1.3.1.1 Bacterial Kidney Disease Appendix 1 ELISA for Detection of *Renibacterium salmoninarum*

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Important considerations before starting

Glassware: It is advisable to dedicate a separate set of glassware for use in preparing reagents for ELISA. Proteins can remain on container surfaces after conventional detergent washing and autoclaving. Disposable containers can also be used to decrease risk of contamination.
The working concentrations of the coating antibody (anti-*R. salmoninarum*) and the conjugate (HRP-anti-*R. salmoninarum*) must first be determined by checkerboard titration. See Section IV for complete checkerboard instructions. Typical concentrations are around 1 µg per mL for the coating antibody and 1:4,000 (v/v) for the conjugate antibody.

Samples: See Section V of this protocol for complete processing instructions. Homogenized fish tissues and ovarian fluid should be diluted with PBS-T20 and stored in 2 mL screw cap centrifuge tubes with O-rings at -20 or -70°C until needed. To prepare samples for the ELISA, heat each sample at 100°C for 15 min, and then centrifuge 8,000 to 10,000 x *g* for 10 minutes at 4°C. If the samples were prepared earlier and frozen, thaw before heating. Heated and centrifuged samples can be stored overnight at 4°C, or frozen at -20°C or -70°C for later testing.

Plates: A 96 well EIA/RIA clear flat bottom plate from Corning Inc. (#3590) is recommended.

I. Procedures to do before starting ELISA

1. **Prepare controls** (See Section III of this protocol for details).
   Positive controls:
   1 - 1:100
   2 - 1:1000
   3 - 1:2000
   4 - 1:5000
   Negative control

2. **Assemble reagents** (See Section III of this protocol for details).
   - ELISA plate(s)
   - Wash solution
   - PBS-T20
   - Coating buffer
   - Coating antibody

- Milk diluent
- HRP-conjugate antibody
- Substrate- chromogen
- Stop solution

II. ELISA Procedure

Day 1

1. **Prepare microplate map.** A control plate with replicates of blank (BL), conjugate control (CC), substrate-chromogen control (SC), negative control (NC), and all concentrations of positive control wells should be done for every four plates processed or every new run of ELISA. (See example control plate below). Sequentially number each plate. The purpose of each control is described in section VI of this protocol.

Example control plate

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NC	CC	PC 1:100	PC 1:2000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	BL	CC	PC 1:100	PC 1:2000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	NC	CC	PC 1:100	PC 1:2000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	BL	CC	PC 1:100	PC 1:2000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	NC	SC	PC 1:1000	PC 1:5000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	BL	SC	PC 1:1000	PC 1:5000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	NC	SC	PC 1:1000	PC 1:5000		Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	BL	SC	PC 1:1000	PC 1:5000		Sample	Sample	Sample	Sample	Sample	Sample	Sample

NC= negative control, BL=blank well, CC=coat control, SC=substrate-chromagen control, PC=positive control

2. **Calculate the amount of coating buffer and coating antibody needed to coat each microplate.** Prepare an excess volume of coating antibody and buffer to compensate for solution loss.

Example
Calculate total volume of coating antibody at 1 µg/mL[§] necessary to apply 200 µL of antibody solution to the wells of 5 microplates:

(5 plates) (96 wells/plate) (200 µL/well) = 96,000 µL = 96 mL

To compensate for loss, prepare 105 mL of diluted coating antibody at 1 µg/mL
V_i ** (1000 µg/mL) = 105 mL * (1 µg/mL)*
$\boldsymbol{V_i = 0.105\ mL = 105\ \mu L}$

Combine 105 µL concentrated coating antibody with 105mL coating buffer.

§Concentration determined by checkerboard

3. **Dilute the coating antibody in coating buffer.**

4. **Place 200µL of coating buffer only in the conjugate control (CC) and substrate-chromogen control (SC) wells.**

5. **Coat all other wells in the microplate with 200 µl diluted coating antibody.**

6. **Seal each plate with an adhesive plate sealer.**

7. **Place each plate in a humid chamber to incubate at 4°C for 16+ hrs.**

8. **Prepare wash buffer solution for Day 2.** Wash buffer can be stored at 4°C overnight, but should remain at room temperature during the ELISA.

9. **Prepare test samples for Day 2**. Heat each sample at 100°C for 15 min, centrifuge 8,000 to 10,000 x *g* for 10 minutes at 4°C. If the samples were prepared earlier and frozen, thaw before heating. Heated and centrifuged samples can be stored overnight at 4°C (see section V of this protocol). Remember to also prepare positive and negative controls along with test samples (see section III of this protocol).

Day 2

1. **Wash wells five times with wash buffer**. Carefully remove the plate sealer, fill each well with wash buffer, and allow the buffer to soak for 30 seconds before removing the solution. Shake out any excess buffer left over after all five washes are complete. An autowasher is highly recommended for this step.

2. **Add 200 µL of only PBS-T20 into blank (BL), conjugate control (CC), and substrate-chromogen control (SC) wells.**

3. **Add 200 µL of prepared negative tissue or ovarian fluid sample into the negative control (NC) wells**. Seal negative control wells with a strip of adhesive plate sealer before loading positive controls.

4. **Add 200 µL of each positive control into the appropriate wells**. Seal positive control wells with another strip of adhesive plate sealer before loading test samples.

5. **Add 200 µL aliquots of each test sample (supernatant only) in the appropriate wells.** Record any discrepancies on your microplate map (e.g. sample wells that did not receive a full 200 µL or samples with tissue debris in the supernatant). Samples are usually tested in duplicate if volume is sufficient.

6. **Cover each plate with a full adhesive plate sealer.** Write time of completion of the loading of each plate on the sealer flap of that plate and on the microplate map.

7. **Incubate for 3 h at 25°C in a humid chamber.**

8. **Wash wells five times as described in step 1.**

9. **Calculate amount of HRP-conjugate antibody and milk diluent needed for 200µL/well.** The HRP-conjugate should be diluted to the concentration previously obtained in the Checkerboard titration (see section IV of this protocol).

Example
Calculate the volume of a 1:4,000 dilution[§] of the HRP-conjugate antibody for 5 microplates.

(5 plates) (96 wells/plate) (200 µL/well) = 96,000 µL = 96 mL
Make 105 mL to account for loss

1/4000 = x/105 mL
x = 0.0263 mL = 26.3 µL

Combine 26.3 µL of the stock HRP-conjugate with 105 mL milk diluent
§Dilution determined by checkerboard

10. **Add the appropriate amount of HRP- conjugated antibody to 1X blocking solution (also called milk diluent).**

11. **Add 200 µL of the diluted conjugate to the appropriate wells.** Substrate-chromogen control wells (SC) receive 200 µL of milk diluent **without** HRP-conjugate.

12. **Seal each plate with an adhesive plate sealer**.

13. **Incubate in a humid chamber for 2 h at 25°C**.

14. **Wash wells five times as described in step 1**.

15. **Calculate amount of substrate-chromogen needed for 200µL/well.** The substrate-chromogen solutions should be at room temperature before use. Remove from refrigerator during HRP-conjugate incubation step.

Example
Calculate the volumes needed of substrate-chromogen for 5 microplates:
equal volumes ABTS solutions A and B necessary for 5 microplates.

*(5 plates) * (96 wells/plate) * (200 µL/well) = 96,000 µL = 96 mL*
Make 105 mL to compensate for loss

52.5 mL of A + 52.5 mL of B = 105 mL

16. **For each plate, set a timer for 20 min (but do not start)**. The timing of the substrate-chromogen reaction is critical. The reaction must be stopped after exactly 20 minutes.

17. **Prepare substrate-chromogen solution and add 200 µL to each well**. Start the appropriate timer immediately when you begin to apply the solution. Fill all plates in numerical order.

18. **Immediately after all the wells have received the substrate-chromogen solution, seal the plate and put it in the humid chamber at 37°C**.

19. **Calculate the amount of stop solution needed**. Dilute 5x concentrate 4:1 and use 50 uL of 4x solution per well.

Example
Calculate the volume of 4X stop solution needed for 5 microplates.

(5 plates) (96 wells/plate) (50 µL/well) = 24,000 µL = 24 mL
Make 34 mL to compensate for loss

$5X * V_i = 4X * 34$
$V_i = 27.2$ *mL concentrate*
Combine 6.8 mL of nH2O with 27.2 mL of concentrated stop solution to get 34 mL total

20. **Apply 50 µL of the stop solution to each well immediately after 20 minute incubation period is complete**. Wells should receive the stop solution in the exact sequence used to apply the substrate-chromogen solution.

21. **Immediately measure absorbance at 405nm wavelengeth in a microplate reader**. Wipe any condensation off the bottom of the plate with a kimwipe before reading.

Data Interpretation

Note: The optical density values for control and test reagents may vary between laboratories using different equipment or manufacturer models. Here we will provide general guidelines for interpreting data.

1. **Quality Control**
   a. Variation between replicates (coefficient of variation) should be less than 10%.
   b. The blank wells should have low OD values (e.g. < 0.08 OD).
   c. The SC and CC controls should have similar values and low OD values (e.g. < 0.08 OD).

2. **Negative-Positive Threshold Determination**
   Note: In some circumstances, such as when fish are to be transported to a watershed or hatchery that has no known *R. salmoninarum* in resident fish stocks, a conservative negative-positive threshold value is often used. However, the use of a conservative threshold increases the likelihood of false-positive ELISA OD values, and may not be desirable for monitoring of fish populations that are known to be *R. salmoninarum*-positive. In circumstances such as the culling of gametes from fish in *R. salmoninarum*-positive populations to reduce vertical transmission of the bacterium, a less conservative threshold OD is often used, although this increases the likelihood of false-negative results, Please consult Meyers et al. (1993) for further discussion of ELISA threshold values.

   a. A conservative negative-positive threshold is often calculated as the mean of the negative control wells (NC) plus two standard deviations. These metrics are easily calculated within Microsoft Excel.

Example
NC 1 = 0.072 OD *NC 2 = 0.069 OD* *NC 3 = 0.067 OD*
Then the mean is 0.069 and the standard deviation is 0.003 *The positive – negative threshold is 0.069 + (2 x 0.003) = 0.074*

III. Reagent Preparation

Notes: Use dedicated ELISA glassware; all protocols use nanopure or reagent grade water

PBS-T20: Phosphate-buffered saline, pH 7.4, 0.05% (v/v) Tween-20 & 0.01% (w/v) Thimerosal

For 1 liter:

NaCl	8.00 g
KH2PO4	0.20 g
Na2HPO4	1.09 g
KCl	0.20 g
Thimerosal	0.10 g
-Confirm pH is 7.4	
Tween-20	0.5 mL

PC: *Renibacterium salmoninarum* Postive Control BacTrace®

- Purchase commercially (KPL[§] part # 50-96-91)
- Reconstitute 5 vials of positive control with 5 mL of PBS-T20*
- Prepare PC dilutions
  - 1:100 Add 5 mL of reconstituted antigen to 495 mL of PBS-T20
  - 1:1000 Add 40 mL of 1:100 to 360 mL PBS-T20
  - 1:2000 Add 20 mL of 1:100 to 380 mL PBS-T20
  - 1:5000 Add 8 mL of 1:100 to 392 mL of PBS-T20
  - *Note: Dilutions should give a standard curve between OD_{405} 0.10 and 2.25*
- Aliquot into 2 mL screw-cap micro tubes* and freeze at -70º C
  - **Notes: Rehydration instructions differ from manufacturer*
  - *Different color caps can be used to distinguish positive controls*
  - *2 mL screw cap tubes with O-rings that can withstand boiling*

NC: Negative Control

- Confirmed negative kidney or ovarian fluid for negative control

Coating Buffer: Coating Solution Concentrate (10X)

- Purchase commercially from KPL[§] (Part # 50-84-01)
- Store at 4°C after opening and is usually stable for a minimum of one year
- Dilute to 1X concentration for use (1 mL concentrate to 9 mL water)

Coating Antibody: Affinity Purified Antibody to *Renibacterium salmoninarum* BacTrace® Antibodies produced in Goat

- Purchase commercially from KPL[§] (part # 01-96-91)
- Vial contains 1.0 mg of lyophilized immunoglobulin
- Rehydrate antibody in 1 mL of 50% sterile-filtered (0.2 μm) glycerol
- Store at 4°C, usually stable for a minimum of one year

Wash Solution: Wash Solution Concentrate (20X)

- Purchase commercially from KPL[§] (part # 50-63-01)
- Store concentrate at 4°C after opening; usually stable for a minimum of one year
- Dilute according to manufacturer's instructions (1 mL concentrate to 19 mL water)
- Prepare fresh for each ELISA

Blocking Solution: 20X Blocking Solution Concentrate / Milk Diluent

- Purchase commercially from KPL[§] (Part # 50-82-00)
- Store concentrate at 4°C after opening; usually stable for a minimum of one year
- Dilute following manufacturer's instructions (1 mL Diluent/Block + 19 mL water)

Antibody Conjugate: Peroxidase-Labeled Affinity Purified Antibody to *Renibacterium salmoninarum* BacTrace® Antibodies produced in Goat

- Purchase from KPL[§] (Part # 04-96-91)
- Vial contains 0.1 mg
- Rehydrate according to manufacturer's instructions (Procedure A: 1 ml of 50% glycerol)
- Store at 4°C in a dark or foil wrapped tube; usually stable for a minimum of one year

Substrate-Chromogen: ABTS® Peroxidase Substrate System (2 Component)

- Purchase ABTS Peroxidase Solution A from KPL[§] (Part # 50-64-02)
  2,2'-azino-di[3-ethyl-benzthiazoline sulfonate in a 0.3g/L glycine/citric acid buffer
- Purchase ABTS Peroxidase Solution B from KPL[§] (Part # 50-65-02)
  0.02% hydrogen peroxide in a proprietary buffer
- Store in dark bottles at 4°C

Stop Solution: ABTS® Peroxidase Stop Solution

- Purchase from KPL[§] (Part # 50-85-02)
- Store at room temperature; stable for a minimum of one year.
- Combine 4 parts stop solution with 1 part water immediately before use*
  **Note dilution differs from manufacturer's recommendation*

[§]Kirkegaard and Perry Laboratories Inc. (KPL)
910 Clopper Road, Gaithersburg, Maryland 20878 USA
Ph: 800.638.3167; www.kpl.com

IV. Checkerboard Titration

Background

Each time new vials of the coating antibody or conjugate are rehydrated, slight variations between lots of the manufacturer's antibody preparations, the rehydration procedure, etc. may affect the amounts of those stock preparations used in the ELISA. To determine the optimal (working) concentration of each reagent, several dilutions of antibody must be evaluated together with a prescribed set of test samples. From these data one can choose a coating antibody-conjugate combination that produces acceptable absorbance values. If rehydrated antibodies have not been used for an ELISA for several months, it is wise to do another checkerboard titration before testing new tissue or ovarian fluid samples.

Procedure

Day 1

1. **Prepare checkerboard plate map**. Suggested format for evaluating one concentration of the coating antibody with four concentrations of conjugate antibody is shown below. A separate microplate is used for each concentration of coating antibody tested.
    a. Suggested concentrations for **conjugate antibody** are 1:2,500, 1:3,000, 1:4,000, and 1:4,500 (v/v) dilutions.
    b. The optimal **coating antibody** is usually 1.0 µg/mL but 1.5 and 2.0 µg/mL concentrations can be tested on separate plates.
    c. **Controls** include: blank wells (BL), the negative control (NC), the conjugate control (CC), substrate-chromogen control (SC), and the 4 positive control (PC) dilutions (1:1000 - 1:5,000). The purpose of the controls are described in section VI of this protocol.

Checkerboard plate: Each quadrant uses a different concentration of conjugate antibody.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	BL	SC	CC	PC 1:100	PC 1:2000	NC	BL	SC	CC	PC 1:100	PC 1:2000
B	NC	BL	SC	CC	PC 1:100	PC 1:2000	NC	BL	SC	CC	PC 1:100	PC 1:2000
C	NC	BL	SC	CC	PC 1:1000	PC 1:5000	NC	BL	SC	CC	PC 1:1000	PC 1:5000
D	NC	BL	SC	CC	PC 1:1000	PC 1:5000	NC	BL	SC	CC	PC 1:1000	PC 1:5000
E	NC	BL	SC	CC	PC 1:100	PC 1:2000	NC	BL	SC	CC	PC 1:100	PC 1:2000
F	NC	BL	SC	CC	PC 1:100	PC 1:2000	NC	BL	SC	CC	PC 1:100	PC 1:2000
G	NC	BL	SC	CC	PC 1:1000	PC 1:5000	NC	BL	SC	CC	PC 1:1000	PC 1:5000
H	NC	BL	SC	CC	PC 1:1000	PC 1:5000	NC	BL	SC	CC	PC 1:1000	PC 1:5000

2. **Rehydrate lyophilized antibody and conjugate according to manufacturer's instructions.** See section III of this protocol for more information.

3. **Calculate the amount of coating buffer and coating antibody needed for the checkerboard plate.** Prepare an excess volume of coating antibody to compensate for solution lost during preparation and pipetting.

Example
Calculation of the total volume of coating antibody at 1 µg/mL is necessary to apply 200 µL of antibody to the wells of 1 microplate.

(64 wells/plate) (200 µL/well) = 12,800 µL = 12.8 mL
Prepare 16 mL coating antibody at 1 µg/mL

X (1,000 µg/mL) = 16,000 µL (1 µg/mL)
X = 16 µL

Combine 16 µL concentrated anti-R. salmoninarum coating antibody with 16 mL coating buffer.

4. **Dilute the coating antibody in coating buffer.**

5. **Place 200µL of coating buffer only in the conjugate control (CC) and substrate-chromogen control (SC) wells.**

6. **Coat all other wells in the microplate with diluted anti-R. salmoninarum coating antibody and coating buffer.**

7. **Seal each plate with an adhesive plate sealer.**

8. **Place each plate in a humid chamber to incubate at 4°C for 16+ h.**

9. **Prepare wash buffer solution for day 2.** Wash buffer can be stored at 4°C overnight, but should remain at room temperature during the ELISA.

10. **Prepare controls for Day 2.** Heat controls at 100°C for 15 min, centrifuge 8,000 to 10,000 x g for 10 minutes at 4°C. If the samples were prepared earlier and frozen, thaw before heating. Processed and heated controls can be stored overnight at 4°C.

Day 2

1. **Wash wells five times with wash buffer**. Fill each well with wash buffer, allow the buffer to soak for 30 second before removing the solution. Shake out any excess buffer left over after all five washes are complete. An autowasher is highly recommended for this step.

2. **Add 200 µL of only PBS-T20 into blank (BL), conjugate control (CC), and substrate-chromogen control (SC) wells.**

3. **Add 200 µL of prepared negative tissue or ovarian fluid sample into the negative control (NC) wells**. Seal control wells with a strip of adhesive plate sealer before loading positive controls.

4. **Add 200 µL of each positive control into the appropriate wells**. Seal positive control wells with another strip of adhesive plate sealer before loading test samples.
   Positive controls:
   1 - 1:100
   2 - 1:1000
   3 - 1:2000
   4 - 1:5000

5. **Cover plate with a full adhesive plate sealer**.

6. **Write time of completion on the sealer flap on each plate and on the template sheet.**

7. **Incubate for 3 h at 25°C in a humid chamber.**

8. **Wash wells five times as described in step 1.**

9. **Calculate amount of conjugate antibody and blocking solution needed for 200µl/well, for four concentrations (1:2,500, 1:3,000, 1:4,000, and 1:4,500 (v/v)).**

Example
Calculate the volume of a 1:2,500, dilution of the HRP-conjugate antibody for 1 microplate.

(20 wells) (200 µL/well) = 4,000 µL = 4 mL
Make ~ 6 mL extra, or 10 mL.

1/2500 = X /10 mL
X = 0.0040 mL = 4.0 µL

Combine 4.0 µL of the stock conjugate antibody with 10 mL blocking solution

10. **Prepare the blocking solution and add the appropriate amount of conjugate antibody, for each of the four concentrations.**

11. **Add 200 µL of the diluted conjugate for each concentration to the appropriate wells in each quadrant.**

12. **Substrate-chromogen control wells (SC) receive 200 µL of blocking solution without conjugated antibody.**

13. **Seal plate with an adhesive plate sealer and incubate in a humid chamber for 2 h at 25°C.**

14. **Wash wells five times as described in step 1.**

15. **Calculate amount of substrate-chromogen needed for 200µl/well.**

Example
Calculate the volume substrate-chromogen needed (equal volumes ABTS solutions A&B) necessary for 1 microplate.

(1 plates) (96 wells/plate) (200 µL/well) = 19,200 µL = 19.2 mL
Again, make ~10 mL extra, or 30 mL.

15 mL of A + 15 mL of B
= 30 mL

Combine 15 mL of solution A and 15 mL of solution B to get 30 mL total.

16. **Set a timer for 20 min (but don't start).**

17. **Prepare substrate-chromogen solution and add 200 µL to each well**. Start the appropriate timer immediately when you begin to apply the solution. Fill all plates in numerical order. The timing of the substrate-chromogen reaction is critical. The reaction must be stopped after exactly 20 minutes.

18. **Seal the plate immediately after all the wells have received the substrate-chromogen solution**.

19. **Put plates in the humid chamber at 37°C.**

20. **Prepare 4X stop solution**.

Example
Calculate the volume of 4X stop solution needed for 1 microplates.

(1 plates) (96 wells/plate) (50 µL/well) = 4,800 µL = 4.8 mL
Make 15 mL to compensate for loss

$5X * V_i = 4X * 15$
$V_i = 12$ *mL concentrate*
Combine 3 mL of nH2O with 12 mL of concentrated stop solution to get 15 mL total

21. **Begin to apply 50 µL of the 4X stop solution to each well immediately after the incubation period is complete**. Wells should receive the stop solution in the exact sequence used to apply the substrate-chromogen solution.

22. **Place plate in microplate reader and measure absorbances at 405 nm**. Wipe any condensation off the bottom of the plate with a kimwipe before putting into reader.

V. Collection and Processing of Tissue or Body Fluid Samples for ELISA

A. Sample Collection and Handling

- Field containers should be labeled, sterile, stomacher-type bags with a top closure, for minimal handling.
- All samples should be treated similarly, e.g., dissect fish, store on ice, freeze-thaw, add diluent and homogenize/transfer sample into microcentrifuge tube, etc.

- Store processed tissues at -20°C or -70°C.
- Use PBS-T20 as sample buffer.
- Recommended sample tubes are 2 mL screw-cap microcentrifuge tubes with O-rings that can withstand boiling.
- All fish taken from a group of fish must be processed identically. You cannot change the dilution of test samples within, or among, test groups.

B. Sample collection from adult fish

- Kidney: Collect a 2 to 5 gram sample; when sampling the kidney, it is recommended that this sample consist of a pool of tissue pieces from the anterior, mid, and posterior kidney.
- Ovarian fluid: collect approximately 1mL.
- Keep tissue and body fluid samples on ice during collection.
- Take care to avoid cross-contamination between fish and contamination of tissue sample from body fluids.

C. Sample collection from juvenile fish

- Kidney: Remove the entire kidney (and/or spleen) from each fish. It is preferable to test tissues from individual fish. Two-fish pools are recommended when an insufficient amount of tissue is recovered from an individual fish. If you cannot adhere to these guidelines, then the fish are too small for testing.
- Juvenile fish are often collected as whole fish and dissected upon return to the laboratory.
- As a minimum, need approximately 0.08 g of sample for testing.
- Keep fish or tissues on ice during collection and processing.

D. Sample Processing

- **Important Considerations**
    - Use dedicated ELISA glassware.
    - Avoid rupturing the stomach or intestine. Commercial fish food frequently contains antigens of *R. salmoninarum* which can be detected by the ELISA, resulting in a false positive reading.
    - When tissues are removed in the laboratory, instruments should be flamed between fish in a given group, and mechanically cleaned with detergent between groups of fish. For field sampling, use disposable instruments when possible to remove organs, or have a sufficient number of pre-cleaned instruments so that there is a separate instrument for each fish. Instruments can be cleaned using detergent, dried and ideally autoclaved

- **Processing kidney or kidney / spleen**

    - Adult fish: Tissue can be placed into bags and homogenized with print roller or stomacher in bag.
        - Dilute tissues 1:4 (w/v); 1 part tissue + 3 parts PBS-T20.
        - Store processed tissues at -20°C or -70°C before testing.
        - Samples are heated at 100°C for 15 min. and centrifuged (8,000-10,000 x g for 10 minutes) to remove debris. The supernatant is used in the ELISA.
        - This step can be done just prior to an ELISA or the day before (spun samples can be stored overnight at 4°C).

    - Juvenile fish: Place tissue from individual fish directly into a pre-weighed microcentrifuge tube and homogenize with a stirring rod.
        - Recommend 1:4 (w/v) dilution or use 1:8 (w/v); 1 part tissue + 7 parts PBS-T20.

- Store tissues at -20°C or -70°C before testing.
- Samples are heated at 100°C for 15 min. and centrifuged (8,000-10,000 x g for 10 minutes) to remove debris. The supernatant is used in the ELISA.
- This step can be done just prior to an ELISA or the day before (spun samples can be stored overnight at 4°C).

NOTE: If you are also going to prepare a tissue smear for the FAT, homogenize the tissue without dilution and make a smear from this material, then dilute the sample for the ELISA.

- **Processing ovarian fluid.**
  - Dilute 1:2 (v/v); 1 volume ovarian fluid + 1 volume PBS-T20.
  - Combine 1000 µL of ovarian fluid and 1000 µL of PBS-T20 in a microcentrifuge tube, store fluid at -20 or -70°C or heat at 100°C for 15 min, remove debris by centrifugation (again, 8,000-10,000 x g for 10 minutes), and test supernatant.
- **Processing plasma**
  - Adult fish: 1:5 (w/v); 1 volume whole blood + 4 volumes PBS-T20.
  - Juvenile fish: 1:10 (v/v); 1 volume whole blood + 9 volumes PBS-T20.
  - Collect blood in a syringe or capillary tube, then dispense the correct volume of blood into the appropriate volume of PBS-T20, remove the cellular fraction by low speed centrifugation, decant the supernatant, store fluid at -20 or -70°C or heat at 100°C for 15 min, and test supernatant.

VI. **Description of controls**

Control Group	Purpose	Step in the ELISA				
		Coating Antibody	**Sample Application**	**Conjugate**	**Substrate-Chromogen**	**Stop Solution**
Blank (BL)	Determine background absorbance levels in the absence of a test sample	Yes	PBS-T20 Only	Yes	Yes	Yes
Positive Controls (PC) [1]	Internal control to ensure that predictable absorbance values are produced by certain levels of antigen	Yes	Yes	Yes	Yes	Yes
Negative Control (NC)[2]	Measure absorbance produced by sample from a negative control fish	Yes	Yes	Yes	Yes	Yes
Conjugate Control (CC)	Ensure that there is no nonspecific binding of the conjugate to well surfaces or to the coating buffer	Coating Buffer Only	PBS-T20 Only	Yes	Yes	Yes
Substrate-Chromogen Control (SC)	Test for nonenzymatic production of the color reaction	Coating Buffer Only	PBS-T20 Only	Diluent Only	Yes	Yes

[1] Sample buffer containing a known level of *R. salmoninarum* (e.g. 1:100, 1:1,000, 1:2,000, and 1:5,000 (v/v) dilutions of control antigen).

[2] Appropriate tissues or body fluids with no, or undetectable levels of *R. salmoninarum* antigen. Store at -70°C; thaw morning of use; heat at 100°C for 15 min; and centrifuge 8,800 *x* g for 5 min.

Disclaimer

The use of trade, firm, or corporation names in this protocol is for the information and convenience of the reader. Such use does not constitute an official endorsement by the U.S. Geological Survey or the Department of the Interior of any product or service to the exclusion of others that may be suitable.

References

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