signs of CPE, toxicity, and contamination. Plates will be monitored twice during the following week. Total observation period will be 21 d.
If no CPE, toxicity or abnormalities are observed within 21 d, the samples are discarded and recorded as negative.

A. Re-Inoculation

If toxicity, abnormal pH or CPE is observed, one of the replicate wells of that sample will be aseptically aspirated, diluted 1:10 with MEM-0, filtered through a 0.45µm filter and re-inoculated onto another 24-well test plate in duplicate and monitored for CPE an additional 14 d. All observations will be documented and recorded by the observer and kept on file with the laboratory records. If no CPE is observed in 14 days after re-inoculation, the sample is discarded and recorded as negative. If CPE is observed, proceed to corroborative methods to confirm identity of suspect virus (Chapter 12).

B. Cytopathic Effects (CPE) of Virus Infection in Tissue Culture Cells

In addition to the descriptions of CPE given here, CPE descriptions including photographs can be found in Standard Procedures for Aquatic Animal Health Inspections (USFWS and AFS-FHS 2004).

1. IHNV-induced CPE

   a.. Rounded and granular cells in grape-like clusters.

   b. Margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened).

   c. Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque.

   d. CPE typically appears in 2-10 d. Blind passes should be performed at 10-14 d and monitored for an additional 14 d.

2. IPNV-induced CPE

   a. Spindle-shaped or "balloon-on-a-stick"-shaped cells.

   b. Pyknosis of nuclei (nuclei shrink in size and chromatin condenses).

c. Plaques are stellate in a confluent cell monolayer and contain not only live cells but also normal looking cells (these are persistently infected and will reform a normal monolayer that is virus positive).

d. Little cellular debris.

e. CPE typically appears in 2-10 d. Blind passes should be performed at 10-14 d and monitored for an additional 14 d.

3. Herpesvirus-induced CPE

a. Pyknosis of nuclei and cellular fusion (syncytia).

b. Syncytia produce multinucleated giant cells.

c. Plaques tend to elongate and follow whorl lines of growth if on RTG-2 cells. They have relatively clear interiors, but living cells extend into the open area.

d. Little cellular debris.

e. CPE may be evident in 14-30 d. Frequently, CPE does not appear on initial culture and samples must be blind passed at 21-28 d and monitored for an additional 28 d.

4. VHSV-induced CPE

a. The North American VHSV isolates plaque very similarly to IHNV in EPC cells forming rounded and granular cells in grape-like clusters.

b. The European VHSV isolates differ from IHNV on RTG-2 cells by having more regular plaque margins with uniformly distributed granular debris within the plaques. Also, affected cells do not show margination of chromatin.

c. Number of days following infection with virus that CPE is usually observed in freshly monolayered fish cell cultures.

d. CPE typically appears in 2-10 d. Blind passes should be performed at 10-14 d and monitored for an additional 14 d.

5. LMBV-induced CPE

a. CPE within 48 h after inoculation.

b. Initial CPE - few pyknotic cells, which develop to form circular, cell free areas, with rounded cells at the margins.

c. Advanced CPE - Pyknosis, rounding and detached cell sheet. Entire cell sheet affected.

d. CPE typically appears in 2-10 d. Blind passes should be performed at 10-14 d and monitored for an additional 14 d.

Note: Toxicity can sometimes mimic viral CPE. Observing the gradual development of plaques over several days is the best way to distinguish viral CPE from toxicity.

C. Intensity of CPE

Monolayers are examined with an inverted light microscope at low power (125X) to determine intensity of CPE. This general scoring scheme is used to record CPE intensity:

+1 = Only one field observed contains CPE
+2 = Two or more fields observed contain CPE
+3 = All fields observed contain CPE
+4 = CPE throughout entire monolayer or monolayer no longer attached to flask/plate. (Note: Separation or retraction of cell monolayer from flask/plate edge can be due to toxicity rather than viral CPE.).

D. Corroborative Testing

Every attempt should be made to confirm the identity of suspect viruses isolated with the preceding methods. Refer to Chapter 12 - Corroborative Testing of Viral Isolates – for appropriate methods to corroborate results of cell culture screening. Be aware that the preceding screening methods may result in the isolation of other viral pathogens not currently listed in the Survey. In cases where replicating viral agent can not be confirmed with methods in Chapter 12 they should be transferred to an appropriate laboratory for further identity testing.

E. Notification

Refer to Appendix 1.B for OIE notification procedures when a replicating viral agent is isolated.

X. Storing, Freezing and Thawing Viral Isolates

A. Preparation of Virus Isolates for Freezing (Archive)

1. Virus samples - suspected virus isolates from all fish species are frozen after completion of viral assays. At least 2 viral isolates (if 2 or more samples produce CPE) are frozen per date, location and species, preferably from wells having 4+ CPE.

2. Aseptically pipette 1.5-2 mL of tissue culture fluid and cell debris from the wells representing each isolate into four freezer vials. Seal tightly and label.

B. Freezing Virus Isolates

1. Freeze vials at -80°C. Virus should not be frozen in the liquid nitrogen dewer that contains the stock cell lines unless a herpesvirus is strongly suspected (i.e., the virus in whole cells could be more easily lost at -80°C).

2. Label each freezer vial per isolate with the case number, isolate number, number of passages through which cell line, fish stock and species, original sample type (ovarian fluid or tissue sample) and date frozen.

3. Log each isolate in the freezer notebook.

C. Thawing Virus Samples

1. One vial of the virus should be thawed and tested for viability before freezing all samples if the identity and stability of the isolate is unknown. However, this may not be feasible for unknown virus isolates requiring long incubation times to produce CPE.

2. Always thaw virus isolates rapidly in lukewarm water, removing the vial just before the last of the ice in the vial has melted.

3. Decant MEM-10 from the required number of 1 to 2-day-old monolayers in 25-cm^2 flasks. Pipet 0.1-mL virus sample onto each cell monolayer.

4. Allow virus to adsorb for 30 min at 15°C.

5. Add 5 mL MEM-10 to each flask and incubate at the appropriate temperature until all cells lift off each flask (4+ CPE).

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Appendix 11.A - Glossary of Terms used in Cell Culture & Virology

Blind passage - transfer of supernatant and inoculated tissue culture cells which are not demonstrating CPE to another plate containing fresh cells in order to dilute out possible inhibitors of viral expression and/or allow possible early viral replication due to low concentrations of virus particles to progress to detectable CPE.

Closed System - a system of incubating cells that is sealed against the transfer of air.

Confluent Monolayer (100%) - a single layer of tissue culture cells in which the cells have filled in all the spaces between them.

Controls

A. Monolayer control: tissue culture cells are grown in presence of growth medium MEM-10. If CPE appears in monolayer control wells, test is invalidated and must be repeated.

B. Sham control: diluent (MEM-0) used for suspension of samples or dilution blanks is added to cells. After adsorption, an overlay medium or MEM-10 is added. If CPE appears in sham control wells, test is invalidated and must be repeated.

Cytopathic Effects (CPE) - changes in the morphology and metabolism of tissue culture cells due to suspected viral infection.

Defective Interfering Particles (D.I. Particles) - defective or incomplete virus particles which cannot replicate but may prevent expression of the infectious virus by attaching to the tissue culture cell receptor sites thereby blocking infectious particles. This can be a problem at low dilutions of tissue or ovarian fluid, particularly with the North American strain of VHSV.

FBS - fetal bovine serum taken from unborn calves in utero.

Monoclonal Antibody (MAb) - antibody produced by tissue culture cell lines derived from the spleen lymphocytes of immunized mice that have been fused (hybridoma) with mouse myeloma tumor cells. Hybridoma cells are cloned to select specific populations of cells, each producing a single antibody against one epitope or antigenic determinant site on one antigen molecule among those used to immunize the mice.

Open System - a system of incubating tissue culture cells that is open to the transfer of air, i.e., a plate. Requires a medium that is buffered against rising pH from air exchange. Common buffering systems are TRIS and HEPES.

Overlay - a medium used in the plaque assay that is placed over a virus-inoculated cell monolayer to prevent physical spreading of viral particles except by cell-to-cell release of infectious particles. The overlay contains a semisolid medium such as methylcellulose or gum tragacanth.

Plaque - a hole or focus of degenerate or dead tissue culture cells in the cell monolayer caused by viral replication. One discrete plaque is assumed to be caused by infection with one infectious particle or aggregate (called one plaque-forming unit = pfu).

Polyclonal Antibody - the entire population of antibodies produced in the sera of immunized animals that are directed against many epitopes on many of the antigenic molecules used for immunization. Most immunogens injected are whole cells or viruses that are composed of many different antigen molecules. Each antigen molecule may have more than one epitope. See "Monoclonal Antibody".

Serum neutralization - antibody molecules in the antiserum neutralize or block the antigenic receptor sites or otherwise degrade the protein coat (capsid) on the corresponding virus (antigen). This prevents virus attachment to and subsequent penetration of host tissue culture cells or virus replication once inside the cell. Neutralization of viruses by antibodies is specific and used to confirm viral identity. Neutralization may be reversible.

Subculture - transfer of inoculated tissue culture cells and supernatant from one plate to another that contains fresh cells. Used for suspected positive cultures to confirm presence of viral CPE as opposed to toxicity or contamination. Also used to replicate more viruses for storage, etc.

$TCID_{50}$ - denotes fifty percent tissue culture infective dose. This is the reciprocal of the highest dilution of virus that causes CPE in 50% of the wells inoculated with that dilution of infectious materials. This is determined by the Reed and Muench (1938) method.

Tissue Culture-Grade Water - High quality water (low in ions, minerals and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells.

Titer - the number of infectious units or plaque-forming units (pfu) per unit of sample, i.e., per gram or mL.

Toxicity - changes in cell morphology or metabolism caused by toxic substances in the medium or inoculum. This can either cause cell death or interfere with cell metabolism, thereby reducing or preventing replication of the virus. These effects may have arisen through sample toxicity, bacterial or fungal contamination, improper glassware cleaning or improper media preparation. Usually toxicity can be distinguished from viral CPE by how rapidly it occurs (1 day), abnormal cell appearance without cell death, absence of the typical pattern of CPE for the test virus and, in the case of contamination, turbidity of the medium or visible contaminant colonies.

NOTE: Inoculation of very high-titer suspensions of certain viruses can cause an apparent toxic effect within 24 h. If there is any doubt to whether disruption of the cell layer was caused by

toxicity or CPE, a subculture should be made. This is especially true for some inocula that can produce toxic effects that may take 5-7 d for development.

Triturating - The act of dispersing tissue culture cells for transfer by repeatedly drawing the cell suspension into a pipet and expelling it back into the flask. This should be done until the cells are in clumps of no more than three when examined with an inverted light microscope.

Trypsin - a proteolytic enzyme used to disperse cells and causes their release from the culture surface. Serum proteins neutralize it and its action is slowed by low temperature. Trypsin will cause release of the cells more readily than versene.

Versene (EDTA) - ethylene di-amine tetra-acetic acid is a chelating agent that binds divalent cations active in forming cell cement (hyaluronic acid) causing cells to round and release from the culture surface.

Appendix 11.B – Reagents and Media Used in Cell Culture and Virology

All chemicals should be reagent or tissue culture grade. Use only glassware which is dedicated to tissue culture, is new, or has been acid washed. Media used in tissue culture must be sterile. This may be accomplished by mixing all the ingredients and filtering with a 0.2µm filter, or, by mixing of the stable ingredients, autoclaving, and then aseptically adding the labile ingredients such as L-glutamine, serum, and antibiotics.

A. Sample dilution medium – made with Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 mL
Tissue Culture Grade Water	895.3 mL
$NaHCO_3$ (7.5%)	4.7 mL

Mix and ensure sterility. Store at 4°C.

B. Antibiotic incubation medium (anti-inc) made with HBSS for sample disinfection

10X HBSS	100.0 mL
Tissue Culture Grade Water	575.0 mL
$NaHCO_3$ (7.5%)	5.0 mL
Penicillin/Streptomycin	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	160.0 mL
250 µg/mL Amphotericin B	
205 µg/mL desoxycholate	
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix and ensure sterility. Store at 4° C.

B.2. HBSS <u>transport medium</u> (only)

10X HBSS	100.0 mL
Tissue Culture Grade Water	to bring final volume to 1 L
$NaHCO_3$ (7.5%)	5.0 mL
*Penicillin/Streptomycin	at a minimum of 10 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	at a minimum of 10 mL
250 µg/mL Amphotericin B	
205 µg/mL desoxycholate	
*Gentamicin (50 mg/mL) (optional)	at a minimum of 2 mL
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix and ensure sterility. Store at 4° C.
*Use either Penicillin/Streptamycin or Gentamycin for antibiotics but not both in the transport medium.

C. Antibiotic incubation medium (anti-inc) made with Minimum Essential Medium (MEM-0) for sample disinfection

10X MEM (Eagles Modified Medium)	100.0 mL
Tissue Culture Grade Water	540.0 mL
L-Glutamine (200 mM)	10.0 mL
$NaHCO_3$ (7.5%)	30.0 mL
Tryptose Phosphate Broth[a]	100.0 mL
Penicillin/Streptomycin	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	160.0 mL
250 µg/mL Amphotericin B	
205 µg/mL desoxycholate	
NaOH or HCL	as needed to adjust pH to 7.2-7.6

[a] optional, if not used, increase volume of TCG-water to 640.0 mL.
Mix and ensure sterility. This may be stored frozen for approximately 3 months. Avoid freeze-thaw cycles, thaw tubes immediately prior to use.

D. Versene (EDTA) (1:5000)

NaCl	8.0 g
$KHPO_4$	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
Disodium Versenate (EDTA)	0.2 g
Phenol Red (0.5% solution)	2.0 mL
Tissue Culture Grade Water	to 1000 mL

Autoclave and store at room temp.

E. Trypsin-Versene (EDTA)

Trypsin (2.5% solution)	20 mL
Versene (EDTA) (1:5000)	480 mL

Store at -20° C.

F. MEM-5/Hepes (tissue culture medium for all cell lines except ASK and SHK-1)

10X MEM	100.0 mL
Tissue Culture Grade Water	815.0 mL
Fetal Bovine Serum	50.0 mL
Sodium Bicarbonate (7.5% solution)	10.0 mL
L-Glutamine (200 mM)	10.0 mL
Hepes Buffer (1M)	15.0 mL
NaOH or HCL	as needed to adjust pH to 7.2-7.6

If antimicrobials are included, use 801.0 mL of water above instead of 815.0 and add

Gentamicin (50 mg/mL)	4.0 mL
Fungizone	10.0 mL
250 µg/mL Amphotericin B	
205 µg/mL desoxycholate	

Mix and ensure sterility. Store at 4° C.

G. MEM-10/Hepes (tissue culture medium for all cell lines except ASK and SHK-1)

10X MEM	100.0 mL
Tissue Culture Grade Water	765.0 mL
Fetal Bovine Serum	100.0 mL
Sodium Bicarbonate (7.5% solution)	10.0 mL
L-Glutamine (200 mM)	10.0 mL
Hepes Buffer (1M)	15.0 mL
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix and ensure sterility. Store at 4° C.

H. Leibovitz L-15 (enhanced growth formula[a])

Media Components:

Leibovitz L-15 Powder	BIO-Whittaker 12-700F
L-Glutamine 200 mM	BIO-Whittaker 17-605E
Gentamicin 50 mg/mL	BIO-Whittaker 17-518Z
Foetal Calf Serum (FCS, Australian)	BIO-Whittaker 14-506F
2-mercaptoethanol (2-ME) 50 mM	GIBCO BRL 31350-01D

Composition of Medium:

Leibovitz L-15 (reconstituted according to manufacture)	500.0 mL
L-Glutamine (8.0 mM final concentration)	20.0 mL
Gentamicin (final Conc. – 50 µg/mL)	0.5 mL
FCS (final conc. – 15% v/v)	75.0 mL
2-ME (40 µM final concentration)	0.4 mL

NaOH or HCL	as needed to adjust pH to 7.2-7.6

[a] ASK and SHK-1 cell lines typically experience slow growth. Growth performance has been shown to improve when Leibovitz L-15 medium is formulated with FCS from Australia and higher concentrations of L-glutamine (McAllister, P.E. 2003, Pers. comm.).

I. Leibovitz's L-15 (Standard Formula)

1X L-15 with 0.3g/L L-glutamine	1000.0 mL
Fetal bovine serum (5%)	50.0 mL
Gentamicin (50 mg/mL)	1.0 mL
2-mercaptoethanol (0.055 M)	0.7 mL
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix and ensure sterility. Store at 4°C.

J. Methyl Cellulose Overlay (base for Overlay)

Methyl cellulose base:

Methyl cellulose (4000 centipoises)	8 g
Tissue Culture Grade Water	555 mL

1. Heat 225 mL d-H_2O in a 1 L bottle with a stir bar to a near boil.
2. Add methyl cellulose and mix by swirling.
3. Mix on a stir plate and add 330 mL cold d-H_2O washing down sides; stir until cool.
4. Stir at 4°C overnight.
5. Autoclave for 15 min at 121°C; will form an opaque solid.
6. Cool to room temperature and stir at 4°C until soluble; store at 4 °C.

Methyl cellulose Overlay

1. combine the following with aseptic procedures:

Methyl cellulose base	496 mL
10X MEM	100 mL
Tissue Culture Grade Water	300 mL
Fetal Bovine Serum	50 mL
L-glutamine (200 mM)	10 mL
Hepes buffer (1 M)	15 mL
$NaHCO_3$ (7.5%)	10 mL

NaOH (1 M)	5 mL
Fungizone (250ug/mL)	10 mL
Gentamicin (50mg/mL)	4 mL

Store at 4° C.

CHAPTER 12

Corroborative Testing of Viral Isolates

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I. Introduction

Serological testing is an important step in viral identification and confirmation. Several viruses such as IHNV and VHSV can produce similar cytopathic effects on cell cultures making it difficult to determine the identification of the viral agent based on cell culture results alone. Serological tests are highly specific allowing accurate and rapid identification of viruses based on unique antigenic characteristics. The serum neutralization test has long been the standard test for corroboration of viral isolates for these reasons. However, advances in molecular techniques such as DNA probes and Polymerase Chain Reaction (PCR) allow identification of viral isolates based on their nucleotide sequences. These sequences are used to develop primers specific to the viral strain, or group of viruses. These molecular tools provide an increased sensitivity and unsurpassed specificity that allows rapid and accurate identification of viral isolates.

II. Plaque Reduction Serum Neutralization Assay

Serum neutralization is one method of confirming the serological identity of a virus isolate. Then a known concentration of a virus from tissue culture is incubated with a known dilution of specific neutralizing antiserum against that virus, the ability of the virus to then produce CPE when inoculated onto cells is significantly reduced (neutralized). This neutralization is often temporary such that with time the antigen-antibody complex (virus and antibody combined) breaks apart, freeing the virus, allowing it to again infect a cell. This is called "breakthrough" which can confound results if a neutralization test is not read soon enough. Generally, the results of the unknown virus dilutions can be read when the positive control of known virus is significantly neutralized (at least one $\log_{10}$ in titer or 80% plaque reduction).

There are at least two general variations of the virus neutralization test; constant virus concentration exposed to varying antiserum dilutions or varying virus concentrations exposed to a constant antiserum dilution. The latter type of test requires the least amount of antiserum and has less inherent error in preparation. The following method will apply for those viruses that will produce plaques under a semi-solid overlay (rhabdoviruses, birnaviruses, aquareoviruses, and some herpesviruses).

The plaque reduction serum neutralization assay can be used to confirm the identity of suspected IHNV isolates from possible viral epizootics or from fish species. Flat bottom 24-well plates are used for this serum neutralization assay.

A. Preparation of Plates

1. Determine number of plates needed for the assay. You will need three 24-well plates to run one unknown virus against one antiserum.

2. Prepare the plates and allow them to form a monolayer the day before you want to run the assay.

B. Preparing Dilutions of Known Virus, Unknown Virus, Antiserum and Normal Serum

1. Dilute antiserum to appropriate dilution with MEM. Various dilutions of antiserum will have to be tested against the control virus beforehand to determine the optimum neutralizing dilution. You will need 1.5 mL of diluted antiserum to run one unknown against one antiserum.

2. Dilute normal serum with MEM to the same dilution as the antiserum. You will need 1.5 mL of diluted normal serum to run one unknown against one antiserum.

3. Dilute known virus and unknown virus to approximately 1 x 10^5 pfu/mL with MEM. You will need 1.5 mL of diluted known and unknown virus.

C. Setting up Neutralization Test and Controls

1. Label a sterile unused 24-well plate appropriately, as in the example.

2. Aseptically pipette 200 µL of diluted antiserum into appropriate wells.

3. Aseptically pipette 200 µL of diluted normal serum into appropriate wells.

4. Aseptically pipette 200 µL of MEM into appropriate wells. Add 400 µL of MEM into tissue control well (MEM only).

5. Aseptically pipette 200 µL of each known and unknown virus into appropriate wells.

6. Incubate for one hour at room temperature on a rotary shaker.

EXAMPLE

	A Known Virus	B Known Virus	A Unknown Virus	B Unknown Virus	MEM
Antiserum	KV + AS	KV + AS	UV + AS	UV + AS	MEM + AS
Normal Serum	KV + NS	KV + NS	UV + NS	UV + NS	MEM + NS
MEM	KV + MEM	KV + MEM	UV + MEM	UV + MEM	MEM

KV = Known virus
UV = Unknown virus
AS = Antiserum
NS = Normal serum
MEM = Minimum essential media

D. Performing the Assay

1. Label the three 24-well plates to be used in the plaque assay. Three duplicate tests are run on one plate, i.e., plate 1 may contain:

   e.g., KV + AS; UV + AS; KV + NS

KV + AS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}
UV + AS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}
KV + NS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}

   The antiserum, normal serum and MEM controls can be run on the third plate using only 10^{0} and 10^{-1} dilutions.

2. Dilute 0.1 mL of the solution from each test well in the incubated 24-well plate 10^{-0} to 10^{-3} in 0.9 mL MEM dilution blanks. Dilute 0.1 mL of the mixture from each control well 10^{-0} to 10^{-1} in 0.9 mL MEM dilution blanks.

3. Overlay EPC cells with 100 µL of 7% polyethylene glycol (PEG) for a few min prior to inoculations. PEG solution should be made up in MEM-10.

4. Pipette 100 µL of each dilution into appropriate well of PEG-treated EPC cells.

5. Incubate for 30 min at room temperature to allow virus adsorption.

6. Overlay wells with 1 mL of methylcellulose overlay medium.

7. Incubate at 15°C for 7 days in a sealed plastic bag or plastic container.

8. Fix and stain plates by pipetting approximately 1 mL of 0.5% crystal violet in 40% formalin into each well and let stand for 1 hour.

9. Pour off stain, rinse monolayers with water and allow plates to air dry.

10. Count and record numbers of plaques.

E. Interpretation

1. The tissue control wells (MEM only), the AS + MEM wells and the NS + MEM wells should not have any plaques present. Plaques would indicate that the medium, antiserum or normal serum was contaminated with virus and the test must be repeated.

2. An 80% or greater reduction of plaques is considered a positive serum neutralization test and confirms the identity of the virus. The known virus control should always show an 80% or greater reduction for the test to be valid.

3. When determining if there is an 80% reduction of plaques, first look at the virus control wells. Determine the dilution where countable numbers of plaques are present. Calculate the mean plaque-forming units (pfu) of the duplicate wells and compare this value to that of the virus + AS wells at that same dilution. Subtraction of the latter value from the control value will provide the pfu/mL of virus remaining after neutralization.

   a. Calculating pfu/mL: Viral titer for each sample is expressed as mean pfu/mL of test tissue or pfu/g of tissue. The best wells to use for determining titer are those of the highest dilutions with between 20 and 200 plaques.

   b. The following equation is used to express pfu/mL (or gram of tissue) in one well: # plaques x 1/tube dilution x 1/# mL added to well.

      For example:
      The 10^{-4} well of ovarian fluid sample A has 20 plaques in the cell monolayer.
      1/tube dilution = $1/10^{-4} = 10^{4}$
      1/# mL = 1/0.1 mL = $1/10^{-1} = 10^{1}$
      20 plaques x 10^{4} x 10^{1} = 2.0 x 10^{6} pfu/mL ovarian fluid

4. The normal serum + virus wells should not show any plaque reduction as compared to the virus positive MEM wells. If there is significant plaque reduction in the normal serum wells, this indicates that there is some nonspecific neutralization occurring with the virus and the serum.

5. Another method of expressing neutralization is the neutralization index (NI). This value is calculated by subtracting the $\log_{10}$ pfu/mL value of the neutralized virus remaining from the value of the same un-neutralized virus in MEM. Example:

Antisera	Log_{10} pfu/mL Remaining Virus			
	IHNV	**NI**	VHSV	**NI**
IHNV	2.0	**2.8**	4.4	**0.4**
VHSV	4.8	**0**	<1.0	**>3.8**
MEM	4.8	**0**	4.8	**0**

Using Log_{10} pfu, a smaller value denotes greater neutralization.

After conversion to NI the opposite is true, i.e., the larger the NI value the greater the neutralization.

III. Immunoblot

Dot blot is a relatively simple and quick assay to differentiate viruses detected in cell culture; however, it should be employed as an additional test for viral identification. Serum neutralization or PCR is still suggested for confirmation.

Run controls for each assay using known viruses as positive controls; cell cultures and PBS as negative controls. Follow good laboratory techniques by handling all suspect samples as positive for virus. Discard capillary tubes, wash solutions, and all supplies coming in contact with samples into an appropriate biohazard container or a chlorine solution. NOTE: Heat-inactivated IHN and VHS viruses (60°C for 2 hours) work well in dot blot and eliminate the need for biohazard precautions.

A. Blotting Procedures

1. Don't touch the nitrocellulose membrane (paper) with hands. Use forceps and handle the paper by the edges.

2. Draw a grid of 1 cm squares on the paper using a permanent alcohol/waterproof marker. Cut into strips; one strip will be used for each antisera.

3. Pour PBS 1X into a glass staining dish. Slide one edge of strip just under the surface of the PBS and slowly submerge paper until uniformly wet. After soaking for a few min, remove strips and air dry on bibulous paper for 5 min.

4. Make a map of your sample placement. With microcapillary tubes, slowly spot 10µL of each sample on a square of the paper. Air dry, until paper appears completely dry (approximately 5-10 min). If using two antisera, repeat this process, in the same order, on the second antisera strip.

5. In a glass staining dish, immerse the strips for 20 min in the 3% gelatin solution. Keep at 37°C to prevent gelatin from solidifying.

6. Remove each strip and quickly rinse in PBS 1X to remove excess gelatin. Then place each strip in a plastic Ziploc bag or "seal-a-meal" pouch and add 3-5 mL of the antisera of choice (IHN, IPN, or VHS). Seal and incubate at 37°C for 60 min. Agitate (by palpating pouch) every 15 min.

7. The primary antibody is saved for reuse. Open sack and carefully remove all antisera, making sure antisera is returned to its appropriate container.

8. Place the paper in a glass dish with Tween 20 solution (TPBS) and wash for 30 min, changing the TPBS every 10 min. The TPBS should be approximately 3-4 cm deep for each wash. Use a separate dish for each antisera used.

9. Put paper strips in a plastic Ziploc bag or "seal-a-meal" pouch and add 5-10 mL of the secondary antibody [goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP)]. Incubate for 60 min at 37° C. Agitate every 15 min.

10. Open sack and discard the second antibody solution. Wash strips as in step 8 above.

B. Color Development

1. Prepare solutions A and B at room temperature during the final wash. Mix the solutions immediately prior to use.

2. Put paper in a glass dish, add the color developer solution, and place in the dark. Allow to develop 7-10 min.

3. Remove strips from the developer and wash in running water for 15 min. Positive reactions will appear as dark blue spots where samples were blotted. Place strips between two 96-well plate sealers if you wish to keep results as reference or records.

C. Dot Blot Reagents

1. PBS 10X (Calcium & Magnesium Free) - Indefinite shelf life @ room temperature

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	11.5 g
KH_2PO_4	1.0 g
Thimerosal (0.01%)	0.1 g
qs to 1000 mL with cell culture water	

2. PBS 1X - Shelf life at least 1 month @ room temperature

Cell culture water	900.0 mL
PBS 10X (Ca^{++}, Mg^{++} free)	100.0 mL

3. 3% Gelatin - Shelf life stable for at least 1 month @ 4°C

Gelatin [Enzyme Immunoassay (EIA) Grade]	3.0 gm
PBS 1X.	100.0 mL
Heat or microwave to dissolve gelatin.	

4. Tween 20 Solution (TPBS) - Shelf life at least 1 month @ room temperature

PBS 1X	500.0 mL
Tween 20	0.1 mL (5 drops)

5. Primary Antibody – Recommended dilution for the primary antibody

(i.e., rabbit antisera against IHN, IPN, or VHS) is 1:1000.

6. Secondary Antibody - Goat anti-Rabbit Horseradish Peroxidase Conjugate (GAR-HRP)

	1:2000	1:3000
GAR-HRP	10.0 µL	10.0 µL
PBS 1X	20.0 mL	30.0 mL

7. Color Development Solution (CDS)

Solution A -	HRP Color Development Reagent	30.0 mg (0.03 gm)
	Cold methanol (reagent grade)	10.0 mL
Solution B -	Cold hydrogen peroxide (30% stabilized reagent grade) (Recommend J. T. Baker, cat. #JT-2186-1, 500 mL).	30.0 µL
	PBS 1X	50.0 mL

Prepare A and B solutions during final wash period, prior to color development. Mix A and B solutions together immediately prior to use to make color development solution.

IV. Indirect Fluorescent Antibody Test (IFAT) for Viral Identification

Immunofluorescence assay for IHNV is used as another serological test for confirming the identity of a viral isolate as IHNV (LaPatra et al. 1989). The assay uses a primary mouse anti-IHNV monoclonal antibody and a goat anti-mouse IgG FITC conjugate. The assay is performed in microwells of standard FAT slides.

A. Materials

cleaned FAT slides and coverslips (Freed, Inc.)
disposable petri dishes
gauze swabs
airtight plastic container
filtered (0.45 µm) distilled water
MEM-10-3X (3X = 12 mL Gentamycin)
EPC cells
MAb mouse monoclonal anti-IHNV antiserum (or other suitable antiserum)
Goat anti-mouse IgG FITC conjugate (Cappel)
known and unknown virus isolates

B. Setup

1. Work is done in a tissue-culture hood.

2. Clean FAT slides in 70% alcohol and wipe with gauze swabs.

3. Place the clean slides in the sterile petri dishes (chambers), 1 slide per dish. Cut 2 strips from a gauze swab and lay in the dish alongside the slide. (Soak the gauze with distilled water for humidity).

4. Seed each of the necessary wells with EPC cells in MEM-10-3X of sufficient density to monolayer overnight (1 drop per well from 5-mL pipette). Put all chambers into an airtight container and incubate at 21°C overnight.

C. Sample Inoculation and Incubation

1. When cells are ready, the medium is dumped from each slide into a waste beaker containing bleach.

2. Add a tissue-culture isolate of suspect IHNV to cells, 1 drop per well. Replicate slides are prepared, one for each incubation period; 8, 12, 24 and 48 hours. The suspect IHNV isolate should be taken from a culture having 3-4+ CPE.

3. Replicate controls are prepared at the same time, on different slides, for identical treatment and incubation periods. Place experimental and control slides each in separate chambers at 15°C for the prescribed times. Controls should include a known IHNV isolate stained with and without the primary antiserum (these could be on the same slide) followed by the conjugate; uninfected cells stained with all reagents.

4. For a single tested isolate there would be: 4 slides of cells infected with unknown virus, each in a single chamber for the 4 incubation periods; 4 slides of cells infected with known IHNV in another chamber; and 4 slides of uninfected cells in a third chamber. Hence, there would be 3 slides (unknown, known, uninfected) removed for staining at each incubation interval. The optimum sample will be the incubation interval just prior to early CPE.

D. Fixation

1. At scheduled incubation periods, an experimental and the 2 control slides are washed for 5 min in a Coplin jar containing cold PBS.

2. Slides are fixed in methanol for 10 min.

3. Slides can be stored at 4°C until ready to stain.

E. Staining

1. Undiluted MAb is added to appropriate wells (except negative IHNV control) and allowed to incubate for 5 min.

2. Rinse slides in 0.45 µm filtered distilled water for 5 min and gently shake free of water.

3. Goat anti-mouse conjugate diluted 1:160 (or as determined) is added to each well for 5 min.

4. Rinse in filtered distilled water for 5 min as above and shake off excess water.

5. Remove and coverslip with a minimum amount of FA mounting fluid and observe at 1000X for cytoplasmic fluorescence in the known positive control. No fluorescence should be observed in the negative controls. Read results for the unknown samples.

V. Biotinylated DNA Probes for Detection of IHNV and Distinction between the European and North American Strains of VHSV

The DNA Probe detects and identifies isolates of IHNV, North American VHSV, and European VHSV, using a dot blot procedure from infected fish cell cultures. Biotinylated DNA probes for IHNV and both European and North American strains of VHSV were developed by Deering et al. (1991) and by Batts et al. (1993). The probes hybridize with different sequences within the messenger RNAs of the nucleoprotein (N) gene elicited by each of the viruses that are extracted from tissue culture cells that have been infected for 24-48 h. The probe for North American VHSV hybridizes specifically with a nearly unique 28-nucleotide sequence following the open reading frame of the N gene mRNA. The probe recognizing all strains of VHSV binds to a 29-nucleotide sequence near the center of the N gene common to both American and European strains. The IHNV-specific probe (Deering et al. 1991) recognizes a 30 base sequence unique only to IHNV.

A. DNA Probe Set Up

Two days before running DNA Probe Test:

1. Prepare a 24-well microtiter plate with EPC or CHSE-214 cells so that it will be confluent by the next day.

2. If necessary, make up DEPC-treated water at a concentration of 1 mL DEPC to 1 L of distilled water. Mix on stir plate until thoroughly mixed. Make at least 5-6 L for treating glassware.

3. Rinse needed glassware with DEPC treated water and let dry. Store on shelf in an area dedicated for this use.

4. Sterilize distilled water and make up solutions that need to be autoclaved (solutions f, g, l, m, n).

One day before running DNA probe test:

1. Inoculate viral isolates onto cell monolayers in 24-well plate. Use several wells per isolate. Inoculate 2 wells with MEM-10-TRIS to use as a negative control. There should be no CPE when mRNA is extracted. Use dilutions if CPE occurs in 24-48 hrs. Incubation of virus on cells may require up to 48 h for adequate mRNA from certain isolates.

2. Prepare all other solutions needed to run the test. Adjust pH of final products carefully.

3. Fill both water baths and turn on. Adjust to 55°C and 65°C.

4. Get out rotator, Hybridot, and Seal-a-Meal and make sure they are operational.

B. DNA Probe Test Procedure

1. Extraction of mRNA from infected cells:

   a. Preparation

      i. Place crushed ice in a tray with microcentrifuge racks.
      ii. Make sure water baths are at 55°C and 65°C.
      iii. Always wear latex gloves.
      iv. Label tubes to be used.

   b. Pipette off the infectious medium above cells and add 0.5 mL RNAzol B to each well. Replace lid and put on rocker for 5-10 min at room temperature to digest cells.

   c. During step b put 50 µL cold chloroform/iso-amyl alcohol into labeled siliconized 1.7-mL tubes and keep on ice.

   d. Triturate the cell debris in each well with a 1-mL pipette five times and transfer solution into the labeled chloroform/iso-amyl tubes. Vortex the tubes 3 seconds each and store on crushed ice for 5 min to allow phase separation.

   e. Centrifuge the suspension at 10,000 rpm for 15 min. The RNA will remain in the clear aqueous phase and the DNA and protein will be left in the lower blue phenol phase.

   f. During step e, put 0.25 mL of cold absolute isopropyl alcohol into new labeled tubes and store on ice. Keep the alcohol at -20°C until ready for use.

   g. Transfer the aqueous phase containing the RNA (0.25 mL, no blue fluid) into the tube with 0.25 mL absolute isopropyl alcohol. Vortex for 1 second and chill tubes on ice for 15 min to precipitate RNA.

h. Centrifuge for 15 min at 10,000 rpm and remove as much fluid as possible from pellet. When you centrifuge, put the hinge of the microtube on the top. The pellet will be on that side and may be very difficult to see.

i. During step h, prepare nitrocellulose membrane. Wet membrane in distilled water for 1 minute. Wet by capillary action at an angle. Pour water off and soak for at least 5 min in 10X standard saline citrate (SSC).

j. For each probe used, heat approximately 140 µL of North American VHSV, common VHSV and IHNV PCR products for 1 minute in boiling water to denature the DNA. Transfer to ice. If only two probes are used, heat about 250 µL of product.

k. Warm pre-hybridization buffer to 55°C in water bath.

l. Add 170 µL of autoclaved distilled water to RNA pellets. Mix by flicking bottom of tube and warm tubes in 65°C water bath for 15-20 min. RNA pellets should dissolve. Mix again. Pellets appear as small white or brown flakes.

m. Add 170 µL of 20X SSC into tubes containing dissolved RNA pellets and store on ice.

n. During step k put wetted membrane in Hybridot. Attach vacuum pump hoses to blotting device.

o. Add 200 µL of 10X SSC to each well of blotting device. Membrane should not be dry when RNA is added. Try to avoid trapping air in the wells of the Hybridot.

p. Mix gently and add 100 µL of each RNA solution to wells of Hybridot which contain 200 µL of 10X SSC. Blot PCR products last.

q. Apply vacuum at 5 psi. After all solutions are added leave vacuum on 10-15 psi for about 1 minute. Turn off vacuum. Poke holes with pipette tip into empty wells for easy cutting of membrane.

r. Dismantle apparatus and remove membrane with forceps. Transfer membrane to thick filter paper wetted with 10X SSC.

s. Cut membrane into sections and label.

t. Transfer membranes to dry sheet of blotting paper and cover with a second sheet. Microwave for 60 s on high to attach nucleic acids to membrane. Weights can be placed on sides of the blotting paper to keep it from curling up.

2. Hybridization of probes with RNA on nitrocellulose membrane:

a. For pre-hybridization, place membranes spot-side-up into separate Seal-A-Meal® pouches. Add 10 mL pre-hybridization buffer to each pouch, remove air bubbles, and seal. Pre-hybridize for 30 min to 24 h at 55°C in water bath.

b. Thaw the probe solutions and heat to 50-55°C. Cut off edge of pouches and pour off the pre-hybridization buffer. Add 10 mL of each probe solution (pre-diluted in buffer) to the respective pouch and re-seal. React membranes in probe solutions for 1 h to 24 h at 55°C in water bath. If you are using probes that are not pre-diluted, do not pour off the pre-hybridization buffer and add 100 µL of probe.

c. Remove probe solutions from pouches and store in tubes at -20°C for reuse up to 5 times.

d. Transfer membranes into 40 mL post-hybridization solution in a buffer dish. Wipe forceps between each membrane. Discard solution and add 40 mL fresh post-hybridization solution and wash for 15 min on rocker at room temperature (RT). Wash two more times with 40 mL buffer for 15 min each on the rocker at RT.

e. Put dish with membranes and pre-warmed post-hybridization buffer into 55°C water bath for 15 min. Cover dish with parafilm.

f. Warm color development buffer to RT.

g. Rinse membranes briefly with 40 mL of Buffer A.

3. Color development of biotinylated probe:

a. Incubate membranes in a solution containing 40 µL streptavidin/alkaline phosphatase conjugate in 40 mL Buffer A for 30 min on rocker at RT. The conjugate can be used up to five times.

b. Rinse membranes briefly in 40 mL Buffer A and then wash twice in 40 mL Buffer A on the rocker for 7 min at RT.

c. Wash twice in 40 mL Buffer B on the rocker for 7 m at RT.

d. Immediately before use, add 0.4 mL alkaline phosphatase (AP) color reagent A and 0.4 mL AP color reagent B to 39.2 mL color development buffer warmed to RT.

e. Add 40 mL color development solution to the dish containing the membranes. Store in the dark for 15 m on the rocker at RT. The rocker can be placed under a box for this step.

f. Wash membranes in distilled water for 10 m with at least one change of water. Store membranes in distilled water until ready to photograph.

4. Probes for IHNV and VHSV are synthesized from the sequences given in Derring et al. (1991) or Batts et al. (1993). They can be obtained from the USGS, Western Fisheries Research Center, Seattle, Washington.

5. Solutions needed for DNA probe dot blot procedures:

All glassware should be Cleaned with DEPC-treated water and autoclaved before use. This is to prevent RNA-ase contamination. This water is available from Five Prime→Three Prime, Inc. (catalog #5302-336550).

a. Pre-hybridization Buffer

Distilled-deionized water	69.5 mL
10x Denhardt's solution	10 mL of 100x stock
2x SSC	10 mL of 20x stock
1% SDS	10 mL of 10% stock
0.1 mg/mL SSS DNA (Five Prime→Three Prime)	0.5 mL of 20 mg/mL stock

b. Hybridization Solution

Prehybridization buffer	10 mL
Biotinylated DNA probe	100 ng/mL

(Store at -20°C; may reuse up to 5 times)

c. Post-Hybridization Solution

2x SSC	50 mL of 20x stock
0.1% SDS	5 mL of 10% stock
distilled-deionized water	up to 500 mL

d. Denhardt's Solution

(100x stock concentration from Five Prime→Three Prime, Inc. Catalog #5302-213502)

10X Solution
1% bovine serum albumin
1% polyvinylpyrrolidone 360
1% ficoll 400

e. Sonicated Salmon Sperm DNA (SSS DNA)

(20 mg/mL stock solution from Five Prime→Three Prime, Inc. Catalog #5302-754688)

Transfer 0.5 mL of SSS DNA into 10 vials (with gaskets). Place vials into boiling water for 10 m. Cool vials in crushed ice, then transfer to -20°C freezer until needed. When needed, add 0.5 mL to prehybridization buffer (see #1)(final concentration of 0.1 mg/mL).

f. 20X Standard Saline Citrate (20X SSC)

NaCl (Sigma #S-3014, 3 M final concentration)	87.65 g
citric acid (Sigma #C-8532, 0.3 M final concentration)	44.11 g
distilled-deionized water	up to 500 mL

Adjust to pH 7.0 with HCl, autoclave. SSC may be purchased from Five Prime→Three Prime, Inc.Catalog #5302-227160.

g. 10X Standard Saline Citrate (10X SSC)

NaCl (Sigma #S-3014, 3 M final concentration)	43.82 g
citric acid (Sigma #C-8532, 0.3 M final concentration)	22.05 g
distilled-deionized water	up to 500 mL

Adjust to pH 7.0 with HCl, autoclave. Or, dilute 1:2 from 20x SSC by combining equal volumes of 20X SSC with distilled-deionized water. Autoclave.

h. 10% Sodium Dodecyl Sulfate (10% SDS)

Lauryl sulfate sodium salt (Sigma #4390)	10.0 g
sterile distilled-deionized water	up to 100 mL

(Adjust to pH 7.2. Do not autoclave this solution)

i. Streptavidin/Alkaline Phosphatase Conjugate (SA/AP)

0.1 µg/mL streptavidin/alkaline phosphatase conjugate (BRL #9543SA), store vial at 4°C.

Prepare by diluting SA/AP 1:1000 in Buffer A: Example: 30µL SA/AP stock added to 30 mL of Buffer A. Solution can be reused up to 5 times. Store at 4°C.

j. Buffer A

0.1 M Tris (pH 7.5)	50 mL of 1 M stock (#14)
0.1 M NaCl	10 mL of 5 M stock (#12)
2 mM $MgCl_2$ (Sigma #M-1028, 100 mL size)	1 mL of 1 M stock
0.05% Triton X-100 (BIORAD, Catalog #161-0407)	0.25 mL
distilled-deionized water	up to 500 mL

k. Buffer B

0.1 M Tris (pH 9.5)	50 mL of 1 M stock (#13)
0.1 M NaCl	10 mL of 5 M stock (#12)
50 mM $MgCl_2$ (Sigma #M-1028, 100 mL size)	25 mL of 1 M stock
distilled-deionized water	up to 500 mL

l. 5 M NaCl

NaCl (Sigma #S-3014)	146.1 g
distilled-deionized water	up to 500 mL

(Autoclave solution)

m. 1 M Tris Buffer (pH 9.5)

Tris base (Sigma #T-8524)	54.7 g
Tris HCl (Sigma #T-7149)	7.6 g
distilled-deionized water	up to 500 mL

(Adjust to pH 9.5 and then autoclave)

n. 1 M Tris Buffer (pH 7.5)

Tris base (Sigma #T-8524)	11.8 g
Tris HCl (Sigma #T-7149)	63.5 g
distilled-deionized water	up to 500 mL

(Adjust to pH 7.5 and then autoclave)

o. Chloroform/Iso-amyl Alcohol

chloroform (J.T. Baker #9180-03)	24 mL
iso-amyl alcohol (J.T. Baker #9038-1)	1 mL

(store at -20°C)

p. Isopropyl Alcohol

2-Propanol (isopropyl alcohol), (J.T. Baker #9084-03). Use undiluted for precipitation of RNA.

q. RNAzol B

RNA isolation solvent. Store at 2-8°C in dark. Cinna Biotecx Laboratories, Inc. 6023 South Loop East, Houston, Texas 77033. 1-800-535-6286. Catalog #104B is 100-mL size. Contains guanidine thiocyanate, 2-mercaptoethanol, and phenol.

r. Alkaline Phosphatase Conjugate Substrate Kit

(**NOTE:** This product contains dimethylformamide. Use in area with good ventilation (BIORAD Catalog #170-6432).

1. Dissolve AP color development buffer in 1 L volume of distilled-deionized water.

2. Filter-sterilize then store at 4°C until needed.

3. Immediately before use, add 0.3 mL of AP color reagent A and 0.3 mL AP color reagent B to 29.4 mL color development buffer at RT.

RAPID DOT BLOT (7 h)

	CELLS INFECTED WITH VHSV OR IHNV
8:30	
	EXTRACT RNA (2 h)
10:30	
	BLOT RNA, GET READY TO PREHYBRIDIZE (0.5 h)
11:00	
	PREHYBRIDIZE MEMBRANE (1 h)
12:00	
	HYBRIDIZE TARGET RNA ON MEMBRANE IN PROBE SOLUTION (1 h)
1:00	
	COLOR DEVELOPMENT OF SPOTS (2.5 h)
3:30	

VI. Polymerase Chain Reaction (PCR) for Detection of Fish Viruses

Personnel should be knowledgeable of the information in Appendix 12.C - General Procedures for PCR and Appendix 12.E - QA/QC for PCR before using these protocols for confirmation of viral fish pathogens.

A. PCR for Infectious Hematopoietic Necrosis Virus (IHNV)

Infectious Hematopoietic Necrosis Virus (IHNV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) for confirmation of IHNV (Arakawa et al. 1990) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

1. Extraction of RNA from cell culture fluid (Heat RNA release method):

   Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

   a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2µL fluid to 98µL water in microcentrifuge tubes.

   b. Heat tubes to 95°C for 2 min. in a heat block, or thermocycler.

   c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself).

2. Quantify RNA Template in the spectrophotometer (Appendix 12.D). The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5µL/reaction if reading falls below 50 ng/µL.

3. Production of DNA by Reverse Transcription and Amplification by First Round PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. Using Worksheet 12.A.1. (Appendix 12.A) Infectious Hematopoietic Necrosis Virus (IHNV) record case number and date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of $MgCl_2$ for this reaction

should be determined when the procedure or any component of the reaction mixture is altered.

c. First Round Primers for IHNV:

Forward: 5'-TCA AGG GGG GAG TCC TCG A-3'

Reverse: 5'-CAC CGT ACT TTG CTG CTA C-3'

d. First Round Thermocycler Program for IHNV:

1) Pre-dwell at 50°C for 15 min.

2) Preheat or "Jumpstart" sample to 95°C for 2 min.

3) 25 cycles as follows:

i. Denaturing at 95°C for 30 s.
ii. Annealing at 50°C for 30 s.
iii. Extending at 72°C for 60 s.

4) Post dwell at 72°C for 7 min.

5) Hold samples at 4°C after cycling is complete.

4. "Nested" Second Round PCR for IHNV:
If the first round PCR provides insufficient amplified product a nested set of primers is used for additional DNA amplification.

a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR)

b. Again use Worksheet 12.A.1 (Appendix 12.A) for the Second Round (IHNV) to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

c. Second Round Primers for IHNV:

Forward: 5'-TTC GCA GAT CCC AAC AAC AA-3'

Reverse: 5'-GCG CAC AGT GCC TTG GCT-3'

d. Second Round Thermocycler Program for IHNV

1) Preheat or "Jumpstart" sample to 95°C for 2 min.

2) 25 cycles as follows:

   i. Denaturing at 95°C for 30 s.
   ii. Annealing at 50°C for 30 s
   iii. Extending at 72°C for 60 s

3) Post dwell at 72°C for 7 min.

4) Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

5. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.

   Bands occurring at the 786 bp location in the First Round Assay and the 323 bp location in the Second Round Assay are confirmatory for IHNV and are reported as POSITIVE.

   The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IHNV.

6. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

B. PCR for Infectious Pancreatic Necrosis Virus (IPNV)

Infectious Pancreatic Necrosis Virus (IPNV) is a nonenveloped icosahedral shaped bi-segmented double-stranded RNA virus belonging to the *Aquabirnavirus* genus of the *Birnaviridae*. The Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) for confirmation of IPNV (Blake et al. 1995) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

1. Extraction of RNA from cell culture fluid (Heat RNA release method):

   Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

   a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2µL fluid to 98µL water in microcentrifuge tubes.

   b. Heat tubes to 100°C for 10 min in a heat block.

   c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself).

2. Quantify RNA Template in the spectrophotometer (Appendix 12.D). The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5µL/reaction if reading falls below 50 ng/µL.

3. Production of DNA by Reverse Transcription and Amplification by PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. Using Worksheet 12.A.2. (Appendix 12.A) Infectious Pancreatic Necrosis Virus (IPNV), record case number and date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of $MgCl_2$ for this reaction should be determined when the procedure or any component of the reaction mixture is altered.

   c. Primers for IPNV:

      Forward: 5'- AAA GCC ATA GCC GCC CAT GAA C -3'

Reverse: 5'- TCT CAT CAG CTG GCC CAG GTA C -3'

d. Thermocycler Program for IPNV

1) Pre-dwell at 50°C for 15 min.

2) Preheat or "Jumpstart" sample to 95°C for 2 min.

3) 35 cycles as follows:
   i. Denaturing at 95°C for 30 s.
   ii. Annealing at 50°C for 30 s.
   iii. Extending at 72°C for 60 s.

4) Post dwell at 72°C for 7 min.

5) Hold samples at 4°C after cycling is complete.

   PRC Products can be refrigerated (4°C) for one month or frozen (-70°C) for long term storage.

4. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.

   A band occurring at the 174 bp location is confirmatory for IPNV and is reported as POSITIVE.

   The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IPNV.

5. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

C. PCR for Infectious Salmon Anemia Virus (ISAV)

Infectious Salmon Anemia Virus (ISAV) is a spherical enveloped single-stranded RNA virus belonging to the newly proposed *Isavirus* genus of the *Orthomyxoviridae*. The Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) for confirmation of ISAV (Bouchard et al. 1999) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

1. Extraction of RNA from cell culture fluid (Heat RNA release method):

   Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low. See Chapter 14 Non-Lethal Methodology for Detection of Fish Pathogens, ISAV section, for extraction method using the QIAGEN RNeasy® kit and amplification using Invitrogen® One Step RT-PCR System.

   a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2µL fluid to 98µL water in microcentrifuge tubes.

   b. Heat tubes to 95°C for 2 min. in a heat block or thermocycler.

   c. immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).

2. Quantify RNA Template in the spectrophotometer (Appendix 12.D). The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5µL/reaction if reading falls below 50 ng/µL.

3. Production of DNA by Reverse Transcription and Amplification by PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. Using Worksheet 12.A.3. (Appendix 12.A) Infectious Salmon Anemia Virus (ISAV), record case number and date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of $MgCl_2$ for this reaction should be determined when the procedure or any component of the reaction mixture is altered.

   c. Primers for ISAV:

Forward: 5'- GGC TAT CTA CCA TGA ACG AAT C - 3'

Reverse: 5'- TAG GGG CAT ACA TCT GCA TC - 3'

d. Thermocycler Program for ISAV

1) Pre-dwell at 42°C for 15 min.

2) Preheat or "Jumpstart" sample to 94°C for 5 min.

3) 40 cycles as follows:

i. Denaturing at 94°C for 45 sec.
ii. Annealing at 59°C for 45 sec.
iii. Extending at 72°C for 105 sec.

4) Post dwell at 72°C for 7 min.

5) Hold samples at 4°C after cycling is complete.

PRC Products can be refrigerated (4°C) for one month or frozen (-70°C) for long term storage.

4. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). The use of 0.5X TAE buffer is recommended for gel preparation and electrophoresis running buffer (Appendix 12.E). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.

   A band occurring at the 493 bp location is confirmatory for ISAV and is reported as POSITIVE.

   The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for ISAV.

5. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

D. PCR for Largemouth Bass Virus (LMBV)

Largemouth Bass Virus (LMBV) is an icosahedral enveloped double-stranded DNA virus in the *Ranavirus* genus of the *Iridoviridae* family. Protocols for confirmation of Largemouth Bass Virus by PCR were developed by Grizzle et al. (2003). DNA extraction procedures by BuccalAmp kit is a modification provided by J. Woodland (USFWS, Pinetop Fish Health Center). LMBV is a DNA containing virus so DNA is extracted from cell culture fluid, amplified with forward and reverse primers, and the products are then visualized by agarose gel electrophoresis. Note the PCR master mix contains Uracil-DNA glycosylase and the deoxynucleotide dUTP is used in place of dTTP.

1. Extraction of DNA from cell culture fluid. DNA extraction may be completed with one of the following two methods.

   (1). Extract DNA following the procedures outlined in the BuccalAmp DNA extraction kit (Epicentre®). Collect sample by rotating the swab onto infected cells still attached to the well of cell culture plate. To obtain sufficient DNA, swab1-2 wells (24 well plate) or 3-4 wells (48 well plate) containing infected cells. Follow remaining steps provided with the kit.

   (2). Extract DNA following procedures provided for QIAGEN DNeasy® Tissue kit.

      a. Supernatant and cells from suspect sample wells are removed and centrifuged for 5 m at 300 Xg.

      b. Re-suspend pellet in 200µL PBS.

      c. Add 20µL proteinase K solution and 200µL Buffer AL to the sample, mix thoroughly by vortexing.

      d. Incubate for 10 m at 70°C.

      e. Add 200µL of 100% ethanol to the sample, mix thoroughly by vortexing.

      f. Pipet the mixture, including any precipitate into the DNeasy spin column sitting in the 2 mL collection tube provided.

      g. Centrifuge for 1 minute at 6,000 Xg . Discard flow-through and collection tube.

      h. Place the DNeasy spin column in a new 2 mL collection tube, add 500µL Buffer AW1, and centrifuge for 1 minute at 6,000 Xg. Discard the flow-through and collection tube.

      i. Place the DNeasy spin column in a new 2 mL collection tube, add 500µL Buffer AW2, and centrifuge for 3 min at full speed to dry the membrane.

j. Place the DNeasy spin column in a clean 1.5 or 2 mL microcentrifuge tube and pipet 200µL Buffer AE directly onto the DNeasy membrane.

k. Incubate at room temperature for 1 minute, then centrifuge for 1 minute at 6,000 Xg to elute.

l. Repeat steps "j" and "k".

m. Discard spin column and store DNA solution at -20 to -70°C until used for amplification.

2. Quantify DNA Template in the spectrophotometer (Appendix 12.D). The optimum amount of DNA template should be around 10 - 100 µg/mL (10 - 100 ng/µL). Dilute template if more than 350 ng/µL or use up to 5µL/reaction if reading falls below 10 ng/µL.

3. Amplification of DNA by PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. An internal control may be performed using the highly conserved, 632 bp segment of the β-actin gene (GenBank acession L36342), see Worksheet 12.A.4 (Appendix 12.A) for primer set. If β-actin is used an adjustment will be required to the master mix (less water). See also Grizzle et al. (2003).

   c. Using Worksheet 12.A.4. (Appendix 12.A) Largemouth Bass Virus (LMBV), record case number and date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). Note this MM uses dUTP in place of dTTP to make a quasi DNA molecule. DNA is normally made of A, C, G, T nucleotides, however the replicated quasi DNA in this procedure makes DNA with uracil (U) nucleotide in place of thyamine (T). The quasi DNA is degraded when placed in the MM and incubated at 20°C. In turn, the uracil-DNA glycosylase is inactivated at 95°C in the second step of the thermocycler program. Therefore this process allows for degrading of contaminant DNA containing U prior to amplification of new target DNA with U as a component. If running a large number of samples plan a negative control every 10 samples. The optimum concentration of $MgCl_2$ for this reaction should be determined when the procedure or any component of the reaction mixture is altered.

d. Primers for LMBV:

Forward: 5'- GCG GCC AAC CAG TTT AAC GCA A -3'
Reverse: 5'- AGG ACC CTA GCT CCT GCT TGA T -3'

e. Thermocycler Program for LMBV

1) UNG incubation at 20°C for 5 min.

2) UNG inactivation at 95°C for 3 min.

3) 15 cycles as follows:

i. Denaturing at 95°C for 45 s.
ii. Annealing at 60°C for 45 s.
iii. Extending at 72°C for 60 s.

4) 20 cycles as follows:

i. Denaturing at 95°C for 45 s.
ii. Annealing at 60°C for 45 s.
iii. Extending at 72°C for 60 s; 5 s are added to each successive extension step beginning at the 16th cycle and proceeding through the 35th cycle (ie. 65, 70, 75….155 sec). If this routine can not be programmed into your thermocycler, set extension to 60 s for all 35 cycles.

5) Post dwell at 72°C for 7 min.

6) Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated (4°C) for one month or frozen (-70°C) for long term storage.

4. Visualize the DNA by electrophoresis (1X TBE, ~75 volts for 1 – 1.5 hrs) of the product in 1.0% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.

A band occurring at the 248 bp location is confirmatory for LMBV and is reported as POSITIVE.

The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for LMBV.

5. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

E. PCR for *Oncorynchus Masou* Virus (OMV)

Oncorynchus Masou Virus (OMV) is an enveloped double-stranded DNA virus belonging to the *Herpesvirus* genus of the *Herpesviridae*. OMV is considered an exotic pathogen in the United States and the maintenance of live virus for positive controls by serological methods may not be prudent in many laboratories. The Polymerase Chain Reaction (PCR) method may be used to confirm that the cause of the CPE is due to the presence of OMV or suspect samples may be sent to an appropriate laboratory for confirmation. A Polymerase Chain Reaction (PCR) procedure that does not require live positive control material has been developed for this virus (Aso et al. 2001). DNA is extracted from cell culture material, amplified with forward and reverse primers, and then products are visualized with agarose gel electrophoresis.

1. Extraction of DNA from Cell Culture Fluid

   a. Supernatant and cells from suspect sample wells are removed and a pellet is formed by centrifugation of this material at 19,000 Xg (14,800 rpm) for 15 min.

   b. Wash the pellets twice with 1 mL PBS and mix with 200 µL of chelating resin (Sigma).

   c. Incubate the mixture at 56°C for 20 min in a water bath, vortex, and then place in a boiling water bath for 8 min.

   d. Vortex the samples and centrifuge at 8200Xg (10,000 rpm) for 90 s.

2. Amplification of DNA by PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. Using Worksheet 12.A.5. (Appendix 12.A) *Oncorynchus Masou* Virus (OMV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).

   c. Primers for OMV

      Forward: 5'-GTA-CCG-AAA-CTC-CCG-AGT-C-3'

      Reverse: 5'- AAC-TTG-AAC-TAC-TCC-GGG-G-3'

   d. Thermocycler program for OMV

      1) 30 cycles as follows:

         i. Denaturing at 94°C for 30 s.

ii. Annealing at 56°C for 30 s.
iii. Extending at 72°C for 30 s.

2) Hold samples at 4°C after cycling is complete.

Note: PCR Products can be refrigerated (4°C)for one month or frozen at -70°C for long-term storage.

3. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.

   A band occurring at the 439 bp location is confirmatory for OMV and is reported as POSITIVE.

   The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for OMV.

4. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

5. A laboratory capable of confirming the identity of OMV is the Laboratory of Microbiology, Hokkaido University, 3-1-1 Minato-cho, Hokodate, Hokkaido 041-0821, Japan, Phone/fax: (81.138) 40.88.10.

F. PCR for Spring Viremia of Carp Virus (SVCV)

Spring Viremia of Carp Virus (SVCV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Vesiculovirus* genus of the *Rhabdoviridae*. The Polymerase Chain Reaction (PCR) method may be used for confirmation of SVCV (Stone et al. 2003). The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

It should be noted that this PCR assay will also amplify Pike Fry Rhabdovirus (PFRV) and that further testing is required to distinguish isolates of the four genogroups described by Stone et al. (2003). Because several PFRV-like isolates cross-react in both the ELISA and immunofluorescent antibody test using anti-serum raised against SVCV, PCR products should be sequenced to identify the isolate.

1. Extraction of RNA from Cell Culture Fluid (Heat RNA Release method):

   Note: other methods for extraction of RNA are available including the Trizol Reagent™ method described by Strømmen and Stone (1997). Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

   a. Dilute Cell Culture fluid (with some cell scrapings) 1:20 in molecular grade RNase free water by adding 5µL fluid to 95µL water in microcentrifuge tubes.

   b. Place tubes in heat block at 95°C for 2 min.

   c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself).

2. Quantify RNA Template in the spectrophotometer (Appendix 12.D). The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5µL/reaction if reading falls below 50 ng/µL.

3. Formation of cDNA by Reverse Transcription and Amplification by PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. Using Worksheet 12.A.6. (Appendix 12.A) Spring Viremia of Carp Virus (SVCV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed.

(Add 4 to the number of samples so that there is enough to run controls in the assay).

c. First Round Primers for SVCV:

Forward (SVC F1): 5'- TCT TGG AGC CAA ATA GCT CAR RTC -3'

Reverse (SVC R2):5'-AGA TGG TAT GGA CCC CAA TAC ATH ACN CAY-3'

d. Thermocycler Program for SVCV:

1) Pre-dwell at 50°C for 20 min (cDNA synthesis).

2) Preheat or "Jumpstart" sample to 95°C for 2 min.

3) 30 cycles as follows:

i. Denaturing at 95°C for 30 s.
ii. Annealing at 50°C for 30 s.
iii. Extending at 72°C for 60 s.

4) Post dwell at 72°C for 7 min.

5). Hold samples at 4°C after cycling is complete.

4. "Semi-Nested" Second Round PCR for SVCV:
If the first round PCR provides insufficient amplified product a semi-nested assay is used for additional DNA amplification. The semi-nested assay uses the same forward primer used in the first round of amplification.

a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

b. Again use Worksheet 12.A.6 (Appendix 12.A) for the Second Round (SVCV) to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

c. Second Round Primers for SVCV:

Forward (SVC F1): 5'- TCT TGG AGC CAA ATA GCT CAR RTC -3'

Reverse (SVC R4): 5'- CTG GGG TTT CCN CCT CAA AGY TGY -3'

d. Second Round Thermocycler Program for SVCV.

1) Preheat or "Jumpstart" sample to 95°C for two min.

2) 30 cycles as follows:
   i. Denaturing at 95°C for 30 s.
   ii. Annealing at 50°C for 30 s.
   iii. Extending at 72°C for 60 s.

3) Post dwell at 72°C for 7 min.

4) Hold samples at 4°C after cycling is complete.

   PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

5. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.

   Bands occurring at the 714 bp location in the First Round Assay and the 606 bp location in the Second Round Assay are confirmatory for SVCV and are reported as POSITIVE.

   The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for SVCV.

6. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

G. PCR for Viral Hemorrhagic Septicemia Virus (VHSV)

Viral Hemorrhagic Septicemia Virus (VHSV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The European and North American strains of VHSV are indistinguishable by serologic methods but may be separated by PCR methods or the biotinylated DNA probe method presented in this chapter (Section 12.V). Polymerase Chain Reaction (PCR) method for confirmation of VHSV (Einer-Jensen et al. 1995) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

1. Extraction of RNA from cell culture fluid (Heat RNA release method):

   Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

   a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2µL fluid to 98µL water in microcentrifuge tubes.

   b. Heat tubes to 95°C for 2 min. in a heat block, or thermocycler.

   c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).

2. Quantify RNA Template in the spectrophotometer (Appendix 12.D). The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5µL/reaction if reading falls below 50 ng/µL.

3. Production of DNA by Reverse Transcription and Amplification by First Round PCR (refer to Appendix 12.C for General Procedures for PCR).

   a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

   b. Using Worksheet 12.A.7. (Appendix 12.A) Viral Hemorrhagic Septicemia Virus (VHSV), record case number and date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of $MgCl_2$ for this reaction should be determined when the procedure or any component of the reaction mixture is altered.

   c. First Round Primers for VHSV:

Forward: 5'-TCT CTC CTA TGT ACT CCA AG-3'

Reverse: 5'-TTC CGG TGG AGC TCC TGA AG-3'

d. First Round Thermocycler Program for VHSV:

1) Pre-dwell at 50°C for 15 min.

2) Preheat or "Jumpstart" sample to 95°C for 2 min.

3) 25 cycles as follows:

i. Denaturing at 95°C for 30 s.
ii. Annealing at 50°C for 30 s.
iii. Extending at 72°C for 60 s.

4) Post dwell at 72°C for 7 min.

5) Hold samples at 4°C after cycling is complete.

4. "Nested" Second Round PCR for VHSV:

a. QA/QC (see Appendix 12.E for QA/QC considerations for PCR).

b. Again use Worksheet 12.A.7 (Appendix 12.A) for the Second Round VHSV to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

c. Second Round Primers for VHSV:

Forward: 5'-ATG GGC TTC AAG GTG ACA C-3'

Reverse: 5'-GTA TCG CTC TTG GAT GGA C-3'

d. Second Round Thermocycler Program for VHSV:

1) Preheat or "Jumpstart" sample to 95°C for two min.

2) 25 cycles as follows:

i. Denaturing at 95°C for 30 s.
ii. Annealing at 50°C for 30 s.
iii. Extending at 72°C for 60 s.

3) Post dwell at 72°C for 7 min.

4) Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated (4°C) for one month or frozen at -70 ° C for long-term storage.

5. Visualize the DNA by electrophoresis of the product in 1.2% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix 12.C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.

   Bands occurring at the 950 bp location in the First Round Assay and the 558 bp location in the Second Round Assay are confirmatory for VHSV and are reported as POSITIVE.

   The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for VHSV.

6. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix 12.B, "Photodocumentation of the PCR Product Gel").

H. PCR for White Sturgeon Herpesvirus (WSHV)

White Sturgeon Herpesvirus (WSHV) is an enveloped icosahedral shaped double-stranded DNA virus belonging to the *Herpesvirus* genus of the *Herpesviridae*. A PCR method has been developed for this virus, however, the necessary sequences are not available at this time. Therefore, suspect samples must be sent to a reference laboratory for confirmation.

1. A laboratory capable of confirming the identity of WSHV is the Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, Phone: 530-752-3411.

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Additional Reading

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Appendix 12.A - PCR Worksheets: Amplification of Nucleic Acid for the Corroboration of Viral Fish Pathogens

12.A.1 Infectious Hematopoietic Necrosis Virus (IHNV)

12.A.2 Infectious Pancreatic Necrosis Virus (IPNV)

12.A.3 Infectious Salmon Anemia Virus (ISAV)

12.A.4 Largemouth Bass Virus (LMBV)

12.A.5 *Oncorhynchus Masou* Virus (OMV)

12.A.6 Spring Viremia of Carp Virus (SVCV)

12.A.7 Viral Hemorrhagic Septicemia Virus (VHSV)

Worksheet 12.A.1: Infectious Hematopoietic Necrosis Virus (IHNV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 U/Rx[†]	9 U/µL	0.5 µL	
Forward Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
Reverse Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template[a]		-	-	5.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
[†] Rx = Reaction
[a]Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H_2O equal to the volume of template subtracted.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	27.5 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
Forward Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
Reverse Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
Round 1 Product		-	-	2.0 µL	-

Primer Sets for IHNV

	Forward	Reverse
1st round	5'-TCA AGG GGG GAG TCC TCG A-3'	5'-CAC CGT ACT TTG CTG CTA C-3'
2nd round	5'-TTC GCA GAT CCC AAC AAC AA-3'	5'-GCG CAC AGT GCC TTG GCT-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Worksheet 12.A.2: Infectious Pancreatic Necrosis Virus (IPNV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
Forward Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
Reverse Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template[a]		-	-	5.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
[a]Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H_2O equal to the volume of template subtracted.

Primer Sets for IPNV

	Forward	Reverse
1st round	5'-AAAGCCATAGCCGCCCATGAAC-3'	5'- TCTCATCAGCTGGCCCAGGTAC-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Worksheet 12.A.3: Infectious Salmon Anemia Virus (ISAV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent[a]	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	26.75 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
Forward Primer		50 pmoles/Rx	50 pmoles/µL	1.0 µL	
Reverse Primer		50 pmoles/Rx	50 pmoles/µL	1.0 µL	
Taq		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template[b]		-	-	5.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
[a]A MM protocol using Superscript One-Step RT-PCR System® (Invitrogen) is also acceptable. See ISAV section in Chapter 14 Non-Lethal Methodologies for Detection of Fish Pathogens.
[b]Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H_2O equal to the volume of template subtracted.

Primer Sets for ISAV

	Forward	Reverse
1st round	5'-GGC TAT CTA CCA TGA ACG AAT C-3'	5'-TAG GGG CAT ACA TCT GCA TC-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Worksheet 12.A.4: Largemouth Bass Virus (LMBV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 35µL)	Volume for ____samples
d-H_2O[a]		-	-	Add to 35 µL total Rx volume	
PCR Buffer (no $MgCl_2$)		1X	10X	3.5 µL	
$MgCl_2$		2.0 mM	25 mM	2.8 µL	
dNTP's [b]			[b]	0.7 µL	
GC-rich solution[c]		1X	5X	7.0 µL	
Uracil-DNA glycosolase		0.5 U/µL	1.0 U	0.5 µL	
Forward Primer		15 pmol/Rx	15 pmol/µL	1.0 µL	
Reverse Primer		15 pmol/Rx	15 pmol/µL	1.0 µL	
FastStart Taq		1.2 U/Rx	5 U/µL	0.24 µL	
DNA Template		-	-	Adjust to meet target quantity	-

[a] Add nuclease free water to Master Mix first, Taq last. Add to total of 35 µL per reaction.
[b] dNTP mixture contains 10µL of 100 mM dATP, 10µL of 100 mM dCTP, 10µL of 100 mM dGTP, 30µL of 100 mM dUTP, and 40µL d-H_2O. Can be aliquoted to convenient volumes and stored at -20°C.
[c] Use concentration recommended by supplier. MM table uses GC-rich solution supplied with FastTaq® polymerase from Roche Molecular Biochemicals (Indianapolis, IN).

Primer Sets for LMBV

	Forward	Reverse
1st round	5'- GCG GCC AAC CAG TTT AAC GCA A -3'	5'- AGG ACC CTA GCT CCT GCT TGA T -3'

Internal Control Primer Set for 632 bp segment of the β-actin gene (GenBank L36342)

	Forward	Reverse
1st round	5'-TGC GTG ACA TCA AGG AGA AG -3'	5'-AAT CCA CAT CTG CTG GAA GG-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)
1%			

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Worksheet 12.A.5: Oncorhynchus Masou Virus (OMV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	15.5 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		1.5 mM	25 mM	3.0 µL	
dNTP's		0.8 mM	10 mM	4.0 µL	
TMAC		40 µM	100 µM	20.0 µL	
(+)Primer		50 pmoles/Rx	100 pmole/µL	0.5 µL	
(-)Primer		50 pmoles/Rx	100 pmole/µL	0.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
DNA Template[a]		-	-	1.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
[a]If more template is needed (up to 5.0 µL) substract equal volume of d-H_2O.

Primer Sets for OMV

	Forward	Reverse
1st round	5'-GTA CCG AAA CTC CCG AGT C-3'	5'-AAC TTG AAC TAC TCC GGG G-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Worksheet 12.A.6: - Spring Viremia of Carp Virus (SVCV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.25 mM	2.5 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		10 Units/Rx	40 Units/µL	0.25 µL	
RNA Template[a]		-	-	5.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
[a]Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H_2O equal to the volume of template subtracted.

Master Mix for Semi-Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for ____samples
d-H_2O*		-	-	27.5 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.25 mM	2.5 mM	5.0 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
Round 1 Product		-	-	2.0 µL	-

Primer Sets for SVCV

	Forward	Reverse
1st round	5'- TCT TGG AGC CAA ATA GCT CAR RTC -3'	5'-AGA TGG TAT GGA CCC CAA TAC ATH ACN CAY-3'
2nd round	5'- TCT TGG AGC CAA ATA GCT CAR RTC-3' (same as 1st round)	5'-CTG GGG TTT CCN CCT CAA AGY TGY-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)
1.5%			

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot #_____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Worksheet 12.A.7: Viral Hemorrhagic Septicemia Virus (VHSV)

Case Number_____Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (50µL total)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template[a]		-	-	5.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
[a]Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H_2O equal to the volume of template subtracted.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (50µL total)	Volume for ____samples
d-H_2O*		-	-	27.5 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5.0 µL	
$MgCl_2$		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
Round 1 Product		-	-	2.0 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for VHSV

	Forward	Reverse
1st round	5'-TCT CTC CTA TGT ACT CCA AG-3'	5'-TTC CGG TGG AGC TCC TGA AG-3'
2nd round	5'-ATG GGC TTC AAG GTG ACA C-3'	5'-GTA TCG CTC TTG GAT GGA C-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Appendix 12.B – Photodocumentation of Agarose Gel Electrophoresis of PCR Products

Case Number_____ Date:_____ Initials_____

Samples_____

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

Appendix 12.C – General Procedures for PCR Protocols

I. Preparation of Amplification Reaction Mixture

Specific amplification protocols may require one or two amplification reactions.

Note: samples and reagents should be kept cold either on ice or in a frozen cryo-rack during all assembly procedures.

A. Under a UV cabinet, prepare "Master Mix" (MM) using pathogen-specific protocols in the worksheets of Appendix 12.A. Calculate the amount of each reagent to go into the MM according to the number of samples to be processed. Add PCR reagents, except for sample DNA**,** in the order listed on the worksheet, adding water first and Taq polymerase last. Keep all reagents cold in frozen cryo-rack or on ice during mixing and return them to freezer immediately after use. Prepare enough MM for 2-4 more samples, including controls, than actually being tested to compensate for retention of solution in pipette tips and tube.

B. Place specified volume of MM into each PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

C. In the sample preparation area, load specified volume of each sample DNA to the appropriately labeled PCR tubes. To avoid cross contamination, always change tips between samples and avoid touching the sides of the tube. Close caps tightly.

II. Running the PCR

All general considerations should be employed including the following:

A. Thermocycler should be programmed for the specific PCR condition used for each pathogen (see thermocycler programs in Section VI).

B. Before loading into thermocycler, give tubes a "quick-spin" to ensure that all reagents and sample are drawn down from sides of tube.

C. Load the sample tubes into the wells following manufacturer's recommendations.

D. Program thermocycler for appropriate cycle conditions and run reaction.

E. After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes, perform a "quick-spin" to draw this fluid down into the reaction area of the tube and reduce the possibility of aerosol contamination upon opening tubes.

F. PCR products can be refrigerated for up to a month following amplification or for longer storage they may be frozen at -20°C.

III. Detection of Product

A. Assemble the gel tray and position well comb in the tray according to manufacturer's recommendations. Note gel box combs are available in several sizes and will affect the quantity of DNA product that can be loaded. Verify that the comb you use will be sufficient for the amount of product you choose to separate by electrophoresis (generally 10µL).

B. Prepare buffer (Appendix 12.F - Reagents) with distilled water to volume adequate for agarose gel and running buffer.

Either TAE or TBE buffer is acceptable. Generally a concentration of 1X is used however some protocols suggest a concentration of 0.5X. Use 1X unless specified in Visualization of PCR Product by Electrophoresis section for each pathogen.

C. Prepare agarose gel according to percent recommended (generally 1–2%) under pathogen section and the volume recommended for specific gel forms used.

1. Weigh appropriate amount of agarose and add to proper volume of buffer.

   Note: the same type of buffer and buffer concentration must be used in both the preparation of agarose gel and as a running buffer in the electrophoresis chamber.

2. Heat solution to near boiling until agarose is completely dissolved.

3. Allow solution to cool to about 65°C and then pour agarose solution into gel tray. Avoid the formation of bubbles especially around the comb. Bubbles may be removed with a hot flame-sterilized needle.

4. Allow gel to cool completely for about 30 min and then carefully remove the comb.

5. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode.

6. Slowly fill the chamber with the remaining buffer solution until the top of the gel surface is submerged.

D. Load samples into wells as indicated for each assay:

1. For each tube of PCR product to be visualized, mix 2 µL of gel loading dye (Appendix 12.F - Reagents) to every 10 µL of PCR product needed to fill each well formed in the gel. Mix the sample and the dye by repeated expulsion prior to loading. Parafilm® provides a good surface for performing this procedure.

2. When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the agarose gel, and load the well

with the sample. Repeat this procedure for all the wells, being sure to include the DNA molecular weight standard (one with bands at 100 bp increments in the 100 to 1,000 bp range) for base pair (bp) reference and positive and negative controls.

3. Document gel lane assignments for each sample and control on the Amplification of Nucleic Acid by PCR worksheet (Appendix 12.A) and allow for at least one lane for a DNA ladder reference (when possible one on each side of the gel is preferred).

IV. Electrophoresis

Set power supply to approximately 80 volts and run for 30 - 90 min or until tracking dye front approaches the edge of the gel. The rate of electrophoresis may be affected by gel characteristics such as width, length, depth, and agarose concentration. Refer to manufacturer's recommendations for electrophoresis system specifics. Generally, small gel chambers can be run faster than larger gels.

Tracking dye mobility chart.

	Base pair size corresponding to dye in 1X TBE	
Percent agarose	XC	BPB
1.00	4100	400
1.25	2500	260
1.50	1800	200

V. Staining the Gel

Remove gel and tray and place in ethidium bromide (EtBr) solution (Appendix 12.F - Reagents) for 15 to 20 min.

Note: EtBr solution can be reused and should be stored in a dark plastic tray container with a secure lid. EtBr is very toxic and binds with all DNA (including yours); follow appropriate manufacturer warnings! For safe proper disposal of expired EthBr solutions see Sambrook et al. or check with your local biotech supply retailer for specific products designed to remove EthBr from solution for disposal.

VI. De-Staining the Gel

De-stain in clean water for 5 to 60 min. De-stain water should be handled and disposed of appropriately (see Section V above "Staining the Gel").

VII. Visualize the DNA

A. Place gel on a UV light source and carefully record locations of bands on positive control samples in relation to the DNA molecular weight standard. Band locations of positive controls should be at anticipated locations according to primers used (these are designated in each viral pathogen section).

Use UV protective goggles or face shield when working with UV light.

B. Note any unusual band occurrences. Negative controls should not have any bands. Contamination suspicions indicate the samples should be re-run from template DNA tube.

VIII. Photo Documentation

A. Photo document all PCR gels. Record case number on the photo and attach to the Photodocumentation of PCR Product Gel worksheet (Appendix 12.B), or provide reference for finding the photo documentation.

B. If using a computer-based photo documentation system it is advisable to create and save an image file of the gel.

Appendix 12.D – Analysis of Extracted DNA Using a Gene Quant UV Spectrophotometer

1. Turn on Gene Quant spectrophotometer before using (no need to let it warm up).

2. Dilute DNA isolation 1:100 (add 5 µl of isolate to 495 µl of sterile water).

3. Take Reference Measurement first*.

   a. Add 100 µl of sterile water to cuvette. Check that there are no bubbles or meniscus visible in the optical path window. Wipe sides of cuvette with lens paper.

   b. Press [Set Ref] button. Screen should say " Please wait ".

   c. Wait for tone. Tone sounds, screen will display " Insert reference ". Quickly insert cuvette into slot.

   d. Wait for the second tone and remove cuvette when it sounds and screen displays "Remove reference".

   e. Once removed, display should show: "Absorbance": 260nm 0.000AU

   f. Empty cuvette onto paper towel by gently knocking it upside down. Dry cuvette with canned air (keep air can level to prevent spraying liquid into cuvette).

   *Note: Once reference is set, there is no need to set it again (i.e. between samples).

4. Sample Measurement

   a. Add 100 µl of dilution sample to cuvette; check that there are no bubbles or meniscus in optical path, wipe sides with Kim-wipe.

   b. Press [Sample] button. Screen will display: " Please wait."

   c. When tone sounds, insert sample. Screen will display: "Insert sample."

   d. When second tone sounds, remove cuvette. Screen will read: "Remove sample."

   e. Screen will automatically show Absorbance at 260nm.

   f. Press the [RNA / DNA] button. Screen will now show: "dsDNA conc 1"
   X is the sample conc. in µg/mL (***X*** µg/mL)
   RECORD THIS NUMBER ON DATA SHEET!

   g. Press the [Select] button. Screen should now read: "dsDNA conc 2"

X is the conc. in μg/μL. (***X*** μg/μL)
RECORD THIS NUMBER ON DATA SHEET!

h. If reading multiple samples, rinse cuvette with sterile water at least 3 times, then dry with canned air.

i. Repeat steps a – g for each sample.

5. Calculate the amount of QIAGEN isolate sample to add per PCR reaction tube.

a. First look at the two numbers for concentrations.

The range for dsDNA conc. 1 is: 1-4,000 μg/mL.
The range for dsDNA conc. 2 is: 0.001-0.2 μg/μL.

b. Decide which of your two readings best fits within its range, use that conc. number.

c. Take your concentration "***X*** " number and multiply it by the dilution factor used.

(***X***) x dilution. factor = actual concentration in tube.

Note: **If DNA sample is too dilute** to measure at 1:200, **make a less dilute solution** for spec analysis. For example, make a 1:50. Record this dilution factor on worksheet.

d. Use this formula for dsDNA conc. #2:

$$\frac{300\text{ ng}}{(\text{dil. factor}) \times \boldsymbol{X}} \cdot \frac{1\ \mu\text{L}}{1000\text{ng}} \cdot \frac{1\ \mu\text{g}}{} = \text{\# of } \mu\text{l of DNA isolate to add per rxn tube.}$$

e. Use this formula for dsDNA conc. #1:

$$\frac{300\text{ ng}}{(\text{dil. factor}) \times \boldsymbol{X}} \cdot \frac{1\text{ mL}}{1000\text{ng}} \cdot \frac{1\ \mu\text{g}}{1\text{ mL}} \cdot \frac{1000\ \mu\text{L}}{} = \text{\# of } \mu\text{l of DNA isolate to add per rxn tube.}$$

f. Remember if the sample is at a 1:200 dilution then the dilution factor is 200.

g. Record the amount to add per reaction tube on the data sheet.

h. If the amount to add is less than 0.5 μL you can make small dilutions of the sample DNA with sterile water to get a number between 1 μL – 10 μL.

6. Shutdown

a. Clean cuvette with sterile water and dry it with canned air, place cuvette in storage case.

b. Turn machine off.

c. Note: It is safe to keep the machine on all day. The deuterium lamp goes into standby mode which does **not** shorten its life. Reference reading taken earlier in the day is still good after the instrument has been left in standby mode.

Appendix 12.E – Quality Assurance/Quality Control for PCR

I. General Considerations

A. Quality control is critical to all steps of the PCR process, beginning with collection of samples in the field. It is important that the person performing sample collection use the precautions discussed in Chapter 2, Sample Collection and Submission, to avoid cross-contamination.

B. Work surfaces should be decontaminated by washing with 10% chlorine (or commercial reagents like "DNA Away") to hydrolyze possible DNA contaminants. All sample racks and reusable equipment should be washed in DNA-away and autoclaved after use. Spray/wipe pipettors and working areas with DNA or RNase -Away and turn UV on for at least 30 min after use (UV light damages DNA).

C. Wear and change gloves often. This helps prevent spread of amplified DNA or contamination of sample DNA with nucleases naturally occurring on the skin that will degrade the sample DNA. Always change to a fresh pair when leaving and entering PCR reagent mixing areas. Change gloves whenever contamination between samples is possible.

D. Employ aerosol resistant pipette tips and/or positive displacement pipettors during all extraction and amplification procedures. Separate pipettors should be dedicated for use with reagents only and another set for use with amplified products only.

E. Mix and aliquot pre-amplification ingredients under bench top UV cabinet and NEVER contaminate this area with sample material or amplified DNA product.

F. One aerosol drop of PCR product may contain thousands of strands of DNA, which can easily contaminate reagents! Therefore, three separate areas of lab space are necessary to reduce the risk of contamination.

1. Master Mix (MM) Area with UV Hood:
   For mixing and aliquoting master mix reagents. Supply area with dedicated pipettes, ideally positive displacement pipettor/tips. **No samples or amplified DNA is to be handled in or near this area!**

2. Sample Loading Area with UV Hood and Dedicated Pipettor:
   For loading of extracted (template) DNA from samples**.**

3. Amplified DNA Area:
   Supplied with pipettes dedicated for **amplified PCR product ONLY.** Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with "DNA Away" type solutions after working with amplified DNA.

G. Provide separate storage areas for RNA and DNA samples, amplified DNA, and PCR reagents.

H. Controls:

1. Extraction controls.
   A known positive tissue sample (or tissue spiked with target pathogen DNA) and a known negative tissue should be processed with the test samples to ensure that the DNA extraction was successful and contamination did not occur.

2. PCR Controls
   Sterile water (negative) and the known positive DNA and negative controls from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.

I. Primers: Newly received primer batches should first be tested on known positive and negative controls.

J. Dispose of trash containing amplified DNA products frequently.

II. Sample Processing

A. Tissue samples should be collected on a clean bench top, which has been disinfected using a 10% chlorine (or "DNA Away") solution if possible. If samples are collected in the field, use a disposable work surface between each lot of tissue collected (paper towel, foil etc.)

B. Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between samples will effectively remove contaminants from previous samples.

C. Keep samples cold and freeze as soon as possible at or below -20°C until processing can be accomplished.

D. RNA is extremely sensitive to enzymes present in sample tissues. Samples collected for RT- PCR should be frozen immediately and transported on dry ice. An RNA stabilizing buffer can also be used and does not require that samples be frozen immediately.

III. Extraction of DNA or RNA from Samples

Individual protocols will vary in specific steps for extraction of genetic material, however, the following general considerations should be employed:

A. Use micro centrifuge tubes with locking or screw-cap lids. Heating of extraction solutions causes unlocked caps to pop open, releasing aerosols that can cause cross-contamination between samples and controls. Pulse spin in the microcentrifuge before opening DNA sample tubes so that the lids are dry before opening them. This will help in preventing cross-contamination.

B. Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.

C. Always run positive control samples as well as negative (water and negative tissue samples) from the start of the extraction process, through amplification to electrophoresis. These controls will allow for detection of contamination as well as assure that the extraction was successful. This is the only means of assuring validity of the assay and its results.

IV. Quantification of DNA

If the protocol used advises that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted, refer to quantification guidelines in Appendix 12.D, Analysis of Extracted DNA using a Gene Quant UV Spectrophotometer.

V. Interpretation of PCR Results

Use of the appropriate controls should allow you to assess the integrity of your PCR result.

A. False-negative reactions may result from insufficient DNA extraction, excessive amounts of DNA, PCR inhibition, improper optimization of the PCR, or human error (e.g. loading errors).

B. False-positive reactions may result from contamination either directly from the sample lot being tested or from previously amplified target DNA.

C. For further help in troubleshooting, see PCR Protocols: A Guide to Methods and Applications (Ennis et al. 1990).

Appendix 12.F – Reagents

I. Electrophoresis Buffers

A. Tris-Acetate-EDTA (TAE) Buffer:

1. 10X TAE stock solution: Sigma T-4038 or comparable product. Comes in prepared packets, add DI water and qs to 1.0L. Label as 10X- Stock and store at room temperature.

2. 1X TAE working solution: Dilute 1:10 from 10X stock. Label as 1X Working Solution and store at room temperature.

B. Tris-Borate-EDTA (TBE) Buffer:

1. 5X TBE stock solution: Sigma T-3913 or comparable product. Comes in prepared packets, add DI water and qs to 1.0L. Label as 10X- Stock and store at room temperature.

2. 1X TBE working solution: Dilute 1:5 from 5X stock. Label as 1X Working solution and store at room temperature.

II. PCR Loading Buffers

Loading buffer can be purchased from a commercial supplier or made from the following recipe (B).

A. 6X concentrate, ready to use. Sigma P-7206 or comparable product.

B. 6X Loading Dye Recipe:
Bromophenol blue 0.25%
Xylene cyanol 0.25%
Glycerol 30.0 %
store at 4°C

III. Ethidium Bromide

Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

Note: USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH "CHEMICAL CARGINOGEN."

A. EtBr Stock Solution (10mg/mL)
100 mg Ethidium Bromide
10 mL DI water
Protect from light (store at RT)

B. EtBr Working Solution (4.0ug/mL*)
Add 200 µL Stock Solution to 500mL water
Protect from light (store at RT)

*References may suggest weaker working solutions (0.5 µg/mL) and staining periods of 45 to 60 min.

CHAPTER 13

Histology of Finfish

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I. Introduction

Histology is an important field of fish health that can often detect subtle conditions or early signs of disease not easily recognized on gross examination. Changes at the cellular level and in the function of key organs are the first indications of mal-adjustment to stressors that may eventually lead to poor health or disease. Histology in the National Wild Fish Health Survey supplements general observations and pathogen screening of wild fish populations. Special projects such as water quality and contaminant studies can be augmented with histology to provide better insight into the environmental and/or physiological demands presented to fish in their natural environment.

This particular chapter of the manual is unique in that for the purposes of the National Wild Fish Survey histologic examination of the tissues is ancillary, and thus up to the discretion of the Fish Health Biologist. In addition, the variation in equipment among the labs (especially tissue processors) and individual histologists' preferences make a specific standardized approach impossible. Instead, this chapter is intended to be a general guideline and offer tips.

II. Acknowledgements

The instruction and comments by John Morrison, Beth MacConnell and Sandy Pidgeon were invaluable for this chapter. This chapter was based on information provided by Short, Sally and Meyer, Ted. Alaskan Department of Fish and Game, Juneau and Anchorage and AK.

III. Preparation of Finfish Tissue

NOTE: Only live or moribund fish will be suitable for processing. Do not collect and process dead fish. Tissues in dead fish autolyze quickly and will mask ante-mortem changes. Keep fish alive as long as possible during transport to the site of necropsy. Animals are euthanized in a solution of MS-222, preferably buffered (with baking soda) to pH of water the fish came from, or other appropriate anesthetic.

Do not over-ice fish such that fixed tissues freeze while in transit. Frozen tissues result in artifacts that make interpretation of the results difficult.

A. Fixation

1. Tissues should be preserved in Davidson's fixative, 10% neutral buffered formalin, or non-formalin, alcohol-based fixative such as Prefer® or Safe-Fix®.
2. Davidson's contains acetic acid in it which results in some decalcification.
3. Place the tissues into the fixative immediately after euthanasia.
4. Handle the tissues carefully with forceps as excessive pressure can cause cellular damage which is visible microscopically in the tissue sections.
5. The volume of fixative should be ten times the volume of tissue. This is important since less fixative may result in tissue autolysis, compromising the evaluation of the tissues.

6. For most tissues, after 24-48 hours, the Davidson's should be poured off and replaced with 70% ethyl alcohol for transport and storage to prevent tissues from becoming too hard and brittle which occurs when left in acid fixatives for long periods. This transfer should be done in a fume hood.
7. Tissues can be left in alcohol-based fixative for longer periods.

B. Handling of Samples

1. In order to get the fish tissues in to fixative as quickly and with as little tissue damage as possible, you may want to pre-label your containers or cassettes.
2. When labeling cassettes with the fish identification/case number, use a soft lead pencil or a marker specifically designed to withstand the solvents used in tissue processing. When handling the tissues, use tissue-saving instruments (i.e. not rat-toothed forceps) and minimize pulling on organs.
3. Work quickly so that the tissues do not dry out.

C. Fixation of Small Fish

1. Fish less than 3 cm may be fixed whole by dropping into preservative.
2. NOTE: Whole swim up fry do not need to be decalcified and become too brittle unless removed after 4-8 hours of fixation. Store samples in 70% alcohol.
3. Slightly larger fish can still be fixed whole if the caudal peduncles are removed.
4. NOTE: Important! Remove egg yolk from sac fry before fixation.

D. Fixation of Medium Sized Fish

1. Fish 4 cm-10 cm should have the abdomen slit with a scalpel or scissors.
2. The intestines should be detached at the vent.
3. The internal organs should be pulled out slightly for proper fixative penetration.

E. Fixation of Larger fish

1. Fixation >11 cm will require on-site excision of 0.3-cm sections of major tissues and internal organs as listed.
2. Do not send whole fish.
3. Depending on the situation in the field, the collector may elect to do the "cutting in" (preparing the samples for placing in cassettes) or perform the gross dissection in the field, to later be "cut in" at the lab. In any case, the tissues should be no thicker than 0.3 cm in at least one dimension, allowing for adequate penetration of fixative into the organ.

4. Exceptions to this rule would include heads and brains, among others.
5. When cutting in organs that have a capsule, try to include some of the capsule for orientation when embedding and reading the slides.

F. Sampling Tissues

1. At the discretion of the fish health biologist, the number and type of tissues collected may vary. If the sampler is unsure as to how many tissues to take, more is better as tissues can be discarded later if they are not necessary.
2. Using the necropsy procedures outlined in Chapter 4, open the body cavity of fish (if histo preparations only, sterility is not essential). Where possible, try to get samples approximately 0.5-cm x 0.5 cm X 0.3 cm of liver, head and mesonephric kidney, spleen, GI tract (esophagus, stomach, pyloric caecae, anterior and posterior intestine with attached adipose tissue and pancreas), heart, gonads.
3. Use a sharp scalpel or razor blade to cut organs. Using scissors, cut the top and bottom of the gill arch(s). Carefully remove one or more gills, taking care to only handle them at the cut edge. Using the scalpel blade, cut a 0.5 x 0.5 x 0.3-cm square of musculature and attached skin intersected by the lateral line midway between the head and tail on the right side of the fish. Excise the head (from just behind the opercula opening).
4. If brain is desired, Using a scalpel, shave off the skull just on the dorsal aspect of the brain, taking care not to disturb the brain.
5. If the entire head is desired, for fingerling size fish or smaller, cut the head sagittally off center, to allow for adequate penetration of the fixative. Refer to the AFS Bluebook for diagrams, if needed.
6. Organs and tissue samples from a single fish should be placed in tissue-processing cassettes, up to 4 to 5 tissue samples to one cassette (depending on size).
7. If tissues are being compacted by the cassette or lid, then reduce the number and/or size of the tissues in the cassette.
8. In general, multiple tissues, especially of unlike textures, will make cutting the blocks more difficult. try to group tissues of like textures in the same cassette. Gills should be in cassettes of their own (unless whole fish or heads) to facilitate cutting.

G. Tracking and Recording

1. External and internal abnormalities must be noted on the Submission Form (Appendix 2.B) and the particular fish sample identified.
2. Be sure and include tissues from a lesion area if there is one observed.
3. Lesions should include normal and abnormal tissue.
4. The Submission Form should also contain the label information below and should accompany the samples in a separate sampling bag.
5. Do not mix samples of different fish species within the same jar of fixative. Each species requires a separate sample jar.
6. Individual fish can be kept separate by cassette or individual jars.

H. Shipping Considerations

1. If shipping collected material, place sample jars containing alcohol and tissues and the high quality sample bag containing sample submission data into a suitable shipping package with adequate packing material to prevent breakage.
2. Plastic jars or containers for fixative and samples work best.
3. Be sure lids are on tight and do not leak.
4. Wrap electrical tape around the lid seal several times.
5. Make sure everything is double bagged with a high quality sampling bag.

NOTE: Any quantity of alcohols and formalin solutions are dangerous goods and need to be shipped in accordance with special packaging and shipping requirements. Check with Federal Express, or the commercial carrier used, for specific instructions on shipping dangerous goods

I. Special Procedures - for fingerlings, fry, and sac fry:

1. Decalcify fingerlings and fry – If the yolk sac is not removed, chelate sac fry with EDTA to soften the yolk sac.
2. Larger fish heads may need to fix for up to 72 hrs.

NOTE: Proper sectioning of the brain and eye in smaller fish will require that the head be halved longitudinally after fixation using a very sharp razor blade. Both halves are laid face down in the cassette for embedding after decalcification.

IV. Fixation and Decalcification Solution Recipes

J. Davidson's Fixative (Kent and Poppe 1998)

95%Ethanol	300	mL
Acetic acid	200	mL
Formalin	400	mL
Deionized water	600	mL

K. 10% Buffered Formalin (Luna 1992)

Formalin	100.0	mL
Sodium phosphate (monobasic) ($NaH_2PO_4H_2O$)	4.0	g
Sodium phosphate (dibasic) (Na_2HPO_4)	6.5	g
dH_2O	900.0	mL

L. Decalcification Solution (Luna 1992)

Solution A:

Sodium citrate ($Na_3C_6H_5O_7 . 2H_2O$)	50.0	g
dH_2O	250.0	mL

Solution B

Formic acid 90% (HCOOH)	125.0	mL
dH_2O	125.0	mL

1. Mix A & B in equal portions for use - (leave tissues in for 8-12 hours or until decalcification is complete).
2. Wash in running tap water for 30 minutes-1 hour.
3. Place tissues in tissue processor for usual cycle.
4. Alternately, place in Cal-Ex® for 24 hours. Rinse in running water 3-4 hours and process as usual.

NOTE: Always wear gloves when handling fixatives, all contain cancer causing chemicals.

V. Tissue Dehydration and Infiltration

After the tissues are in cassettes, preserved with fixative and moved into 65%- 70% ethanol, they are dehydrated in a tissue processor. Isopropyl alcohol may be used if ethanol is not available, but may compromise tissue integrity. The solutions and times can be processor dependent, refer to the manufacture's instructions of your equipment. The following is a general procedure for processing.

#1 70% alcohol
#2 70% alcohol
#3 95% alcohol
#4 95% alcohol
#5 95% alcohol
#6 100% alcohol
#7 100% alcohol
#8 100% alcohol
#9 Pro-Par®or other comparable clearant
#10 Pro-Par® or other comparable clearant
#11 Paraplast extra ® or other comparable melted paraffin
#12 Paraplast extra® or other comparable melted paraffin

A. Maintenance of Reagents

1. These solutions will evaporate over time and should be topped up as needed and replaced after processing a set number of cassettes or a specified time period (see manufactures' instructions)
2. The temperature for the paraffin baths should be set at 1 -2º C, no higher than 3-4°C above the melting point of the paraffin you are using. (59-60°C for Paraplast extra®. Do not exceed 62ºC or polymerization will occur. This will produce hard blocks resulting in difficult or impossible sectioning.)

B. Equipment Variability

1. The exact program times may be dependent on the type of equipment used at each lab. Do not allow the tissues to remain in melted paraffin any longer than necessary. Excessive time or heat in melted paraffin can cause tissues to become brittle and they become difficult to cut.

VI. Embedding Tissues into Paraffin Blocks

A. Preparation

1. Turn on the paraffin bath in the embedder prior to the end of the processing cycle in sufficient time to allow the paraffin to completely melt (may be several hours). (You may be able to set the internal timer to automatically turn the embedder on at the appropriate time.)
2. Shortly before the scheduled embedding, turn on the cold plate so that it can become well chilled.

B. Transferring from Processor to Embedder

1. When the tissue processor cycle ends, remove the basket from the final paraffin bath and put the cassettes into the melted paraffin bath or the cassette chamber of the embedder. It is important to start embedding ASAP to prevent the tissues from becoming brittle. Try to embed all of your blocks within at least 2 hours of the processor finishing.
2. Place the cassettes flat on the bottom of the holding chamber to keep the tissues warm until they are embedded with wax. Do not leave them in the chamber too long without wax as they will become brittle.

C. During Embedding

1. Dispense enough paraffin into an embedding block mold to cover the bottom. Place the mold on the hot plate of the embedder.
2. Remove a cassette from the paraffin bath/ of the embedder and place it on the hot plate.
3. Open the cassette and discard the lid. Using forceps, gently transfer the tissue sample(s) from the cassette to the mold.
4. Keeping in mind the orientation of the block on the microtome, try not to place hard tissues (boney or containing grit or scales) above or below other tissues. This placement will make cutting easier. (Ideally, use separate cassettes for hard tissues).
5. Keep the tissues away from all the edges of the mold. Having plenty of wax around the tissues allows for a better ribbon when cutting.
6. Consult your histologist on how the tissues should be oriented (i.e. the hollow viscera can be oriented in cross-section, sagittally or longitudinally).
7. Place the mold on the cold plate. Using rounded-tipped forceps, gently press each tissue piece to the bottom of the mold. This must be done quickly. Allow the paraffin to set up just enough to hold the tissues in place. DO NOT ALLOW THE PARAFFIN TO COMPLETELY HARDEN.

8. Quickly move the mold back to the hot plate and place the cassette bottom onto the mold like a cap.
9. Fill the mold tot the top of the mold with melted paraffin from the dispenser of the embedder (paraffin will shrink as it hardens). If the bottom layer of wax is allowed to cool too much, the block will likely split when cutting.
10. Return the mold to the cold plate to cool.
11. Once the block has completely solidified, it may be popped out of the mold. If the cassette cannot be easily popped out, do not force it. The block may break, if it does, then it can still be re-embedded. Note: re-embedding increases the tissue brittleness and should be avoided if possible. If the blocks are difficult to remove, try cooling the blocks down more, or go to a commercially available mold release spray prior embedding. Once the block is removed, use a scraper to remove any excess wax along the edges of the block. If this wax is not removed than it may be difficult to hold the block in the chuck of the microtome in a consistent manner.
12. At this point, if blocks are going to be cut that day they can be stored in the freezer until you are ready to cut. For longer term storage, store in a cool, dry place where the temperature will not exceed the melting point of the wax.

VII. Cutting Paraffin Blocks and Mounting Sections on Glass Slides

A. Preparation of materials

1. The blocks should be cold prior to starting. If they are not cold from the embedding center, they can be placed in the refrigerator prior to trimming. The water bath should be set at 45ºC-48ºC (below melting point of wax). If air bubbles in water bath are a problem, use a paint or make-up brush to remove the bubbles prior to cutting sections. In areas where there are excessive minerals or chlorine in the tap water, consider using de-ionized water.
2. Standard Glass slides can be used or if tissues do not remain attached to the slides during the staining process, electrostatic (Plus) or Poly-Lysine treated slides may be used. Pre-labeling maybe helpful.
3. Place blocks to be trimmed on the cold plate or ice block.
4. Check the blade in the microtome and replace if nicked or scratched. Use disposable blades, they are always sharp and clean. There is little tissue loss when re-facing a block if re-cutting is required if blades are held at a fixed angle.

B. Cutting sections

1. Clamp a block securely into the microtome chuck and begin cutting. Ideal sections will be between 2-6 microns thick. Try to be consistent on which way they block label is placed in the chuck (i.e. always up or always to the right). This consistency will be helpful when you go to re-cut blocks…less re-facing waste.

2. You may choose to “face off” (trim off the wax until you have exposed the tissues to be cut and the blade comes in contact with the entire block) all the blocks to be cut for the day prior to actually cutting any sections. This allows you to use one blade for all the trimming. In addition, it gives time for your tissues to become re-hydrated on the block of ice with water. Once the blocks have been faced off, add a small amount of water to your ice block to allow rehydration of the tissues. Rehydration will improve tissue sectioning and ribbon integrity. The tissues require varying amounts of time for re-hydration, but 20-30 minutes for most tissues is adequate. If you decide to do this, keeping the block label in the same place in the chuck and removing all the excess wax around the block is even more important. Use a fresh blade when starting to section your blocks.
3. After the tissues are re-hydrated, you are ready to cut your sections. Once you are able to cut ribbon of whole sections, transfer the ribbon to the water bath. Possible ways of making the transfer include two small paint brushes, coverslipping forceps, and fingers. If you are using fingers, consider wearing gloves to prevent your epithelial cells from ending up on your sections. Regardless of the technique, try to gently stretch out the wrinkles as the ribbon makes contact with the warm water surface. If bubbles occur in the sections, reduce heat and gently dab the bubble with a paintbrush to remove.
4. Separate the desired sections from the ribbon by gently pulling the ribbon apart using two small, fine bristled paint brushes. Sections may also be separated with tool (knife) heated in an open flame or heating block which will “cut “the sections apart. Do not put the heated tool too close to the tissues. You can also eliminate the need for separating the sections in the bath by dipping the slide under the ribbon and wiping the excess ribbon off, after allowing the slide to dry.

C. Mounting sections

1. Submerge a clean, labeled glass slide into the water bath under the desired sections. Gently pull the slide out of the water at a slight angle. Consider cutting at least two sections per block to allow for H&E staining and one additional stain if needed.
2. Lean the slide upright to dry. Use racks designed to hold the slides at an angle to facilitate drying with no water underneath the sections.
3. Once the slides are dry. Place the slides in rack (which may be a staining rack)to be placed in oven.
4. All the slides now in racks should be dried for 30-40 minutes, up to an hour at 40-50ºC prior to staining. Note: Heat is detrimental to the tissues. This step helps to prevent wash-offs during the staining process. If the paraffin in the sections melts, the temperature is too high and tissue artifact will occur. Alternatively, you can allow the slides to dry overnight prior to staining.

VIII. Routine Staining of Paraffin Sections - Hematoxylin and Eosin

There are many H&E stains and protocols that are readily available either as components or part of a kit. Here is an example of one that has worked well for fish tissues:

A. General Staining

1. Hematoxylin Solution (Harris Formula) and Eosin Y.

2. Purchase already prepared; it is inexpensive and gives reproducible results. Ordering information and references containing recipes if preferred are listed at the end of this chapter.

3. The basic procedure includes removal of the paraffin in the sections (deparaffinization) and rehydration of the tissue so that the H & E stains may be used. This is followed by dehydration again so that the stained section may be mounted in a permanent medium under a glass coverslip.

   Standard H&E schedule (Adapted from Sheehan and Hrapchak 1980)

#1 Clearant - (i.e. Propar®)	5 minutes in each container
#2 Clearant - (i.e. Propar®)	5 minutes
#3 100% Ethanol	1 minute with agitation
#4 100% Ethanol	1 minute with agitation
#5 100% Ethanol	1 minute with agitation
#6 95% Ethanol	5 minutes with agitation
#7 Tap Water	1 minute
#8 Hematoxylin	5 minutes
#9 Running water	1 minute
#10 Acid Alcohol	Dip 2-3 times
#11 Tap Water	1 minute
#12 Bluing solution	30-40 seconds.
#13 Tap Water	2-3 minutes
#14 70% Ethanol	1 minute with agitation
#15 Eosin Y	30 to 60 seconds
#16 100% Ethanol	1 minute in each container
#17 100% Ethanol	1 minute
#18 100% Ethanol	1 minute
#19 100% Ethanol	1 minute
#20 Clearant – (Propar®)	3 minutes in each container
#21 Clearant – (Propar®)	3 minutes
#22 Clearant – (Propar®)	3 minutes

   See Note Below

Note: Staining times will vary with thickness of sections, age of stain, and animal species. Thinner sections will require increased staining times. This staining schedule is based on sections of fish tissues 5 µ thick.

B. Special staining

Examples of special stains include but are not limited to Giemsa, Steiner, Silver, Periodic Acid Schiff, Methylene Blue, and Gram Stains. Protocols for these stains can be obtained in one of the histology texts listed on the reference page.

IX. Cover Slipping

A. Try to coverslip in a hood, even the xylene substitutes are not "non-toxic"

B. One of the many ways to coverslip (Adapted from Bozeman Fish Health Center unpublished):

1. Put a clean paper towel down.
2. Have slides, appropriate sized coverslips (No. 1 thickness), and mounting media available. There are many different mounting medias, consider trying a low viscosity media compatible with the xylene substitutes since the media is not as soluble in Propar® or other xylene substitutes as it is in xylene.
3. Keep the slides waiting to be coverslipped in clearant.
4. Wipe the back of the slide to be coverslipped with a paper towel.
5. Place the slide on the paper towel.
6. Put the coverslip down directly in front of the slide and centered on the sample. The coverslip should be flat on the paper towel.
7. Put a few drops of mounting media on the center or the slide or in a line, depending on the size of the coverslip.
8. Pull the back corners of the slide upward and over the coverslip until the side is face down on the coverslip. This method will pull the mounting media over the slide by using low angle capillary action. Placing the coverslip flat on the slide will induce air bubbles.
9. If the mounting media is too thick, gently press on the coverslip with a pencil eraser, or put the slide face down on the paper towel and gently press over then entire surface of the slide.
10. Air bubbles can also be removed with a pencil eraser. Check for air bubbles 30 minutes after coverslipping.

X. Reagent Source List

ExCell plus	FXEXPCS	American Master Tech
Paraplast extra	15159-486	VWR
Propar	511	Anatech
Permount	SP15-100	Fisher
Harris Hematoxylin	HXHHELT	American Master Tech
Harris Hematoxylin	842	Anatech
Eosin Y	STEOSLT	American Master Tech
Eosin Y	832	Anatech
Giemsa Powder	11700	Sigma
Jenners Dye	861197	Sigma

XI. Manufactures websites (this is not an exhaustive list)

www.mastertechs.com	American Master Tech
www.polysciences.com	Polysciences
www.fisher.com	Fisher
www.vwr.com	VWR
www.sigmaaldrich.com	Sigma
www.rallansci.com	Richard Allan
www.sakura.com	Sakura
www.anatechltdusa.com	Anatech

XII. Maintenance of Equipment

The amount and type of maintenance required will be depend on the specific piece of equipment in each lab (refer to manufacture's instructions) and the volume of tissues processed. However, there should be some schedule for changing of reagents and wax as physical/chemical changes can occur even with light usage.

References

Luna, L. G. 1992. Histopathologic methods and color atlas of special stains and tissue artifacts. American Histolabs Inc., Gaithersesburg, MD, pp 10:107

Sheehan, D., and B. B. Hrapchak. 1980. Theory and Practice of Histotechnology, 2nd edition, C. V. Mosby Company, St. Louis, MO, pp 143-144.

Additional Reading

Bozeman Fish Health Center. Tips on how to have successful tissues in histology, unpublished, Bozeman, Montana.

Luna, L. G. (Editor) 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd Edition. McGraw-Hill Book Company, NY.

Yasutake, W., 1983. Microscopic anatomy of salmonids: an atlas. United States Department of the Interior, Fish and Wildlife Service Resource Publication 150, Washington DC.

CHAPTER 14

Non-Lethal Methodology for Detection of Fish Pathogens

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I. Introduction

Fish health plays a key role in monitoring, evaluating and protecting the health of all aquatic animals within an ecosystem whether it relates to restoring depleted populations or the recovery of threatened and endangered (T&E) species. For this reason, fish species involved in special recovery projects and those of special management concern (T&E) have been targeted as a high priority for the Fish Health Program to address in the National Wild Fish Health Survey (Survey).

A. Endangered Species Act

The Endangered Species Act of 1973 was enacted to protect and enhance the recovery of endangered (in danger of becoming extinct) and threatened (likely to become endangered) species. The Act prohibits harmful actions to any endangered or threatened plant or animal species. Increasing numbers of fish species are being listed under the Endangered Species Act (ESA). Biologists participating in the Survey will be responsible for the proper treatment of T&E species that are captured for disease sampling and/or other reasons, such as population monitoring.

B. Permitting

The Secretary of the Interior, through the Regional Directors of the U.S. Fish and Wildlife Service, may issue permits for the taking and possession of T&E species, under certain circumstances**.**

Fish Health work, whether lethal or non-lethal to the fish collected, may be considered a harmful action which is governed by this permitting process. All personnel involved in collection of samples for the Survey should contact their regional ESA permitting office or recovery team to determine how the Act applies to the work they will be performing, should T&E species be encountered. Decisions on lethal or invasive sampling techniques discussed herein should be made with the participation of the collecting field biologists and the specific recovery team involved.

For more detail, see 50 CFR 17, Code of Regulations, 50 CFR 17.22-17.32 and the Endangered Species Act.

C. Validity and Sensitivity

Many concerns must be considered when evaluating results from non-lethal versus lethal fish health sampling protocols:

1. Sensitivity - in many cases detection of pathogens from blood serum is less sensitive than internal tissues. In other cases, detection of an organism may be enhanced by utilization of mucus or serum.

2. Validity - specific protocols for detection of fish pathogens using non-lethal sampling means are scarce, and many of those that are utilized have not been validated.

Clearly, more research must be accomplished to improve sensitivity and validity of non-lethal detection protocols available in the literature**.**

3. Statistical concerns - most of the stocks being considered for non-lethal fish health sampling are valuable and/or few in number, further reducing the sensitivity for detection of any particular pathogen which may be carried by members of that population**.**

II. Non-lethal Assay Methods to Employ

A. General Considerations

1. Most of the tissues and materials listed in the table in Section E below can be processed and analyzed in the laboratory according to the procedures described within the chapters referenced in the chart. Several procedures are not described in detail within this manual. An attempt will be made, therefore, to detail them within this chapter of the manual.

2. The collection of blood and other tissue biopsies for use in PCR or RT-PCR assays should be done with consideration of the integrity of the DNA or RNA during collection, processing, and performing assays. Samples should be frozen on dry ice immediately after taken. In the case of samples to be assayed by RT-PCR, special commercially available buffers can be used, instead of immediate freezing, to help preserve the integrity of the sample RNA until the assay can be run (RNAlater® is a buffer available from Ambion, Inc. Telephone: 800-888-8804, cat# 7020).

B. Considerations for Virology

1. Cell Lines: An impressive number of fish cell lines have been and continue to be developed and established by professionals in the fish health community. Many of these lines are catalogued in the American Type Culture Collection (ATCC). The ATCC database can be queried on the Internet for availability and purchasing information at http://www.atcc.org.

2. Additional cell lines, which may not be available at ATCC, are reviewed by Fryer and Lannan (1994).

   NOTE: The detection of virus from water samples using an adsorption-elusion technique (as listed in the chart) is labor intensive, and therefore only the literary reference for this procedure is given (McAllister and Bebak 1997).

C. Considerations for Bacteriology

1. Culture on Selective Media - a brief list of several media, which are considered selective for culturable bacterial fish pathogens, is given in Section IV of this chapter.

2. Part V and VI of this Chapter describe, in detail, procedures for detecting bacteria from fish mucus and water samples**.**

D. Considerations for Parasitology

Any material collected non-lethally can be examined microscopically for parasites. Tests using PCR can be conducted to detect Survey target parasites such as Myxobolus cerebralis and Ceratomyxa shasta. See manual chapters referenced in the chart below (Section E).

E. General Non-lethal Sampling Considerations

Below is a chart which lists seven main forms of fish tissue and other material that can be collected non-lethally and examined for fish pathogens targeted for the Survey. Chapters in which protocols can be found are referenced.

Sample Material	**Target Pathogens**	**Protocols**	**Chapter Reference**
Coelomic Fluid	Viral pathogens *R.salmoninarum* Bacterial Pathogens	Cell culture/ PCR ELISA/ PCR M-FAT/PCR BHIA culture/ serology	Chapter 10 & 11 Chapter 6 & 7 Chapter 14 Chapter 5
Blood and Blood Serum*	Viral pathogens *R. salmoninarum* Bacterial pathogens Parasites	Cell culture/ PCR ELISA/ PCR BHIA culture/ serology Microscopy/PCR	Chapter 10 & 11 Chapter 6 & 7 Chapter 5 Chapter 8
Fecal /Intestinal Lavage	IPN *R. salmoninarum* Bacterial Pathogens Parasites (C. shasta)	Cell culture/PCR KDM culture/PCR BHIA culture/ serology Microscopy/PCR	Chapter 10 & 11 Chapter 5 Chapter 5 Chapter 8
Mucus*	IHN, IPN Bacterial Pathogens Parasites	Cell Culture/PCR BHIA culture/ serology Microscopy	Chapter 10 & 11 Chapter 5 Chapter 8
External Lesions	Viral pathogens Bacterial Pathogens Parasites	Cell Culture/PCR BHIA culture/ serology Microscopy	Chapter 10 & 11 Chapter 5 Chapter 8
Water/Sediments**	IPN* Bacterial Pathogens* M. cerebralis	Adsorption-elusion Filtration/culture/ serology Microscopy/PCR	References Chapter 5 Chapter 8
Tissue Biopsy: Gill*, fin, opercula	Parasites (Including M.cerebralis)	Microscopy/PCR	Chapter 8

* with special considerations explained within this Chapter.
** although detection does not come directly from fish, examination of water and sediments can indicate the presence of a pathogen in a particular watershed.

III. Collection of Fish Blood for Pathogen Assays

Blood can serve as the ideal non-lethal tissue for detection of systemic infections. The preservation and storage methods for blood collection depend largely upon the target pathogen(s) of interest.

A. Collection Procedure

1. It is most advantageous to use Vacutainer® collection tubes with accessory needles and holders, available through most scientific supply catalogs. Vacutainer® tubes come in a variety of capacities, but the 2 or 5 mL draw are the most appropriate for pathogen detection purposes. Vacutainer® tubes are also available with a variety of preservatives and anti-coagulants (EDTA, heparin, clot activator,), depending upon the intended use of the sample. Some assays are not compatible with these additives (these substances can act as inhibitors in PCR), so untreated tubes are available with no additive.

2. Blood should be collected only from fish large enough to withstand the procedure. Collection of blood from small fish can be lethal. No more than 1 mL of blood can be obtained from a 100g fish without lethal results.

3. Fish should be thoroughly immobilized by anesthetic prior to handling to avoid injury by the needle.

4. Place the anaesthetized fish on a non-slip surface, or have an assistant hold large fish so that the collector has access to the ventral peduncle surface.

5. Affix a properly sized needle onto the Vacutainer® holder, and carefully place the stopper end of the Vacutainer tube down into the holder, but do not puncture the rubber seal. It is necessary to maintain vacuum inside the tube in order to obtain blood. If the vacuum is broken, a new tube must be used.

6. Blood is removed from the caudal vein located ventral to the spinal column. Insert the needle into the side or ventral surface of the caudal peduncle and approach the area with the needle. When the needle is near the vein, push on the end of the Vacutainer so that the rubber stopper is punctured and blood will flow into the tube. It may be necessary to move the needle tip slightly to locate the vein. Once located, the blood will flow freely into the tube. When enough volume is obtained, remove the needle from the fish and remove the tube from the holder. It is necessary to properly preserve the blood sample by freezing or distribution into appropriate buffers before the blood clots.

B. Whole Blood Samples for Microscopic Examination

1. Blood is collected in anti-clotting agent (heparin) and smears are prepared according to individual assay protocols (wet mount for immediate observation or dried and fixed for hematological staining).

C. Whole Blood Samples for Virology and Bacteriology

1. Blood is collected without a preservative, and immediately diluted 1:10 with buffered saline.

2. Store samples on ice until processing.

3. Samples can be homogenized by stomacher, or by repeated expulsion with a small gauge needle and syringe.

4. The samples are further diluted and inoculated according to standard protocols for the detection of the target pathogen.

D. Whole Blood Samples for RT-PCR and PCR

1. RNA viruses – samples must be collected in tubes with no additives.

2. Immediately after collection, a small amount of blood is frozen on dry ice, or placed in a preservative buffer solution for RNA (RNAlater®, Ambion, Inc.).

3. PCR: DNA or RNA are extracted according to tissue protocols for PCR and RT-PCR.

E. Serum Samples

Whole blood is allowed to clot or settle, and the serum is carefully aspirated from the remainder of the clot.

1. In case blood cells remain suspended in the serum, the sample can be centrifuged for 10 minutes at 3,000 rpm. Serum can be stored for 1 week at 4°C, or for one year at -20°C. The addition of thimersal (1:10,000) will preserve the samples for extended storage, as long as this additive does not interfere with intended assays.

2. Serum can be used in a variety of assays including immunological antigen and antibody detection.

IV. Non-lethal Detection of Infectious Salmon Anemia Virus in Blood

Infectious salmon anemia virus can be detected from whole fish blood using both tissue culture and reverse transcriptase polymerase chain reaction technology (RT-PCR), as reported by Giray et al. (2005). Refer to Section III for blood collection procedures. Blood is immediately diluted in physiological saline or PBS, processed, and inoculated onto plates containing both SHK-1 and ASK cell lines (Chapters 10 & 11). Any CPE detected by 28 days can be confirmed as ISAv by RT-PCR (Chapter 12).

Blood has been determined to be a suitable material for direct use in RT-PCR for detecting RNA from ISAv as well, and is already used for screening of wild anadromous salmonids during upstream migration. The following is a detailed protocol for RNA extraction and RT-PCR on whole blood preserved in RNAlater® (Section III):

A. RNA EXTRACTION

Using QIAGEN® RNeasy Kit with optional vacuum manifold:

1. Materials and Reagents:

   RNeasy Mini Kit if Tissue is used (QIAGEN # 74104)
   QIAGEN Viral RNA Kit (QIAGEN # 52904)
   Qia-shredder spin columns (QIAGEN # 79654)
   QiaVac-24 vacuum manifold -Optional (QIAGEN # 19403)
   Vacuum Pump- Optional
   Microcentrifuge
   RNALater® (Ambion, Inc. # 7020).
   QIAGEN Viral RNA Mini Kit if Cell culture or serum is used (QIAGEN #52904)
   Ethanol (Absolute 97-100%)
   Ethanol (70%)
   β-Mercaptoethanol (14.5 M)
   Pipettor (100-200 µL and 0.5mL)
   Aerosol Barrier Tips
   1.5mL centrifuge tubes
   Micro centrifuge
   heat block(s) (70°C and 95°C)
   latex or nitrile gloves

2. General QA/QC - Wear and change gloves often. This prevents spread or contamination of sample RNA/DNA and polymerase naturally occurring on the skin. Review Chapter 12 for important QA/QC considerations before proceeding with this protocol.

3. Assay Preparation:
   Mix Beta-mercaptoethanol (ME) into Buffer RTL before starting (10 µL ME to 1 mL RLT).Ensure ethanol is added to Buffer RPE. Prepare 70% ethanol solution for step 4. Label QIAshredder and collection tubes. Set up QuaVac-24 unit with VacValves

and/or VacConnectors, and place labeled spin columns into VacConnectors so they are ready for lysates. Label 1.5 mL mc tubes for lysing samples (make sure that a positive and negative controls are included). Fish blood should be stored refrigerated or frozen in RNAlater.

4. Procedure (adapted from RNeasy Handbook):

   a. Pippette 30 µL blood into a 1.5 mL mc tube.

   b. Add 600 µL Buffer RTL, and vortex for 1 minute.

   c. Transfer lysate to a QIAshredder spin column.

   d. Homogenize the tissue by centrifuging the QIAshredder for 2 minutes at maximum speed.

   e. Discard the QIAshredder spin column and retain the filtered lysate.

   f. Either place a cap on the collection tube containing the lysate, or transfer to a new 1.5 mL microcentrifuge tube.

   g. Add 600 µL of 70% ethanol to the cleared lysate, and mix well by pipetting. A precipitate may form, but should not affect the outcome of the procedure.

   h. Apply 700 µL of the sample at a time (including any precipitate) to an RNeasy spin column that has been placed on the QiaVac 24 manifold. Vacuum until all lysate has passed through filter.

   i. Apply the remainder of the sample to the spin column and vacuum filter again.

   j. Pipette 700 µL Buffer RW1 into the spin column, and vacuum filter.

   k. Be sure ethanol is added to Buffer RPE, then pipette 500 µL Buffer RPE to spin column and apply vacuum.

   l. Add another 500 µL Buffer RPE to the spin column and vacuum.

   m. Place spin column in a clean collection tube. Centrifuge for 3 minutes at maximum speed (14,000 X g). This step ensures drying of the filter.

   n. Transfer spin column to an RNase free 1.5 mL microcentrifuge tube and pipette 50-100 µL RNase-free water directly into the spin column. Centrifuge for 1 min at 8000Xg to elute. Repeat elution with fresh water (50-100 µL) into the same collection tube. Measure and dilute RNA to 10 to 100 ng/µL.

B. RT-PCR Procedures (ISAV) (adapted from Keleher et al. 2003)

1. Materials and Reagents:

   Superscript One-Step RT-PCR System (GIBCO #10928-018)
   Rnasin RNase Inhibitor (Promega)
   Upstream primer for ISAv 1D: 5' GGC TAT CTA CCA TGA ACG AAT C
   Downstream primer for ISAv 2: 5' TAG GGG CAT ACA TCT GCA TC
   Molecular Grade (RNase free) d-H_2O
   0.5 and 1.5 mL microcentrifuge tubes (RNase/DNase Free)
   Thermal cycler
   0.5-25µL and 20-200µL pipetters: (positive displacement with matching lunger/tips and/or regular with aerosol barrier tips)
   gloves (latex or nitrile)
   Bench top UV cabinet.

2. Procedures:

   a. Master Mix Preparation using One-Step RT-PCR Systems (Invitrogen): Add water first and RT/Taq mix last. Keep all reagents cold in frozen cryo-rack during mixing, and return them to freezer immediately after use. Refer to Chapter 12 for general procedures and considerations when performing PCR. The following are final concentrations of each component to be added into each reaction tube:

Component	Final Concentration
2X Reaction Mix	1X
Sense primer	50 pMole
Antisense primer	50 pMole
RT/TAQ Mix(Gibco)	1 µL
Rnasin(Promega)	10 units
RNase free Water	added to above to 49 µL total

   b. Place 49µL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes with MM to sample loading area. Add 1 µL template RNA.

   c. Thermocycler should be programmed for the following regime:

Reverse Transcription:	42°C 15 min.
Pre-dwell	94°C 5 min.
30 Cycles of:	94°C 45 sec.
	59 °C 45 sec
	72 °C 1 min.45 sec.
Post dwell	72°C for 7 min.

   Hold to 4°C chill at end of program.

   d. Retain amplified product for electrophoresis as described in Chapter 12. The resulting cDNA product will produce a 493 base pair band after electrophoresis in a 2% agarose gel, indicating a positive finding for ISAv.

V. Non-lethal Detection of Bacterial Pathogens in Mucus of Fish

The bacterial fish pathogen Aeromonas salmonicida can be readily isolated from the mucus of salmonids using the following techniques. Some success in isolating Yersinia ruckeri and Flavobacterium columnare has also been reported by field personnel. Bacteria can be detected using simple swab/streaks onto agar media, or they can be quantified through serial dilution in PBS. Quantification can help reflect the level of systemic infection in some fish. The following methodologies have been adapted from Cipriano et al. (1992).

A. Non-quantitative Method

1. Mucus is scraped gently from the lateral surface of the fish with a 10µL inoculating loop, and streaked directly onto the agar media of choice, according to the target pathogen(s) for detection.

2. Alternatively, commercially prepared transport swabs (see source list) can be used to collect mucus specimen, for later streaking onto agar media. Follow manufacturer's instructions on use of the swabs. Be sure to store transport swab samples cold and streak the sample onto appropriate media within 24 hours of specimen collection.

B. Dilution Plate Counts from Non-lethal Mucus Samples

Small samples of mucus can be weighed and diluted in PBS, then plated on an appropriate agar medium. After incubation, colonies of target bacterial pathogens can be quantified and reported as colony forming units per gram of mucus (cfu/gm).

1. With a sterile scalpel or bladed instrument, gently collect a small amount of mucus from the lateral surface of the fish.

   a. Place into a pre-weighed sterile culture tube and keep sample cold until processing can be accomplished.

   b. Determine the weight of the sample by subtracting the tube weight from the gross weight after sample is collected.

2. Make a 1:10 dilution according to tissue weight with phosphate buffered saline(PBS). Homogenize tissue with rigorous pipetting motions.

3. Make serial $\log_{10}$ dilutions of the 1:10 dilution in PBS: Fill micro titer plates with 90µL PBS per well (4 wells per sample will be needed), or any other vessel with appropriate volume to accomplish 10 fold dilutions.

4. Add 10 µL of the 1:10 dilution tube to the first micro titer well.

5. Make 10 fold dilutions by taking 10µL from the first well and placing it in the second.

a. Change pipet tips in between dilutions.

b. Do the same from the second to third to fourth wells or tubes of PBS.

6. Prepare media plates with appropriate labels. It is helpful to space the numbers 1 through 5 around the edge of the plate and in the middle to aid in placement and tracking of each dilution.

7. Plate all dilutions, using the same tip but working from the fourth dilution backwards to the higher dilutions. 10µL drops are most readily absorbed by the agar, but larger volumes can also be plated and spread onto individual media plates.

8. Keep plates upright until the fluid of every drop has been absorbed. Then turn them over and incubate appropriately according to optimal conditions required by the target pathogen.

9. Quantify and isolate bacterial colonies produced from samples:

   a. Drops will vary in bacterial load depending upon the sample.

   b. Pick a spot that contains a countable number of colonies.

   c. Pick representative isolates of each colony type observed for characterization and identification.

   d. Count each colony type and record on media tubes as dilution number times number of colonies counted (example: 1 X 16).

10. Calculate colony forming units per gram mucus (cfu/gm):

    a. cfu/gm = colonies counted X dilution factor.

    b. example: 30 colonies counted in 10^{-2} dilution =30 X 10^{2} per 0.01 gm (10^{-2} contains 0.01gm of mucus)or 30 X 10^{4} per 1.00 gm (decimal moved)3 X 10^{5} cfu divided by original sample weight = cfu/gm tissue.

11. Quantification of bacteria on the surface of fish can give a good indication of the level of internal infection that may exist. For instance, on salmonids, when A.salmonicida exceeds 10^{3} cfu/gm mucus, there is a strong possibility that the fish has a systemic infection that could be lethal to the fish. This work has been done under normal fish culture circumstances, however, and adjustments to determine lethal versus carrier infections in wild fish may be necessary.

C. **Materials and Sources**

BACTI-SWAB™ Modified Stuart's Transport Medium available from Remel (800-255-6730).

VI. Procedures for the Detection of Bacteria in Filtered Water Samples

Water can be examined for a variety of bacterial species, including those that can serve as pathogens to fish. Water can be sampled directly from streams, ideally in areas where more fish are congregated, such as below pools, logs, and other habitat fish use for shelter. Sampling at effluents of fish culture facilities can also provide information on cycles of pathogens being shed into the environment. Bacteria can be enumerated on the media plates and reported as colony forming units (cfu) per milliliter (mL). The following methodologies have been adapted from Ford (1994).

A. Methods

1. Collect water sample in sterile 200 mL container.

    a. Take care not to contaminate sample with hands.

    b. Keep sample cold until filtering and plating can be accomplished.

2. Wipe filter unit with alcohol and carefully load with .45µm pore filter paper (grid side up). Take care not to contaminate filter by touching - use forceps that have soaked in alcohol.

3. Filter several dilutions of sample with enough sterile distilled water to make a 100 mL total volume.

    a. Start with the most dilute volume of sample. For example: if plating 1,10 and 100 mL of water, fill filter unit with 99 mL d-H_2O and transfer 1mL sample. The next dilution (10 mL sample in 90 mL d-H_2O) can be done without disinfecting filter unit. However, wipe the filter unit with alcohol between different water samples.

    b. The number and volume of dilutions should be adjusted with the quality of water samples: murky water will contain a lot of bacteria, and therefore, the smallest filtered volume may have to be 0.5 or 0.1 mL

    c. Always dilute with sterile distilled water to bring total filtered volume to 100 mL for consistency and even distribution of bacteria.

4. After filtering, plate samples onto appropriate media:

    a. With disinfected forceps, gently grasp each filter paper from unit and place grid side down onto agar media surface, removing any air bubbles with the forceps.

    b. The filter need remain for only a few minutes before it can be removed and discarded with clean forceps.

5. Incubate plates for appropriate time and temperature depending on target organisms.

6. Perform bacterial counts and isolations directly from plates. Frequency plots and histograms of the major bacterial genera can be plotted as well as the number of cfu/mL of target pathogen in a particular sample.

B. Materials and Sources

Sample Containers (Sterile) 240 mL (Thomas Scientific/6186-M40)
Nalgene Filter Apparatus (Thomas Sci./4618-N60)
Nalgene Filter Apparatus (Thomas Sci/ 4618-N62)
Filters (Sterile), 47mm, 0.45□m pore (Thomas Sci./4626-J20)
Filter Pump (aspirator type - VWR/ 28610-008) or a vacuum pump
1 mL Pipets (Sterile-Fisher Sci./13-678-11A)
Coomassie Brilliant Blue (R250-Sigma/B-0149)
Alcohol (70% isopropyl)
Sterile Distilled Water
Agar Plates (depending on target pathogens)

C. Media Options

The following are media and components which select for, or enhance isolation of the given target pathogen. A literary reference is provided for each.

1. *Aeromonas salmonicida*:

   a. Tryptic soy agar (TSA)-commercial media - follow preparation instructions.

   b. Coomassie Brilliant Blue agar (CBBA) - (Cipriano and Bertolini 1988).

TSA	40 g
Coomassie brilliant blue R-250(CBB)	0.1 g
d-H_2O	1.0 L

   Autoclave for 15 min at 15 psi (121°C) and pour into petri plates. *A. salmonicida* colonies will appear as dark blue, friable colonies after 48 hours at 20°C.

2. *Flavobacterium psychrophilum, F. columnare*:

   a. Tryptone Yeast Extract Supplemented (TYES) - (Holt and Amandi 1989)

Tryptone	4.0 g
Yeast Extract	0.4 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.5 g
Agar	10.0 g
d-H_2O	1.0 L

   Dissolve ingredients and adjust pH to 7.2. Heat to boiling for 1 minute. Autoclave for 15 min at 15 psi (121°C) and pour into petri plates.

b. Tryptone Yeast Gelatin (TYG) - (Bullock et al. 1986)

Tryptone	2.0 g
Yeast Extract	0.5 g
Gelatin	3.0 g
Agar	15.0 g
d-H_2O	1.0 L

Dissolve ingredients and adjust pH to 7.0. Heat to boiling for 1 minute. Autoclave for 15 min at 15 psi (121°C) and pour into petri plates.

3. *Yersinia ruckeri*:

a. TSA, BHIA - both commercially prepared

b. Shotts-Waltman (SW) -(Waltman and Shotts 1984)

Sodium Chloride	5.0 g
Tryptone	2.0 g
Yeast Extract	2.0 g
Tween 80	10 mL
$CaCl_2 \bullet 2H_2O$	0.1 g
Bromthymol Blue	0.003 g
d-H_2O	50 mL
pH to 7.4 and add:	
Agar	15 g

Heat to boiling. Autoclave for 15 min at 15 psi (121°C). Add 10 mL of 0.5g/mL sucrose solution which has been filter sterilized. Refrigerate poured plates until use.

Y. ruckeri will produce a green colony with a zone of hydrolysis (precipitation of calcium oleate from Tween 80). Always confirm colony identity with biochemical characterization of isolates. (Type II will not hydrolyze Tween 80).

4. *Renibacterium salmoninarum*:

a. Kidney Disease Medium (KDM2) - (Evelyn 1977)

Peptone	10.0 g
Yeast Extract	0.5 g
L-Cysteine HCl	1.0 g
Distilled water	1000 mL
Adjust pH to 6.5 and add:	
Agar	15.0 g

Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

FBS	200.0 mL

b. The following volumes of antibiotics can also be added to the KDM2 (SKDM) to reduce overgrowth from other bacterial organisms (Austin et al. 1983).

Cyclohexamide	4.0 mL (see below)
D-cycloserine	1.0 mL
Polymyxin B-sulfate	2.0 mL
Oxolinic Acid	1.0 mL

Prepare antibiotics following these formulas:

Cyclohexamide	1.2 g + 96 mL d-H_2O
D-Cycloserine	0.3 g + 24 mL d-H_2O
Polymyxin B-sulfate	0.3 g + 24 mL d-H_2O
Oxolinic Acid	0.06 g + 24 mL (5% NaOH)

c. Researchers found that variable lots of peptone could adversely affect the ability to successfully culture R.salmoninarum using these media. Evelyn et al. (1990) reported on the use of a metabolite solution from KDM broth containing R.salmoninarum (autoclaved or filter sterilized using 2 µm pore size) and added to the KDM media at 2% (v/v). The addition of metabolite solution to the media has shown to improve success in culturing this organism, and seems to negate the adverse effects of poor peptone lots used in the media.

5. *Edwardsiella ictaluri*

a. S-W E. ictaluri Selective Media - (Shotts and Waltman 1990)

Tryptone	10 g
Yeast Extract	10 g
Phenylalanine	1.25 g
Ferric ammonium chloride	1.2 g
Bromthymol blue	0.003 g
Bile salts	1.0 g
Agar	15.0 g
Distilled water	980 mL

Dissolve ingredients by boiling, then cool to 50°C and adjust pH to 7.0. Autoclave for 15 min at 15 psi (121°C). Cool to 50°C again and add mannitol (filter sterilized) to 0.35% (v/v) and colistin sulphate to 10 ug/mL.

Proteus species will produce brown colonies (caused by phenylalanine and ferric ammonium chloride). Serratia and Aeromonas will ferment mannitol producing yellow colonies. Edwardsiella ictaluri will produce translucent, colorless colonies.

VII. Method for Non-Lethal Gill Biopsy

Gill filaments can be removed from fish while under anesthesia with little injury to the fish. The tissue can be examined directly under microscopy for parasites; preserved for histology; or frozen for examination using other diagnostic methods, such as PCR for Myxobolus cerebralis. The following protocol has been adapted from methods used for collection of gill tissues for gill Na^+, K^+-ATPase activity measurements in salmonids (McCormick, U.S. Geological Survey, personal communication).

A. Materials

Chamois Cloth
Rounded forceps
Fine point scissors
(Vannas eye scissors - 7mm curved blade, Sicoa-phone 201-941-6500, Cat.# OM-1401)
Collection vials
Fish anesthetic

B. Methods

1. While fish is under anesthesia, place on a moistened chamois cloth to minimize scale loss and damage. A right handed individual should place the fish on its right side so that the head is to the left and tail to the right.

2. Gently pull back the operculum with rounded forceps. A cartilaginous septum (present in some species such as salmonids, but not in others) holds filaments together for one-half their length.

3. Using a fine pointed scissors, remove 4-6 filaments just above the septum from a fish weighing between 20 and 80 grams (remove more or less tissue for larger and smaller fish, respectively).

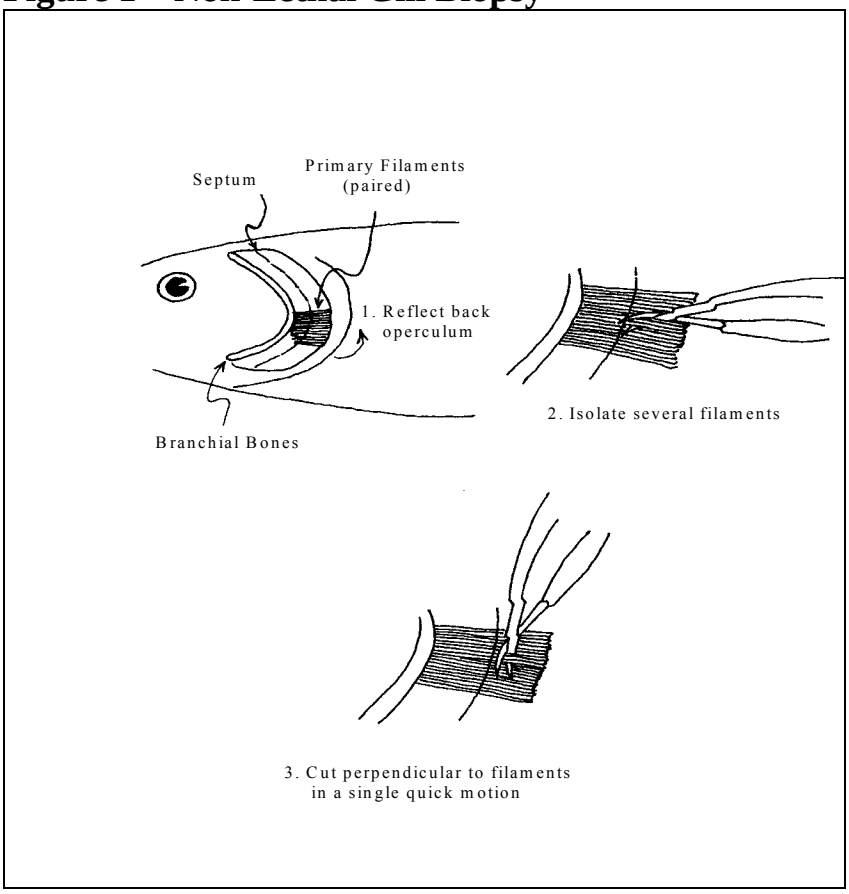
    a. With the operculum reflected, isolate several filaments with the open blades of the scissors (see Figure 1).
    b. Turn scissors so that they are perpendicular to the filaments and cut in a single quick motion.
    c. To retain the filaments on the scissor blades, turn the scissors slightly as you finish cutting.
    d. Take care not to crush sample or remaining filaments. If there is any movement from the fish, be sure to retract forceps and scissors quickly to avoid injuries. Return fish to fresh water immediately.

4. Transfer filament samples to appropriate containers for storage until assays cane be performed.

C. Recovery of Fish Subject to Gill Biopsy

Upon return to the water, the fish may bleed slightly for up to one minute. Excessive bleeding, beyond one minute is usually associated with cutting to deeply into the filaments (i.e. below the septum). Even excessive bleeding does not usually result in mortality.

Figure 1 – Non-Lethal Gill Biopsy



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CHAPTER 15

Procedure for Revisions to the National Wild Fish Health Survey Laboratory Procedures Manual

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I. Introduction

The National Wild Fish Health Survey Laboratory Procedures Manual is intended to provide optimum detection methods and standardized protocols for all aspects of the Survey. This document is intended to be adaptive to follow both the changing body of scientific knowledge and to address emerging pathogens. The following chapter outlines the mechanism for revising to the NWFHS Manual.

II. Format of the Manual

A. Title and Citation

National Wild Fish Health Survey Procedures Manual – Third Edition (June 2005).

Woodland, J., (ed.) 2005 National Wild Fish Health Survey Laboratory Procedures Manual, Third Edition. U. S. Fish and Wildlife Service.

B. Chapters

The Manual consists of 15 chapters and necessary appendices. The Quality Assurance Program (Chapter 15 in the 2001 First Edition of the Manual) still pertains to the NWFHS, but is now contained in a separate volume of the USFWS Handbook:

Chapter 1	**Overview of the National Wild Fish Health Survey**
Appendix 1. A	Glossary of Terms
Appendix 1. B	APHIS Memorandum No. 567.6 regarding OIE Notifiable Diseases
Chapter 2	**Sample Collection and Submission**
Appendix 2. A	Shipping Addresses and Contacts for the Fish Health Centers
Appendix 2. B	NWFHS Submission Form
Chapter 3	**Sample Receipt and Laboratory Tracking**
Appendix 3. A	Case History Record
Appendix 3. B	Chain of Custody Form
Chapter 4	**Standard Necropsy Procedures for Finfish**
Chapter 5	**Bacteriology**
Appendix 5. A	Media Formulations
Appendix 5. B	Reagents
Appendix 5. C	Profiles Obtained with API-20E for Known Fish Pathogens
Appendix 5. D	Flowchart for Targeted Gram Negative Fish Pathogens
Appendix 5. E	Flowchart for Targeted Gram Negative PRI
Appendix 5. F	Some Characteristics of Long Gram-Negative Bacteria
Chapter 6	**ELISA for Detection of Renibacterium salmoninarum Antigen in Fish Tissue**
Appendix 6. A	Quality Control Program for ELISA

Appendix 6. B Standardization of Reagents
Appendix 6. C Collection and Processing of Tissue Samples for ELISA
Appendix 6. D Reagents, Supplies and Equipment Lists

Chapter 7 Corroborative Testing of *Bacteria* by Polymerase Chain Reaction (PCR)
Appendix 7. A Worksheet for DNA Sample Data
Appendix 7. B Worksheet for Initial Amplification of Rs DNA by PCR
Appendix 7. C Worksheet for Nested (Second Round) Rs PCR
Appendix 7. D Photo documentation and Report of Results

Chapter 8 Parasitology
Appendix 8. A Reagents and Solutions
Appendix 8. B Common Parasites of Fish

Chapter 9 Corroboration Testing of Parasites by PCR
Appendix 9. A Equipment, Supplies and Reagents
Appendix 9. B Mc PCR Data Sheet
Appendix 9. C Analysis of Extracted DNA using an UV Spectrophotometer

Chapter 10 Cell Culture of Fish Cell Lines

Chapter 11 Virology
Appendix 11.A Glossary of Terms used in Tissue Culture and Virology
Appendix 11.B Media Used in Tissue Culture and Virology

Chapter 12 Corroborative Testing of Viral Isolates
Appendix 12.A PCR Worksheets: Amplification of Nucleic Acid for Corroboration of Viral Fish Pathogens
Appendix 12.B Photo documentation of Agarose Gel Electrophoresis of PCR Products
Appendix 12.C General Procedures for PCR Protocols
Appendix 12.D Analysis of Extracted DNA using Gene Quant UV Spectrophotometer
Appendix 12.E Quality Assurance/Quality Control for PCR
Appendix 12.F Reagents

Chapter 13 Histology for Finfish

Chapter 14 Non-Lethal Methodology for Detection of Fish Pathogens

Chapter 15 Protocol for Revision to the National Wild Fish Health Survey Laboratory Procedures Manual
Appendix 15.A Third Edition (2005) Manual Revision Committee Members
Appendix 15.B Associate Editors - Previous Contributors to the First Edition (2001)
Appendix 15.C Editor/Chairman of Earlier Editions

C. Layout

The layout of the document will be in outline form as follows:

1. Roman numerals for main chapter headings: I, II, III, IV, V.

2. Sub-heading formatting will follow: A, B, C….1, 2, 3,…..i, ii, iii.

3. The indentation is to be at ¼ inch for each level with the tab to the text at ¼ inch as well (except for tables which are custom designed according to best table expression)

4. Each chapter, or a specific section of a chapter, will have its own reference section and additional reading section.

5. Each chapter will have its own appendices with chapter number and capitol letters.

   a. First appendix of chapter 3 would be titled and referenced as (3.A)

   b. Second appendix of chapter 3 would be titled and referenced as (3.B)

III. Stringency

A. The appropriateness of methodologies shall be determined by the center Directors based on several factors:

The sensitivity of the assay
The specificity of the assay
Availability of reagents
Availability of technology and/or required personnel training
Labor requirements
The cost of the assay

B. The incorporation of additional pathogens into the Manual shall be contingent on the availability of appropriate screening and corroborative tests.

IV. Revision Process

A. The Manual will be reviewed on an annual basis by all USFWS Fish Health Centers.

B. Recommends for revision will be submitted at the annual Fish Health Biologist Meeting.

C. When revisions include proposal of the inclusion of a new pathogen, the Center submitting the proposal will provide the appropriate screening and corroborative test methods, and references for the protocols.

D. Center Directors will adopt revisions by a super majority (2/3 majority) vote.

E. A member from each Fish Health Center will be assigned to the Manual Revision Committee (Manual Committee).

F. The Committee will make appropriate revisions to the Manual and submit the final draft to all Fish Health Centers for review.

G. The revised Manual will be adopted at the subsequent Fish Health Biologist Meeting.

H. A master copy of the Manual will be maintained on the National Wild Fish Health Survey website. In instances where serious typographical or technical errors occur, the web version of the Manual will be updated to reflect immediate changes that cannot wait until the next revision cycle.

I. The National Aquatic Animal Health Coordinator for the USFWS will be responsible for coordinating revisions to the NWFHS Database when new pathogens or procedures are adopted.

V. Committee Members

The Committee shall consist of nine people, the NWFHS representatives from each Fish Health Center. Additional members may be included by a consensus vote of the Committee, if additional expertise is needed. The Committee will have one chairperson, to be nominated and elected by simple majority by the Committee members each year.

VI. Committee Authority

A. The Committee will have the authority to make minor revisions defined as edits that update, optimize, or support current methodologies without changing the screening or corroborative methods. These revisions can be incorporated by consensus vote of the members of the revision committee. If a consensus decision cannot be made, the chair will take the issue to the Center Directors for a final decision by super majority (2/3 majority).

B. Major revisions are changes that involve the addition of new pathogens, new methods, or significant deviations from the current protocol. These revisions will require a super majority (2/3 majority) vote by Center Directors. Proposals for new pathogens, or revisions of current methods will be discussed at the Annual Fish Health Biologists meeting. Center Directors will decide by super majority (2/3 majority) whether to accept, reject, or amend the recommendation(s) for revision.

C. The Committee Chair will maintain a record of all decisions made during the annual meeting and direct the Committee to incorporate these revisions in the annual revision process.

VII. Manual Revisions

A. If the changes are substantive (more than typos or clarifications), then an additional page(s) will be inserted into the Manual, stating the change and the date of the change.

B. The Chair will keep the master copy that indicates the changes, the date, and the rational for change.

C. The Chair will update the web version of the Manual that is maintained on the NWFHS website. This master copy will be the definitive manual.

VIII. Revision Time Line

A. March 1 - April 1 - Changes are proposed and adopted at the annual Fish Health Biologist meeting. Adopted revisions are submitted to the Committee Chair for inclusion in the next Manual revision.

B. September 1 - November 1 - Revision Committee is formed, a new Chair is elected, and Chair distributes proposed changes to the committee members.

C. November 1- December 1 - The Committee meets via teleconference to determine whether to accept or reject proposed changes.

1. If the Committee rejects the proposal, due to lack of appropriate screening and corroborative test, this decision is communicated to the Center Directors, who may provide further direction to the Committee.

D. December 1- January 1 - Committee makes proposed revision to the Manual and any additional minor revisions adopted by the Committee.

E. January 1- February 1 - Committee distributes the revised Manual to Center Directors and Fish Health Center staff for review.

F. February 1- March 1- Centers submit comments to the Committee.

G. April - The Chair or his/her designee(s) distributes final draft to Fish Health Centers and posts final draft on web page.

H. April - A summary of the revisions and future recommendations are presented to the group at the annual Fish Health Biologist meeting.

Appendix 15.A – Third Edition (2005) Manual Revision Committee

Patricia Barbash
Lamar Fish Health Unit
Non-Lethal Methodology

Norm Heil
Warm Springs Fish Health Center
Procedures for Revision

Laura Kessel
Idaho Fish Health Center
Corroborative Testing of Bacteria
by Polymerase Chain Reaction (PCR)

Ken Lujan
Lower Columbia Fish Health Center
ELISA for Detection of Rs,
Corroborative Testing of Bacteria
by Polymerase Chain Reaction (PCR)

Sonia Mumford
Olympia Fish Health Center
Histology for Finfish

Corey Puzach
La Crosse Fish Health Center
Parasitology,
Corroborative Testing of Parasites by PCR

Kenneth Peters
Bozeman Fish Health Center
Cell Culture Section, Virology
Corroborative Testing of Viruses

Kimberly True,
California-Nevada Fish Health Center
Sample Collection and Submission,
Sample Receipt and Laboratory Tracking,
Standard Necropsy Procedures for Finfish

Jason Woodland, Editor
Region 2 Fish Health Unit@ Dexter
Manual Revision Committee Chair
Procedures for Revision, Bacteriology

Appendix 15.B Associate Editors

Fish health biologists from the nine regional Fish Health Centers developed and contributed individual chapters for the initial version of the Manual (Version 1.0 – June 2001). The following individuals made significant contributions:

Patricia Barbash
Lamar Fish Health Unit
Chp 7 - Rs PCR
Chp 14 - Non-lethal Methodologies

Ray Brunson
Olympia Fish Health Center
Chp 11- Virology Section

Norm Heil
Warm Springs Fish Health Center
Chp 2 - Sample Collection and Submission

Becky Lasee
La Crosse Fish Health Center
Chp 8 Parasitology - Section 1
Chp 3 - Sample Receipt
Chp 15 - QA/QC*

Jerry Landye (formerly with)
Pinetop Fish Health Center
Chp 8 Parasitology- Section 3

Beth McCasland (formerly with)
California-Nevada Fish Health Center
Chp 5 - Bacteriology

Kenneth Peters
Bozeman Fish Health Center
Chp 8 - Cs PCR

Terrance Ott
La Crosse Fish Health Center
Chp 10 -Tissue Culture Section

Kimberly True, Editor
California-Nevada Fish Health Center
Chp 5 - Bacteriology
Chp 6 - ELISA
Chp 8 - Parasitology - Section 2

*Quality Assurance/ Quality Control (QA/QC) is now a separate volume of the USFWS Handbook

Other Contributors to the First Edition:
William E. Knapp and Mary Ellen Mueller - Chapter 1
USFWS - Division of Hatcheries
Arlington, Virginia

Theodore R. Meyers - Chapter 4, 12, & 13
Sally Short - Chapter 13
Alaska Fish & Game
C.F. Division
Juneau & Anchorage

James R. Winton and William N. Batts - Chapter 12
Western Fisheries Research Center Seattle, Washington

Appendix 15.C Editor/Chairman of Earlier Editions

Serving as the lead editor and chairman, the following individuals have made significant contributions to earlier editions in compilation and organization of revisions:

Kimberly True, California-Nevada Fish Health Center
In addition to composing individual chapters Kimberly True served as the Editor/Chairman for Editions 1 and 2.