KAMIYA BIOMEDICAL COMPANY

Rat Anti-Dinitrophenol (DNP) IgM ELISA

For the quantitative determination of anti-DNP IgM in rat serum and plasma.

Cat. No. KT-675

For Research Use Only.

PRODUCT INFORMATION

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PRODUCT

The **K-ASSAY**® Rat Anti-Dinitrophenol (DNP) IgM ELISA is an enzyme immunoassay for the quantitative determination of anti-DNP IgM in rat serum and plasma. For research use only.

INTRODUCTION

Haptens such as dinitrophenol (DNP), 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-DNP IgM and IgG in appropriate animal models, researchers can assess the impact of pharmacologic or genetic manipulations on the immune system.

PRINCIPLE

The **K-ASSAY**® Rat anti-DNP IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses DNP-BSA as the capture antigen (coated on the microtiter wells) and horseradish peroxidase (HRP) conjugated anti-rat IgM antibodies for detection. Serum or plasma samples are diluted and incubated alongside calibrators in the microtiter wells for 45 minutes. The wells are subsequently washed, and HRP conjugate is added and incubated for 45 minutes. Anti-DNP IgM molecules are thus sandwiched between immobilized DNP 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-DNP IgM is proportional to the absorbance at 450 nm and is derived from a calibration curve.

COMPONENTS

- DNP-BSA Coated 96-well Plate (provided as 12 strips of 8 wells)
- Anti-Rat IgM HRP Conjugate Stock, 11 mL
- Reference Calibrator (lyophilized), 1 vial
- Diluent, 50 mL
- Wash Solution (20X), 50 mL
- TMB Reagent, 11 mL
- Stop Solution (1N HCl), 11 mL

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes and tips
- Distilled or de-ionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- A microtiter plate reader capable of measuring absorbance at 450 nm with a range of 0-4 OD
- Graph paper (PC graphing software is optional)
- Plate washer

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Please read and understand the instructions thoroughly before using the kit.

3. This kit is designed to measure anti-DNP IgM levels in serum collected ~7 days after immunization with DNP-carrier protein conjugates. Serum collected at post-immunization times greater than 7 days may contain high levels of anti-DNP IgG that compete with anti-DNP IgM for the immobilized DNP, thereby causing interference.

4. Because DNP-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 rats.

5. The optimal sample dilution should be determined empirically. However, our studies suggest an initial sample dilution of 3,000 fold be used. Please do not use dilutions less than 25 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 de-ionized water.

CALIBRATOR PREPARATION
1. The rat anti-DNP IgM calibrator is provided as a lyophilized stock. Reconstitute with 200 µL water. Concentration will be 1,000 units/mL.
2. Label 6 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25 and 3.125 u/mL.
3. Into the tube labeled 100 u/mL, prepare the 100 u/mL working calibrator by diluting 50 µL of reconstituted calibrator with 450 µL of diluent.
4. Dispense 250 µL of diluent into the remaining tubes.
5. Prepare the 50 u/mL calibrator by diluting and mixing 250 µL of the 100 u/mL calibrator 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 calibrators by serial dilution.

Please Note: The reconstituted calibrator remains stable for at least 7 days at 4°C but should be aliquoted and stored frozen at -20°C if future use in intended.

SAMPLE PREPARATION
General Note: In our studies using serum from DNP-KLH immunized rats, levels of 125,561 ± 67,183 u/mL (mean ± SD, n = 10) were observed 7 days after immunization. Levels will vary with the immunization protocol and the DNP carrier protein. We **suggest** that samples initially be diluted 3,000 fold using the following procedure for each sample to be tested but optimal dilutions must be determined empirically. A 3,000 fold dilution may be achieved as follows:
1. Dispense 98 µL and 295 µL of diluent into separate polypropylene or glass tubes.
2. Pipette and mix 2 µL of the serum sample into the tube containing 98 µL of diluent. This provides a 50 fold diluted sample.
3. Dilute 5 µL of the 50 fold diluted sample into the tube containing 295 µL of diluent and mix. This provides a 3,000 fold dilution of the 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 µL of calibrators 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 45 minutes.
4. Aspirate the contents of the microtiter wells and wash the wells 5 times with 1X wash solution using a plate washer (400 µL/well). The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto adsorbent paper or paper towels to remove all residual wash buffer.
6. Add 100 µL 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 µL 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 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 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 calibrators and samples.

2. Construct a calibration curve by plotting the mean absorbance obtained from each reference calibrator 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-DNP IgM in u/mL from the calibration curve.
4. Multiply the derived concentration by the dilution factor to determine the actual concentration of anti-DNP IgM 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 calibration curve when tested at the suggested dilution of 3,000, samples should be diluted appropriately and re-tested.

TYPICAL CALIBRATION CURVE
A typical calibration curve with optical density readings at 450 nm on the Y axis against anti-DNP IgM 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.

Anti-DNP IgM (u/mL)	Absorbance (450 nm)
100	3.097
50	1.920
25	1.033
12.5	0.546
6.25	0.340
3.125	0.196

STORAGE
The kit should be stored at 4°C. The microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable until the expiration date provided that the components are stored as described above.

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.

FOR RESEARCH USE ONLY
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