**Mouse Anti-Trinitrophenol (TNP) IgG ELISA**

**INTRODUCTION**

Haptens such as trinitrophenol (TNP), when attached to carrier proteins such as ovalbumin or keyhole limpet hemocyanin (KLH) and injected into animals, produce a strong immune response. By measuring changes in the levels of anti-TNP IgM and IgG in appropriate animal models, researchers can assess the impact of phamacologic or genetic manipulations on the immune system. Life Diagnostics, Inc. offers a simple ELISA for measurement of mouse anti-TNP IgG. We also manufacture an ELISA kit for mouse anti-TNP IgM (cat. no. TNPM-1).

**PRINCIPLE OF THE ASSAY**

The mouse anti-TNP IgG test kit is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses TNP-BSA as the capture antigen (coated on the microtiter wells) and horseradish peroxidase (HRP) conjugated anti-mouse IgG antibodies for detection. Serum or plasma samples are diluted and incubated alongside standards in the microtiter wells for 1 hour. The wells are subsequently washed, and HRP conjugate is added and incubated for 45 minutes. Anti-TNP IgG molecules are then sandwiched between immobilized TNP and the detection antibody conjugate. The wells are then washed to remove unbound HRP-labeled antibodies, and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of anti-TNP IgG is proportional to the absorbance at 450 nm and is derived from a standard curve.

**MATERIALS AND COMPONENTS**

**Materials provided with the kit:**
- TNP-BSA Coated 96-well Plate (provided as 12 strips of 8 wells)
- Anti-Mouse IgG HRP Conjugate Stock, 11 ml
- Reference Standard1 (lyophilized), 1 vial
- 20x Wash Solution, 50 ml
- Diluent, 50 ml
- TMB Reagent, 11 ml
- Stop Solution (1N HCl), 11 ml

**Materials required but not provided:**
- Precision pipettes and tips
- Distilled or deionized water
- Polystyrene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-plate incubator/shaker mixing speed of ~150 rpm
- Plate washer
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional)

**STORAGE**

The kit should be stored at 2-8°C. The microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

**GENERAL INSTRUCTIONS/PRECAUTIONS**

1. Please read and understand the instructions thoroughly before using the kit.
2. This kit is designed to measure anti-TNP IgG levels in serum collected >14 days after immunization with TNP-carrier protein conjugates. Serum collected at post-immunization times less than 14 days may contain high levels of anti-TNP IgM that compete with anti-TNP IgG for the immobilized TNP, thereby causing interference.
3. Because TNP-modified BSA is used as the capture antigen, it is important that a carrier protein other than albumin (e.g. KLH or ovalbumin) be used to immunize mice.
4. All reagents should be allowed to reach room temperature (18-25°C) before use.
5. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc. suggest an initial sample dilution of 40,000 fold be used. Please do not use dilutions less than 1000 fold.
6. Optimal results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

**WASH SOLUTION PREPARATION**

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.

**STANDARD PREPARATION**

1. The mouse anti-TNP IgG standard is provided as a lyophilized stock. Reconstitute the stock as described on the vial label (the reconstituted reference standard remains stable at 2-8°C for at least one week, but should be aliquoted and frozen at -20°C if use beyond this time is intended).
2. Label 6 polystyrene or glass tubes as 100, 50, 25, 12.5, 6.25 and 3.125 u/ml.
3. In the tube labeled 100 u/ml prepare the 100 u/ml standard as detailed on the stock vial label.
4. Dispense 250 µl of diluent into the remaining tubes.
5. Prepare a 50 u/ml standard by diluting and mixing 250 µl of the 100 u/ml standard with 250 µl of diluent in the tube labeled 50 u/ml.
6. Similarly prepare the 25, 12.5, 6.25 and 3.125 u/ml standards by serial dilution.

**SAMPLE PREPARATION**

General Note: In studies at Life Diagnostics, Inc., using serum from TNP-KLH immunized mice, levels of 1,562,544 ± 707,478 u/ml (mean ± SD, n = 10) were observed 25 days after immunization. Levels will vary with the immunization protocol and the TNP carrier protein used. We suggest that samples initially be diluted 40,000 fold using the following procedure for each sample to be tested but

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1 Mouse anti-TNP IgM levels are measured in nominal units and are calibrated using pooled anti-TNP mouse serum prepared at Life Diagnostics, Inc.
optimal dilutions must be determined empirically. A 40,000-fold dilution may be achieved as follows:

1. Dispense 249 $\mu$l and 318 $\mu$l of diluent into separate polypropylene or glass tubes.
2. Pipette and mix 1 $\mu$l of the serum sample into the tube containing 249 $\mu$l of diluent. This provides a 250 fold diluted sample.
3. Dilute 2 $\mu$l of the 250 fold diluted sample into the tube containing 318 $\mu$l of diluent and mix. This provides a 40,000 fold diluted sample.
4. Repeat this procedure for each sample to be tested.

**ASSAY PROCEDURE**

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 $\mu$l of standards and diluted samples into the wells (we recommend that samples be tested in triplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 1 hour.
4. Aspirate the contents of the microtiter wells and wash the wells 5 times with 1x wash solution using a plate washer (400 $\mu$l/well). The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
6. Add 100 $\mu$l of diluted HRP conjugate into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
8. Wash as detailed in 4 to 5 above.
9. Dispense 100 $\mu$l of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
11. Stop the reaction by adding 100 $\mu$l 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 the optical density at 450 nm with a microtiter plate reader **within 5 minutes**.

**CALCULATION OF RESULTS**

1. Calculate the average absorbance values ($A_{450}$) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in u/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-TNP IgG in u/ml from the standard curve.
4. Multiply the derived concentration by the dilution factor to determine the actual concentration of anti-TNP IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD$_{450}$ values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

**TYPICAL STANDARD CURVE**

A typical standard curve with optical density readings at 450nm on the Y-axis against anti-TNP IgG concentrations on the X-axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns.

<table>
<thead>
<tr>
<th>Anti-TNP IgG (u/ml)</th>
<th>$A_{450}$</th>
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</thead>
<tbody>
<tr>
<td>100</td>
<td>3.141</td>
</tr>
<tr>
<td>50</td>
<td>2.210</td>
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<tr>
<td>25</td>
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<tr>
<td>12.5</td>
<td>0.773</td>
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<tr>
<td>6.25</td>
<td>0.436</td>
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<tr>
<td>3.125</td>
<td>0.243</td>
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</table>

**LIMITATIONS OF THE PROCEDURE**

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
3. Kits are validated using shaking incubators set at 150 rpm and 25°C. Performance of the assay at lower temperatures and/or mixing speeds will likely result in lower absorbance values.

Rev 111816

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