

3.7 Reagents, Media, and Media Preparation

A. Growth Media

Most bacteriological culture medias are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Recipes for those media which are not available in a commercial preparation are included below.

- 1. Brain Heart Infusion Agar (BHIA)** (Difco 1998)
A basic agar for most bacterial cultures.
- 2. Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB)** (Difco 1998)
A basic media for most bacterial cultures.
- 3. Selective Kidney Disease Medium-2 (SKDM-2)** (Austin et al. 1983)
A media used for selective isolation of *Renibacterium salmoninarum*.

Peptone	10g
Yeast extract	0.5g
L-Cysteine HCL	1g
Agar	15g
Distilled water	874mL

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

Fetal Bovine Serum	100.0 mL
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The following antibiotics are added to reduce overgrowth from other bacterial organisms):

- 4.0 mL Cycloheximide (1.2 g Cyclohexamide in 96 mL dH₂O)
- 1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH₂O)
- 2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of dH₂O)
- 1.0 mL Oxolinic acid (0.06 g Oxolinic acid in 24 mL of 5% NaOH)

B. Media to Identify Growth and Biochemical Characteristics

Most of these media are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Unless otherwise specified, these media can also be prepared from basic laboratory ingredients according to recipes found in the following references: MacFaddin 1980, Difco 1998, and MacFaddin 2000.

3.7 Reagents, Media, and Media Preparation - 2

1. Motility Test Medium

A semi-solid media used as a tube test to detect the ability of a microorganism to exhibit motility. Several types are commercially available, including MIO (motility, indole, ornithine) which allows for the detection of motility, and the reaction of two biochemical tests in the same tube.

2. Tryptic Soy Broth (TSB)

A nutrient broth media used to determine motility of a microorganism with the hanging drop method.

3. Triple Sugar Iron Agar (TSI)

A commercially prepared dehydrated media used to evaluate the utilization of glucose and two additional carbohydrates, as well as the production of hydrogen sulfide.

4. Oxidation/Fermentation (OF) Medium

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

- a. To prepare final medium aseptically add 10 mL of a filter-sterilized (0.45 μ m) 10% carbohydrate solution to autoclaved and cooled (50°C) media 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 dH₂O)
10% Rhamnose (1 g Rhamnose to 10 mL in dH₂O)
10% Sucrose (1 g Sucrose to 10 mL in dH₂O)
10% Sorbitol (1 g Sorbitol to 10 mL in dH₂O)*
10% Maltose (1 g Maltose to 10 mL in dH₂O)
10% Glucose (1 g Glucose to 10 mL in dH₂O)
5% Salicin (0.5 g Salicin to 10 mL in dH₂O)

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

5. Nutrient Gelatin

A dehydrated medium for determining the presence of Gelatinase.

6. Tryptone Broth

For use with the indole test.

Tryptone	10 g
Distilled water	1000 mL

Heat gently to dissolve. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Store at 2 to 8°C.

7. Decarboxylase Medium Base

A basal media for use in Lysine test. The basal media, without addition of lysine, serves as the control.

To make L-Lysine media add 5 g L-Lysine to 1 liter of prepare basal decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH = 6.8 ± 0.2 at 25°C. Store at 2 to 8°C.

8. Malonate Broth

A media used for the malonate test.

9. Bile Esculin Agar

A commercially prepared, dehydrated medium used to determine a bacterium's ability to hydrolyze esculin into glucose and esculetin.

C. Media Preparation

1. Plate Media

- a. 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 suspend agar. Use of a stir bar will facilitate mixing of agar.
- b. 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 (consult with the manual provided by the autoclave manufacturer for adjustment of time when large volumes of media are being sterilized).
- c. Cool media to 50°C.
- d. 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.
- e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Mix any added ingredients into the media at this temperature.
- f. Label the bottom of each plate with medium type and date prepared.
- g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per 100 X 15mm petri dish. Replace lids as soon as the plate is poured.
- h. Invert plates when the media has cooled completely (~ 30 to 60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.
- i. 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.
- j. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with the date of preparation.

2. Tube Media

- a. 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 suspend the agar. Use of a stir bar will facilitate mixing of agar.
- b. 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.
- c. Arrange test tubes in racks. Disposable, autoclavable, screw cap tubes can be used for all tube media.
- d. Use an automatic pipetter or Pipette-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 tubes of media that are dispensed. Dispense approximately 5 to 10 mL media in 16 X 125 mm or 20 X 125 mm tubes. Close caps loosely.
- e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.
- f. Loosely place screw caps on tubes. Do not tighten caps. It is necessary to allow pressure to release from tubes while heating in the autoclave.
- g. Follow manufacturer's recommendation for autoclave time and temperature.
- h. 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. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA). Then tighten caps.
- i. Cool completely to room temperature in the slanted position.
- j. Label the tubes or the tube rack with type of medium and date made.
- k. Store at 2 to 8°C, following manufacturer's recommendation for period of long-term storage.

D. Reagents

Most of these reagents are commercially available pre-made. These commercial products are entirely acceptable and should be stored according to the manufacturer's recommendations. The formulations provided below were obtained from the references cited.

1. Gram Stain Reagents

These stains can be ordered as a complete kit or can be prepared as follows:

- a. Crystal Violet

Crystal violet (90% dye content)	20.0 g
Ethanol (95%)	200 mL
Ammonium oxalate	8.0 g
dH ₂ O	800 mL

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

3.7 Reagents, Media, and Media Preparation - 5

ingredients and filter before use.

- b. Gram's Iodine
 - Iodine crystals 1.0 g
 - Potassium iodide 2.0 g
 - dH₂O 300 mL
- c. De-Colorizer
 - Acetone 40 mL
 - Ethanol (95%) 60 mL
- d. Safranin
 - Safranin O 2.5 g
 - 95% ethanol 100 mL
 - dH₂O 900 mL

Filter safranin solution before use.

2. FAT Mounting Fluid (pH 9.0)

- Glycerol 90.0 mL
- DABCO* 2.5 g
- PBS 10.0 mL

Suspend the DABCO in glycerol over low heat. Then add 1X PBS (see below). Adjust pH to 8.6 to 9.0 with 1N hydrochloric acid or 0.1N sodium hydroxide. The pH of the mounting media is important, as an acid pH will quench fluorescence. Check the pH frequently. Store at room temperature.

* Optional ingredient - DABCO is 1,4-diazabicyclo-(2,2,2)-octane. Its addition to mounting fluid can reduce quenching of fluorescence.

3. Phosphate-Buffered Saline for FAT (PBS), pH 7.1

- a. 1x concentration (0.15 M NaCl, 0.01 M phosphate; makes 1 L)
 - NaCl 8.50 g
 - Na₂HPO₄ (anhydrous) 1.07 g
 - NaH₂PO₄·H₂O (monohydrate) 0.34 g
 - DH₂O to 1 L

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

- b. 5x concentration (makes 10 L of 1x PBS)
 - NaCl 85.00 g
 - Na₂HPO₄ (anhydrous) 10.70 g
 - NaH₂PO₄·H₂O (monohydrate) 3.45 g
 - DH₂O to 2 L

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

4. Kovac's Indole Reagent

- Isoamyl alcohol 30 mL

3.7 Reagents, Media, and Media Preparation - 6

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 to 8°C in amber dropper bottle.

5. Counter Stains

a. Rhodamine

Rehydrate the rhodamine to 1 mg/mL in distilled water. If the rhodamine does not completely dissolve, add a small drop of 0.1 M sodium hydroxide. Store at 2 to 8°C.

b. Evans Blue

Prepare 0.1% stock solution with distilled water and decontaminate with 0.45 µm membrane filtration. Store at room temperature. Prepare a 0.01% working dilution with sterile PBS (Section 2, 3.7.D.3 “Phosphate-Buffered Saline for FAT (PBS), pH 7.1”) (Cvitanich 1994).

c. Eriochrome Black T

Prepare a solution at 1:60 (w/v) in PBS (Section 2, 3.7.D.3 “Phosphate-Buffered Saline for FAT (PBS), pH 7.1”), and filter through Whatman #1 and Whatman # 42 filter papers before initial use (Elliott & McKibben 1997).

6. FITC Conjugated Rabbit Anti-X /Rhodamine Counter Stain

Rhodamine stock	10.0 µL
FITC conjugated antibodies	10.0 µL
Phosphate buffered saline	480.0 µL
Store at 4°C.	

Note: Evans Blue or Eriochrome Black T can also be used as counterstain in FAT in a separate step during the staining process, but neither can be added directly to the conjugate solution.

E. Cytochrome Oxidase Spot Test

Individual test strips can be purchased from several suppliers (Catalogue # 38-191, Remel, Tel. 800-255-6730).

F. Determination of Antiserum and Conjugate Working Dilutions for FAT

Commercially prepared anti-sera and conjugates 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 on positive control FAT preparations. 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 how to determine the correct working dilution of FAT conjugate where the manufacturer recommends a working dilution of 1:40.

3.7 Reagents, Media, and Media Preparation - 7

1. 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 by antibody manufacturer.
2. Rhodamine counter stain is added directly to the optimum antiserum working dilution at a 1:50 (alternatively, Evans Blue may be used to counterstain FAT stained slides – do not add Evans Blue to the conjugate directly).
3. 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).
4. Prepared conjugate solutions should be filtered through 0.2 to 0.4 micron filter prior to use and storage. Store frozen in small aliquots. Do not thaw and re-freeze antibodies repeatedly.

G. PCR Reagents

The following are formulations for extraction reagents used specifically in the protocols described in [Section 2, 3.5.B.2 “Nested Polymerase Chain Reaction \(PCR\) for Confirmation of *R. salmoninarum* DNA.”](#)

1. Lysozyme Lysis Buffer

100 mL (this formulation is used for lysis of gram positive bacteria with Qiagen extraction kits).

Lysozyme	2 g
Tris HCl Stock	2 mL
EDTA stock	2 mL
Triton	1.2 mL

Bring these components to 100 mL with sterile distilled water (molecular grade).

2. Tris HCl Stock Solutions

100 mL at 1M pH 8.0 (for use in lysozyme buffer).

Trizma base	5.7 g
Tris HCl	8.9 g
dH ₂ O	85.4 mL

3. EDTA Stock Solution

100 mL at 0.1 M (for use in lysozyme buffer).

EDTA	3.72 g
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Bring to 100 mL with sterile distilled water (molecular grade).