INTRODUCTION

IgM is the most abundant immunoglobulin in trout serum. It is a tetramer; each subunit is comprised of two ~75 kDa heavy chains and two ~25 kDa light chains. In Rainbow trout, levels range from 1 to 8 mg/ml.

PRINCIPLE OF THE ASSAY

The assay uses rabbit polyclonal antibodies generated against a recombinant 12 kDa section of the IgM heavy chain constant region. Unconjugated antibodies are coated on wells of a microtiter plate and used for capture. Horseradish peroxidase (HRP) conjugated antibodies are used for detection. Native Rainbow trout IgM is used as standard material. Standards and diluted samples (100 µl) are incubated in the antibody coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If IgM is present, a blue color develops. Color development is stopped by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of IgM is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-IgM coated plate (12 x 8-well strips)
- HRP conjugate, 11 ml
- IgM stock, 2 vials
- 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

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 plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl 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 IgM in Rainbow trout serum and plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. Reconstitute it with deionized water as detailed on the vial label and prepare the 500 ng/ml standard as described.
2. Label seven polypropylene tubes as 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 500 ng/ml IgM standard into the tube labeled 250 ng/ml and mix. This provides the 250 ng/ml standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

Unused reconstituted IgM is stable overnight in the refrigerator.

HRP CONJUGATE

The HRP diluent is supplied as a concentrated stock. Prepare the working conjugate solution by diluting it with diluent YD50-1 as described on the stock vial label.

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

SAMPLES

In studies at Life Diagnostics, we found IgM levels ranging from 1 to 8 mg/ml in Rainbow trout serum. We suggest that serum or plasma be evaluated at a dilution of 40,000-fold. Ideally, dilutions should be performed in polystyrene 96-well plates (not provided).

1. Pipet 198 µl into two wells and 187.5 µl into a third well.
2. Pipet 2.0 µl of serum into the first well containing 198 µl of diluent and mix. This provides a 100-fold dilution.
3. Pipet 2.0 µl of the 100-fold diluted sample into the second well containing 198 µl of diluent and mix. This provides a 10,000-fold dilution.
4. Pipet 62.5 µl the 10,000-fold diluted sample into the third well containing 187.5 µl of diluent and mix. This provides a 40,000-fold dilution.

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 the wells.
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 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 nm with a plate reader within 5 minutes.

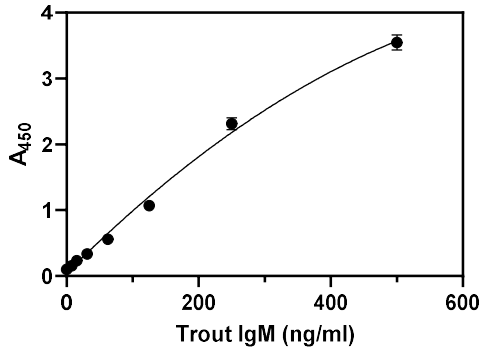
RESULTS

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

TYPICAL STANDARD CURVE

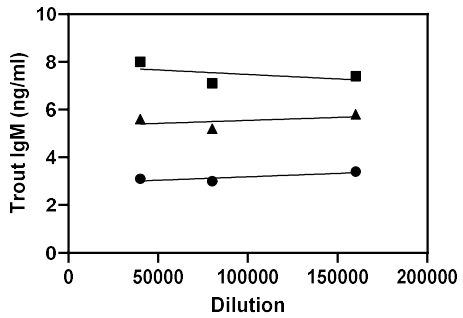
A typical standard curve is shown below. This curve is for illustration only.

IgM (ng/ml)	A_{450}
500	3.544
250	2.314
125	1.072
62.5	0.562
31.25	0.337
15.63	0.236
7.81	0.159
0	0.102



PERFORMANCE

Linearity: To assess the linearity of the assay, three rainbow trout serum samples with IgM concentrations of 3.2, 5.6, and 7.9 mg/ml were serially diluted to produce values within the dynamic range of the assay.



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