

CHAPTER 6

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

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Chapter 6 - Table of Contents

I. INTRODUCTION	6-4
A. Standardization of Reagents	
1. Standardization of Antibody Reagents	
2. Optimization of Antibody Dilutions	
B. Tissue Collection and Processing	
C. Performing the Assay	
D. Interpretation of Data	
E. Quality Control	
II. ELISA - Day 1 Preparation	6-6
A. Coating Immunoplates with Antibody	
B. Preparing Wash Solution and Washer Reservoirs	
C. Thaw and Centrifuge Test Samples	
III. ELISA - Day 2 Running the Assay	6-9
A. Washing Plates	
1. Initial Priming of Washer	
2. Wash Protocol	
B. Loading Tissue Samples and Controls	
C. Washing unbound antigen	
D. Adding Secondary HRP-labeled Conjugate	
E. Washing unbound antibody	
F. ABTS Substrate - Color Development	
G. Stop Solution and Plate Reading	
H. Equipment Maintenance upon completion of assay	
IV. INTERPRETATION OF DATA	6-16
A. Quality Control	
B. Negative-Positive Determination	
V. BIBLIOGRAPHY	6-18

Appendices

APPENDIX A - Quality Control Program for ELISA 6-19

1. Standardization of Antibody Reagents
2. Checkerboard Titration - Optimization of Antibody Dilutions
3. Tissue Collection and Processing
4. Performing the Assay
5. Interpretation of Data and Quality Control
 - A. Controls
 - B. Positive Threshold
6. Corroboration Testing by PCR
7. Annual Testing Program
8. Equipment Calibration and Maintenance

APPENDIX B - Standardization of Reagents 6-23

1. Overview
2. Standardization of Antibodies and Control Reagents
 - A. Pooling of Antibody Reagents
 - B. Positive Control Antigen
 - C. Negative Control Tissue (NCT)
3. Checkerboard Titration Assay
 - A. Reagents and test samples
 - B. Microplate format
 - C. Checkerboard titration procedure
 - D. Determination of antibody dilutions

APPENDIX C - Collection and Processing of Tissue Samples for ELISA 6-29

1. Sample Collection, Transport and Storage
 - A. Field Collection Supplies and Guidelines
 - B. Samples Collected from Adult Fish
 - C. Samples Collected from Juvenile Fish
2. Sample Processing
 - A. PBS-T20 Buffer
 - B. Instruments
 - C. Tissue Dilution and Processing

APPENDIX D - Reagents, Supplies and Equipment Lists 6-32

1. Reagents
 - A. Kirkegaard and Perry Laboratories (KPL) Assay Reagents
 - B. Additional Reagents
 - C. Formula for Phosphate Buffered Saline (PBS) with Tween 20
2. Supplies
3. Equipment

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 (Figure 1). The protocol, appendices, and figures are adapted from the methods of Ron Pascho and Dr. Diane Elliott, at the USGS-Western Fisheries Research Center in Seattle, Washington, with additional revisions by Dr. Theodore Meyers of Alaska Department of Fish and Game, and Kimberly True of USFWS, Ca-Nv Fish Health Center.

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 A-E, 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 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 *R.salmoninarum* 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 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 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 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 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 5-10mL aside for Chromogen Control (CC) and Substrate Control (SC) wells. Add the CAb to the remaining coating Solution and dispense 200µL 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 (1ug/mL) as a standard dilution for CAb as an example only.

Refer to Table 1 for proper placement of reagent controls at each step of the assay. The Control Plate Template (Figure 2) 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:

<u>Number of samples</u>	<u>Plate</u>	<u>Notes</u>
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 **25mL per plate** as a guideline (200µL per well x 96 = 19.2mL). This allows extra volume for priming dispensing cassettes without significant waste of antibody reagent.

<u>Number of samples</u>	<u>Number of plates</u>	<u>CAb Required (1:1000)</u>
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 5mL to be used in Conjugate and Substrate (CC & SC) negative control wells.

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

- b. Remove the 5mL and place in a reagent reservoir or clean test tube. Add 200µL 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: 75µL CAb to remaining 75mL 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 200µL and 12 columns (use the same volume setting for all reagents, except the last step using STOP SLN = 50µL)
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 1. Sample and Control Placement used in ELISA

STEPS>	STEP I COATING ANTIBODY	STEP II SAMPLES/ CONTROLS	STEP III CONJUGATE	STEP IV CHROMOGEN (ABTS)	STEP V STOP SOLUTION
Incubation time & temp>	(16 hr, 4⁰C)	(3hr, 25⁰C)	(2hr, 25⁰C)	(20 min 37⁰C)	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 dH₂O:
- 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 50mL conical tube, and add dH₂O.
 - 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 dH₂O.

- 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 dH₂O. Prepare ~750mL 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 dH₂O.
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.

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 37C for exactly 20 minutes. Stop Solution is added and plates are read immediately using a Spectrophotometer (Plate Reader) set at 405nm 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 dH₂O 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 μ L 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.

PLATE # 1 - Full Control Plate (Refer to Table 1 on page 9, and Figure 2 on page12):

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

<u>Sample</u>		<u>Well Address</u>
#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 10)**. 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 (5mL) aside for Substrate Control (SC) wells. Prepare the conjugated HRP-Ab (.5ug/mL or 1:2000) in remainder of Milk Diluent and dispense

200 μ L 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 (.5 μ g/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 dH₂O. (Example: Add 5 mL Milk Diluent to 95 mL dH₂O).
 - c. Remove 5mL to be used in SC wells as a negative reagent control, and place in a reagent reservoir.
 - d. Manually pipet 200 μ L of MILK DILUENT ONLY to each SC well on Plate #1 (and any additional control plates).
2. Dilute HRP-Conjugate 1:2000 (.5 μ L/mL) in the remaining volume of Milk Diluent
 - a. Pipette HRP-Ab into the remaining Milk Diluent at 1:2000 (For example, 45 μ L HRP-Ab to 90mL 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 μ L 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 dH₂O
6. Turn on incubator and set temperature for 37C at this time. (Most incubators will not require two hours to reach and hold a stable temperature of 37C, 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 μ L 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 25mL 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 μ L 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 37C incubator.
7. Allow approximately 1 minute, then proceed with the next plate. The minute between plates allows adequate time to add Stop Soln and read each plate before the next plate has completed the 20-minute incubation period.
8. Rinse the dispensing cassette with dH₂O.

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 μ L 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 μ L, 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 dH₂O (5% diluted 1:2 = 2.5% concentration), then deliver 50 μ L of this diluted Stop Solution to each well, which contains 200 μ L 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 μ L.

1. Prepare stop solution by mixing 1 part Stop Solution Concentrate to 1 part dH₂O. The volume needed for a single plate is 4.8mL (50 μ L 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:

<u>Number of plates</u>	<u>Stop Sln :dH₂O</u>		<u>Total Volume(mL)</u>
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 μ L.**
- 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 Sln).
- 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 dH₂O. 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 500ppm Chlorine. The Wash Solution and Rinse reservoirs can be cleaned with soapy water. Rinse all reservoirs with dH₂O 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:

$$0.078 + 2(.002) = 0.082$$

2. All test sample values above a mean OD value of .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 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-E provide detailed instructions for each aspect of the methodology used for ELISA.

1. 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 B - Standardization of Antibody Reagents.

2. 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 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 B).

3. 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 C – Collection and Processing of Tissue Samples for ELISA.

4. **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.

5. **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 12).

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:

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.

Negative Control Tissue (NCT) - Negative kidney tissue, tested by ELISA and PCR. Statistically establishes a negative-positive threshold for evaluating test results.

Blanks (BLK) - wells receive PBS-T20 diluent (in place of test tissue) to determine background absorbance levels in the absence of test sample.

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.

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.

<u>Individual Plates</u>	<u>NC OD values</u>	<u>OD MEAN for all Plates</u>
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 : $.00196 \times 2 = .00392...$

THRESHOLD: $.07525 + .00392 = .07918...$

ROUNDED OD THRESHOLD = .079

Samples with mean OD values above .07918 are reported as positive for Rs antigen.

6. 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.

7. 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.

8. EQUIPMENT CALIBRATION & 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

1. 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 μ L 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 -70C 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.

2. 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 dH₂O.
 - b. Optional: For each mL of the above solution prepared, add 10 μ L 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 μ L 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 μ L 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 salmoninarum* 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.

3. 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 1.A.
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 $\mu\text{L}/\text{mL}$ (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 $\mu\text{L}/\text{mL}$ (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).

A	1	2	3	4	5	6	7	8	9	10	11	12	
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	1	2	3	4	5	6	7	8	9	10	11	12	

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 dH₂O.

EXAMPLE: 2.5 mL Coating Solution : 22.5 mL diH₂O

- 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 2.0ug/mL (or 1:500 dilution).

$$1/500 = X/22,000 \mu\text{L}$$
$$X = 44.0 \mu\text{L}$$

The second plate will receive CAb at 1.0ug/mL (or 1:1000 dilution)

$$1/1000 = X/22,000 \mu\text{L}$$
$$X = 22.0 \mu\text{L}$$

The third plate will receive CAb at .67ug/mL (or 1:1500 dilution)

$$1/1500 = X/22,000 \mu\text{L}$$
$$X = 14.6 \mu\text{L}$$

- 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 H₂O 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 4C for 16 hours.
2. On Day 2 - proceed with ELISA following the standard protocol steps III.B on page 12.
 3. Proceed with the addition of HRP-Ab (step III.D) 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 = 14.4mL.
 - b. Prepare the 20 mL of 1X Milk Diluent, for each HRP-Ab concentration, from the 20X concentrate. Remove 2mL 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 (.67ug/mL)

$$1/1500 = X/18,000 \mu\text{L}$$

$$X = 12.0 \mu\text{L}$$

For the HRP-Ab concentration of 1:2000 (.50ug/mL)

$$1/2000 = X/18,000 \mu\text{L}$$

$$X = 9.0 \mu\text{L}$$

For the HRP-Ab concentration of 1:2500 (.40ug/mL)

$$1/2500 = X/18,000 \mu\text{L}$$

$$X = 7.2 \mu\text{L}$$

- 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 CAAb 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

1. Sample Collection, Transport and Storage

A. FIELD COLLECTION SUPPLIES AND GUIDELINES

- a. 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).
- b. 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 -70C) if stored for any extended period of time prior to processing for ELISA, or PCR testing.
- c. 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.
- d. 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

- a. Samples are usually taken following spawning, care is exercised to avoid cross-contamination between fish and contamination of tissue sample with body fluids.
- b. 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.
- c. Keep tissue cool during collection.

C. SAMPLES COLLECTED FROM JUVENILE FISH

- a. Juvenile fish are often collected as whole fish and dissected upon return to the laboratory.
- b. 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.
- c. A minimum sample weight of 0.07 g is needed for testing.
- d. Keep fish or tissues cool during collection and processing.

2. Sample Processing

A. PBS-T20 DILUTION BUFFER (see Appendix D – Reagents, for PBS formula)

- a. All buffer 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

- a. 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* (Scott and Nesbitt 1995).
- b. 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 0.08gm tissue (560 μ L homogenate) is required for sufficient supernatant to inoculate 2 replicate wells.

- a. Collect kidney tissue in whirlpak or stomacher type bags with top closures.
- b. Tare balance with empty collection bag. Kept samples cold during processing.
- c. **Dilute kidney 1:8** (w/v) with PBS-T20 (1 part kidney : 7 parts PBS-T20).
- d. Homogenize until well mixed.

NOTE: individual wooden sticks can be used to macerate small samples collected directly into microcentrifuge tubes. 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).

- e. Dispense or pour into labeled microcentrifuge tubes.
- f. 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.
- g. 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 (True, 1995), 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 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
.07	.49		.30	2.10		.50	3.50
.08	.56		.31	2.17		.51	3.57
.09	.63		.32	2.24		.52	3.64
.10	.70		.33	2.31		.53	3.71
.11	.77		.34	2.38		.54	3.78
.12	.84		.35	2.45		.55	3.85
.13	.91		.36	2.52		.56	3.92
.14	.98		.37	2.59		.57	3.99
.15	1.05		.38	2.66		.58	4.06
.16	1.12		.39	2.73		.59	4.13
.17	1.19		.40	2.80		.60	4.20
.18	1.26		.41	2.87		.61	4.27
.19	1.33		.42	2.94		.62	4.34
.20	1.40		.43	3.01		.63	4.41
.21	1.47		.44	3.08		.64	4.48
.22	1.54		.45	3.15		.65	4.55
.23	1.61		.46	3.22		.66	4.62
.24	1.68		.47	3.29		.67	4.69
.25	1.75		.48	3.36		.68	4.76
.26	1.82		.49	3.43		.69	4.83
.27	1.89					.70	4.90
.28	1.96						
.29	2.03						

References (Sample Processing)

Scott, R., and N. Nesbitt. 1995. Efficacy of decontaminating tool for *Renibacterium salmoninarum* testing. Abstract 1995 Joint Meeting of the Fish Health Section American Fisheries Society and Eastern Fish Disease Workshop; Syracuse, New York, July 19-22, 1995.

True, Kimberly. 1995. Comparison of the Monoclonal and Polyclonal ELISA and Effects of Tissue Processing. Abstract 1995 Joint Meeting of the Fish Health Section American Fisheries Society and Eastern Fish Disease Workshop; Syracuse, New York, July 19-22, 1995.

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.

1. Reagents:

A. KIRKEGAARD AND PERRY LABORATORIES (KPL) ASSAY REAGENTS

The following are available from Kirkegaard & Perry Laboratories (1-800-638-3167):

50-63-00	Wash Solution Concentrate, 4x200mL
50-84-00	Coating Solution Concentrate, 2x25mL
01-96-91	Affinity purified antibody to <i>R. salmoninarum</i> , 1.0mg (<u>Coating Antibody</u>)
50-82-01	Milk Diluent/Blocking Solution Concentrate, 2x100mL
04-96-91	<i>Peroxidase</i> -Labeled Affinity Purified Antibody to <i>R. salmoninarum</i> , 0.1mg (<u>HRP Antibody</u>)
50-62-00	ABTS Peroxidase Substrate System, 6x100mL
50-85-01	ABTS Peroxidase Stop Solution (5X)
50-96-91	<i>R. salmoninarum</i> 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 liter of reagent grade, sterile water, add:

NaCl	8.00 gram (g)
KH ₂ PO ₄	0.20 g
Na ₂ HPO ₄	1.09 g
KCl	0.20 g
Thimerosal	0.10 g
Confirm pH = 7.4	
Tween-20	0.5 mL

2. 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 μ L
- (1) Sample Loading, 40-200 μ L
- (2) Antibody Reagents, 5-50 μ L

3. 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 .01grams
Stomacher 80 Tissue Homogenizer and bags
PBS-Tween 20 (PBS-T20) Buffer (see appendix D – Reagents, for formula)
Wooden stick applicators (for homogenizing small tissue samples)
Screw-cap microcentrifuge tubes, 2.0mL conical with skirting, Perfector Sci. # 6550
Tube caps with O-rings, Perfector Scientific, # 6581
Adjustable pipettors with volume range of 200-1000 μ L 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