

# Rat Anti-KLH IgM ELISA Kit

## ELISA for the Quantitative Determination of Rat Anti-Keyhole Limpet Hemocyanin (KLH) IgM in Serum and Plasma

### INTRODUCTION

Recent studies have demonstrated that suppression of anti-KLH IgM levels by therapeutic agents serves as a useful indicator of immunosuppression<sup>1</sup>. This ELISA allows rapid and quantitative measurement of rat anti-KLH IgM levels in serum or plasma samples.

### PRINCIPLE OF THE TEST

The rat anti-KLH IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses KLH for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat IgM antibodies for detection. Test serum or plasma samples are diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. Anti-KLH IgM molecules are thus sandwiched between immobilized KLH 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-KLH IgM is proportional to the optical density of the test sample.

### MATERIALS AND COMPONENTS

#### **Materials provided with the kit:**

- KLH coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard<sup>1</sup> (lyophilized)
- 20x Wash Solution, 50 ml
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

#### **Materials required but not provided:**

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene 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 OF TEST KIT AND INSTRUMENTATION

The kit should be stored at 2-8°C and 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

1. Please read and understand the instructions thoroughly before using the kit.
2. All reagents should be allowed to reach room temperature (18-25°C) before use.
3. The assay was designed for use with serum or plasma obtained from rats five days after immunization with KLH, at which point the immune response originates almost exclusively from IgM.
4. Serum or plasma samples must be diluted **at least** 500-fold in 1x diluent.
5. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 1000 fold.
6. Optimum 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.

### DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

### STANDARD PREPARATION

1. The rat anti-KLH IgM standard is provided as a lyophilized stock. Reconstitute with 1.0 ml of distilled or deionized water (***the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if additional use is intended***).
2. Label 6 polypropylene or glass tubes as 250, 125, 62.5, 31.2, 15.6, and 7.8 ng/ml.
3. Into the tube labeled 250 ng/ml, pipette the volume of diluent detailed on the anti-KLH IgM standard vial label. Then add the indicated volume of anti-KLH IgM standard (shown on the anti-KLH IgM standard vial label) and mix gently. This provides the 250 ng/ml standard.
4. Dispense 300 µl of diluent into the tubes labeled 125, 62.5, 31.2, 15.6, and 7.8 ng/ml.

5. Prepare a 125 ng/ml standard by diluting and mixing 300  $\mu$ l of the 250 ng/ml standard with 300  $\mu$ l of diluent in the tube labeled 125 ng/ml.
6. Similarly prepare the 62.5, 31.25, 15.6, and 7.8 ng/ml standards by serial dilution.

## SAMPLE PREPARATION

anti-KLH IgM is present in rat serum or plasma at concentrations ranging from approximately 20 to 200  $\mu$ g/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 1000 fold using the following procedure for each sample to be tested:

1. Dispense 196  $\mu$ l and 285  $\mu$ l of 1x diluent into separate tubes.
  2. Pipette and mix 4  $\mu$ l of the serum/plasma sample into the tube containing 196  $\mu$ l of diluent. This provides a 50 fold diluted sample.
  3. Mix 15  $\mu$ l of the 50 fold diluted sample with the 285  $\mu$ l of diluent in the second tube. This provides a 1000 fold dilution of the sample.
  4. Repeat this procedure for each sample to be tested
- Important: Do not use use dilutions lower than 500 fold.**

## 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 duplicate).
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  $\mu$ 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 enzyme conjugate reagent 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 ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.

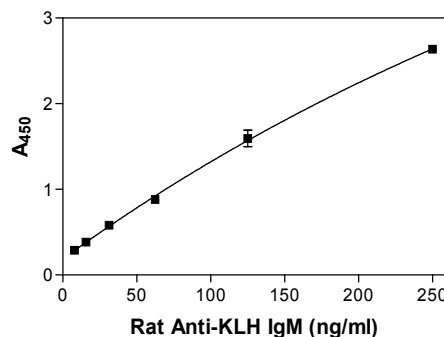
3. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-KLH IgM in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgM in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the  $OD_{450}$  values of samples fall outside the standard curve when tested at a dilution of 1000, samples should be diluted appropriately and re-tested (do not use dilutions lower than 500 fold).

## TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against anti-KLH 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. Each user should obtain his or her data and standard curve in each experiment.

Anti-KLH IgM (ng/ml)	Absorbance (450 nm)
250	2.636
125	1.595
62.5	0.882
31.25	0.583
15.63	0.383
7.81	0.288

**Typical Rat Anti-KLH IgM Standard Curve**



## 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.

## REFERENCES

1. ER Gore et.al. Primary antibody response to Keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. *Toxicology* 197:23-35 (2004)