

INTRODUCTION

Fibrinogen is a protein complex produced predominantly in the liver. It circulates in blood. After tissue injury, it is converted to fibrin, facilitating clot formation. It has been reported that salmon fibrinogen mRNA levels increase three days after *A. Salmonicida* infection (ref 1). We found that plasma fibrinogen levels decreased from 6.51 ± 1.27 mg/ml (mean \pm SD, n=4) in healthy trout to 4.07 ± 0.85 mg/ml in trout infected with *F. psychrophilum* (five days post-infection).

PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies generated against fibrinogen purified from Rainbow Trout plasma. It recognizes Rainbow Trout and Atlantic Salmon fibrinogen. Unconjugated antibodies are coated on wells of a microtiter plate and used for capture. Horseradish Peroxidase (HRP) conjugated antibodies are used for detection. Standards and diluted samples (100 μ l) are incubated in the antibody coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 μ l) is added and incubated for 45 minutes. If fibrinogen molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If fibrinogen is present, a blue color develops. Color development is stopped after 20-minutes by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of fibrinogen is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-Fibrinogen coated plate (12 x 8-well strips)
- HRP conjugate stock.
- Fibrinogen stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the standard stock vials at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate¹. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 μ l aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

WASH SOLUTION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DILUENT

The diluent is formulated for measurement of fibrinogen in trout and salmon serum or plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of pure Rainbow Trout fibrinogen in a stabilizing matrix. Reconstitute it with 200 μ l of deionized water, gently mix, and prepare the 100 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 100 ng/ml fibrinogen standard into the tube labeled 50 ng/ml and mix. This provides the 50 ng/ml fibrinogen standard.
4. Similarly prepare the 25 – 1.56 ng/ml standards by two-fold serial dilution.

If future use is intended, the reconstituted standard stock should be stored frozen at or below -20°C within 15 minutes of reconstitution.

¹ Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

HRP CONJUGATE

The HRP conjugate stock must be diluted with diluent YD50-1 as described on the stock vial label about 5 minutes before use. Use 100 µl of the diluted HRP conjugate per well.

SAMPLES

We found fibrinogen levels of approximately 6.5 mg/ml in healthy trout. To obtain values within range of the standard curve we found it necessary to dilute trout plasma 400,000-fold. A 400,000-fold dilution may be obtained as follows.

1. Pipet 198 µl of YD50-1 into two wells of a 96-well microtiter plate, and 243.75 µl into a third well.
2. Mix 2.0 µl of plasma with 198 µl of diluent in the first well. This gives a 100-fold dilution.
3. Mix 2.0 µl of the 100-fold diluted sample with 198 µl of diluent in the second well. This gives a 10,000-fold dilution.
4. Mix 6.25 µl of the 10,000-fold diluted sample with 242.75 µl of diluent in the third well. This gives a 400,000-fold dilution.

When testing Atlantic Salmon plasma, we found fibrinogen levels of 1-5 to 2.5 mg/ml in healthy fish. We suggest that salmon plasma be tested at a dilution of 100,000-fold (achieved by mixing 25 µl of the 10,000-fold diluted sample with 225 µl of diluent).

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 µl of standards and samples into appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
5. Dispense 100 µl of diluted HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 µl of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20 minutes stop the reaction by adding 100 µl of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm² with a plate reader within 5 minutes.

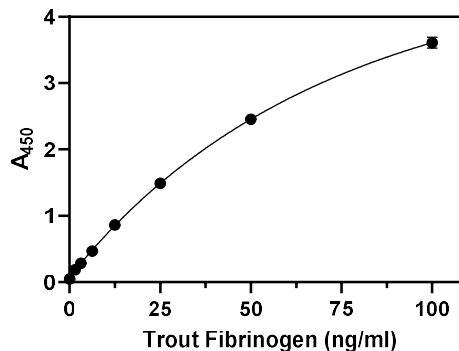
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the fibrinogen concentration. We suggest using a second order polynomial (quadratic) equation.
2. Derive the concentration of fibrinogen in the samples.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
4. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve is for illustration only.

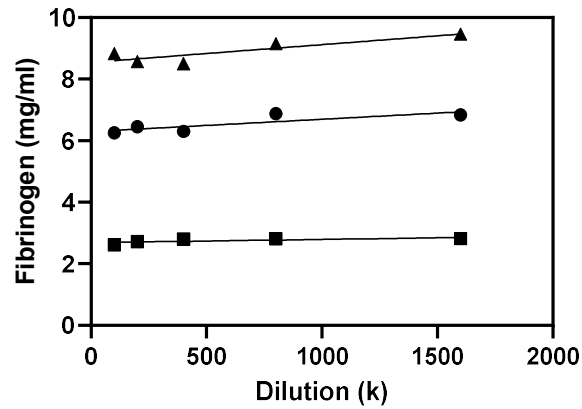
Fibrinogen (ng/ml)	A ₄₅₀
100	3.609
50	2.454
25	1.487
12.5	0.862
6.25	0.467
3.13	0.285
1.56	0.189
0	0.049



² If absorbance of the high standard is ≥ 4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

PERFORMANCE

Linearity: To assess the linearity of the assay, three Rainbow Trout plasma samples with fibrinogen concentrations of 8.9, 6.5, and 2.8 mg/ml were serially diluted from 100,000- to 1,600,000-fold to produce values within range of the assay.



REFERENCES

1. Li X, et al. Identification and expression analysis of a fibrinogen alpha chain-like gene in Atlantic Salmon (*Salmo salar*). *Aquaculture Reports* 22 (2022) 100919

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For technical assistance please email us: techsupport@lifediagnosics.com