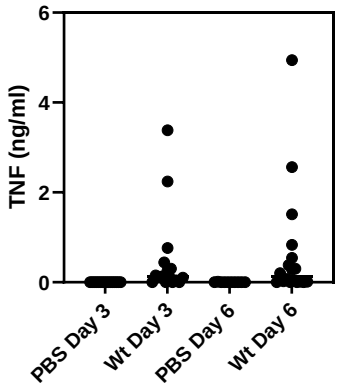
INTRODUCTION

Tumor necrosis factor (TNF) is a cytokine that is a key regulator of the innate immune response. Expression is triggered by bacterial infection. TNF subsequently induces expression of acute phase proteins. At Life Diagnostics, we found that serum levels of TNF increased in trout infected with Weissella tructae (below).



PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies generated against recombinant Rainbow Trout TNF. 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 samples (100 µl) are incubated in the antibody coated microtiter wells for one hour. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If TNF 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 TNF 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 TNF is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

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

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

DILUENT

The diluent is formulated for measurement of TNF in trout serum. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of recombinant Rainbow Trout TNF in a stabilizing matrix. Reconstitute it with 200 µl of deionized water, gently mix, and prepare the 5 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 5 ng/ml TNF standard into the tube labeled 2.5 ng/ml and mix. This provides the 2.5 ng/ml TNF standard.
4. Similarly prepare the 1.25 – 0.078 ng/ml standards by two-fold serial dilution.

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 TNF levels ranging from undetectable in healthy serum to 5 ng/ml in serum from infected trout. We recommend that serum[2] (non-hemolyzed) be tested undiluted. If necessary, use diluent YD50-1 for dilution (do not use other buffers).

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 one hour.
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 nm[3] with a plate reader within 5 minutes.

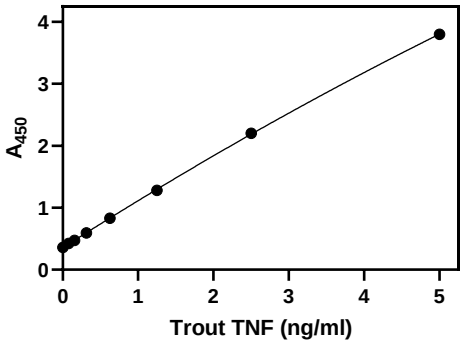
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the TNF concentration. We suggest using a second order polynomial (quadratic) equation.
2. Derive the concentration of TNF 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.

TNF (ng/ml)	A_{450}
5	3.799
2.5	2.201
1.26	1.282
0.625	0.829
0.313	0.592
0.156	0.473
0.078	0.423
0	0.361



[2] The kit was validated using serum. Plasma samples have not been tested.

[3] 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.

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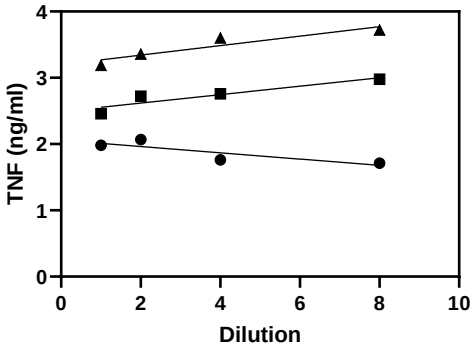
PERFORMANCE

Linearity: To assess the linearity of the assay, three Rainbow Trout serum with TNF concentrations of 1.88, 2.65, and 3.38 ng/ml were serially diluted from 1 to 8-fold to produce values within range of the assay.



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

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