

# Mouse Anti-KLH IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4000-1

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

#### INTRODUCTION

Measurement of KLH induced anti-KLH antibody levels allows quantitative evaluation of the immune response (ref 1). This ELISA is designed for the rapid and quantitative measurement of mouse anti-KLH IgM levels in mouse serum or plasma. A companion ELISA, catalog number 4010-1, can be used for measurement of mouse anti-KLH IgG.

#### PRINCIPLE OF THE TEST

The mouse 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-mouse IgM antibodies for detection. Test serum or plasma samples are diluted and incubated in the microtiter wells for 1 hour. 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
- Diluent (60 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

<sup>1</sup> Mouse anti-KLH IgM levels are measured in nominal units and are calibrated with reference anti-KLH mouse serum at Life Diagnostics, Inc.

- Graph paper (PC graphing software is optional)

#### STORAGE OF THE TEST KIT

- The reference standard should be stored at -20°C for optimal stability
  - All remaining kit components 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 kit 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. **This kit is designed to measure anti-KLH IgM levels in serum collected 5 days after immunization with KLH.** Serum collected at post-immunization times greater than 5 days may contain high levels of anti-KLH IgG that compete with anti-KLH IgM for the immobilized KLH, thereby causing interference.
3. All reagents should be allowed to reach room temperature (18-25°C) before use.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 500 fold. Please do not use dilutions less than 25-fold.
5. 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.

#### STANDARD PREPARATION

1. The mouse anti-KLH IgM standard is provided as a lyophilized stock. Reconstitute the stock as described on the vial label.
2. Label 5 polypropylene or glass tubes as 100, 50, 25, 12.5, and 6.25 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, and 6.25 u/ml standards by serial dilution.

#### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-KLH IgM is present in serum from KLH immunized mice at concentrations of ~10,000 u/ml. In order to obtain values

**within range of the standard curve, we suggest that samples initially be diluted 500 fold using the following procedure for each sample to be tested:**

1. Dispense 48  $\mu$ l and 237.5  $\mu$ l of diluent into separate tubes.
2. Pipette and mix 2  $\mu$ l of the serum/plasma sample into the tube containing 48  $\mu$ l of diluent. This provides a 25 fold diluted sample.
3. Mix 12.5  $\mu$ l of the 25 fold diluted sample with the 237.5  $\mu$ l of diluent in the second tube. This provides a 500 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  $\mu$ 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 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 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

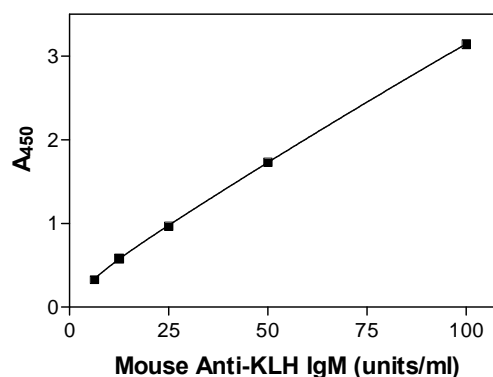
1. Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical 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 u/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 500, 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-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 (u/ml)	Absorbance (450 nm)
100	3.145
50	1.730
25	0.971
12.5	0.581
6.25	0.330

**Representative Mouse 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. Wada H, Noguchi Y, Marino MW, Dunn AR and Old LJ. T-cell functions in granulocyte/macrophage colony-stimulating factor deficient mice. Proc Natl Acad Sci. 94:12557-61 (1997)

# Rat Anti-SRBC IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4200-2

### ELISA for the Quantitative Determination of Rat Anti-Sheep Red Blood Cell (SRBC) IgM in Serum and Plasma

#### INTRODUCTION

Recent studies have demonstrated that suppression of anti-SRBC IgM levels by therapeutic agents serves as a useful indicator of immunosuppression<sup>1,2</sup>. This ELISA allows rapid and quantitative measurement of rat anti-SRBC IgM levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The rat anti-SRBC IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses detergent solubilized SRBC ghosts<sup>2</sup> 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-SRBC IgM molecules are thus sandwiched between immobilized SRBC antigens 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-SRBC IgM is proportional to the optical density of the test sample.

#### MATERIALS AND COMPONENTS

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

- SRBC coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard stock<sup>A</sup> (lyophilized), 2 vials
- 20x Wash Solution, 50 ml
- Diluent (30 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)

<sup>A</sup> The levels of rat anti-SRBC IgM are measured in nominal units and are calibrated with reference rat anti-SRBC serum at Life Diagnostics, Inc.

#### STORAGE OF TEST KIT

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 SRBC, at which point the immune response originates almost exclusively from IgM.
4. Serum or plasma samples must be diluted **at least** 25-fold in diluent.
5. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 200 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.

#### STANDARD PREPARATION

1. Reconstitute one vial of the lyophilized rat anti-SRBC IgM standard stock with distilled or deionized water as described on the standard vial label (***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 100, 50, 25, 12.5, 6.25, and 3.125 u/ml.
3. In the tube labeled 100 u/ml, prepare a 100 u/ml stock by mixing the volume of reconstituted standard stock with the volume of diluent detailed on the reference standard stock vial label.
4. Dispense 250 µl of diluent into the tubes labeled 50, 25, 12.5, 6.25, and 3.125 u/ml.
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:** Studies at Life Diagnostics, Inc., indicate that anti-SRBC IgM is present in rat serum or plasma from SRBC

immunized animals at concentrations in excess of 4000 u/ml. In order to obtain values within the range of the standard curve, we **suggest** that samples initially be diluted 200 fold using the following procedure for each sample to be tested:

1. For each test sample dispense 298.5 µl of diluent into separate tubes.
  2. Pipette and mix 1.5 µl of the serum/plasma sample into a tube containing 298.5 µl of diluent. This provides a 200 fold diluted sample.
  3. Repeat this procedure for each sample to be tested
- Important: Do not use dilutions lower than 25 fold.**

### ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl 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 µl/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 µl 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 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

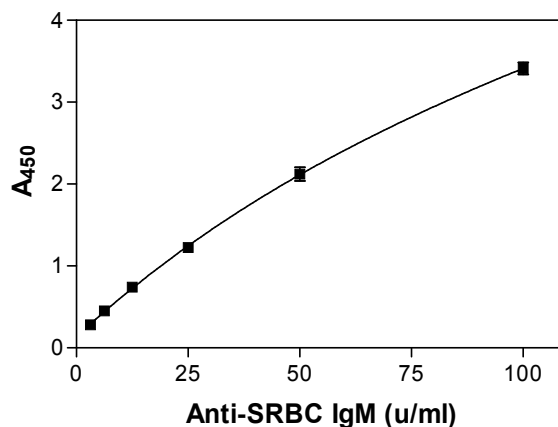
1. Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in u/ml on linear graph paper, with absorbance values on the vertical 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-SRBC IgM in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-SRBC 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 200, samples should be diluted appropriately and re-tested (do not use dilutions lower than 25 fold).

### TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against anti-SRBC 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-SRBC IgM (u/ml)	Absorbance (450 nm)
100	3.411
50	2.122
25	1.224
12.5	0.740
6.25	0.451
3.125	0.282

### Representative Rat Anti-SRBC 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. GS Ladics. Use of SRBC antibody responses for immunotoxicity testing. *Methods* 41:9-19 (2007)
2. L. Temple, T. T. Kawabata, A. E. Munson and K. L. White. Comparison of ELISA and Plaque-Forming Cell Assays for Measuring the Humoral Immune Response to SRBC in Rats and Mice Treated with Benzo[a]pyrene or Cyclophosphamide *Fundamental and Applied Toxicology* 21(4):412-419 (1993)

# Monkey Anti-KLH IgG1 ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4010-4-1

### ELISA for the Quantitative Determination of Monkey Anti-KLH IgG in Serum or Plasma

#### INTRODUCTION

Drug candidates are routinely screened for evidence of immune system regulation during the discovery process. It is important that the immune response is not enhanced or decreased since such effects may lead to hypersensitivity or increased susceptibility to infection. Determination of a drug candidate's effects on anti-KLH antibody levels allows easy assessment of immune system regulation<sup>1</sup>. Animals are immunized with KLH while undergoing drug treatment and serum is collected at appropriate times post immunization. Typically, serum collected 5-7 days after immunization is used for measurement of anti-KLH IgM levels, and serum collected 14+ days post immunization is used to measure anti-KLH IgG levels. Comparison of anti-KLH IgG or IgM levels in drug treated, versus control groups reveals effects on the immune response.

This ELISA allows rapid and quantitative measurement of anti-KLH IgG1 levels in serum or plasma. IgG1 is the major IgG subclass in monkeys<sup>2,3</sup>.

#### PRINCIPLE OF THE TEST

The monkey anti-KLH IgG ELISA is a solid phase enzyme-linked immunosorbent assay. It uses KLH for solid phase (microtiter wells) immobilization and a horseradish peroxidase (HRP) conjugated mouse monoclonal anti-monkey IgG1 antibody for detection<sup>A</sup>. 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 IgG1 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 IgG1 is proportional to the optical density.

#### MATERIALS AND COMPONENTS

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

- KLH coated 96-well plate (provided as 12 strips of 8 wells)
- Anti Monkey IgG1 HRP Conjugate, 11 ml
- Anti-KLH IgG1 Stock<sup>B</sup> (lyophilized)
- 20x Wash Solution, 50 ml

<sup>A</sup> Specificity of the monoclonal antibody was determined in competitive ELISA<sup>§</sup> at Life Diagnostics using recombinant monkey IgG1, IgG2, IgG3 and IgG4 as reference materials. All reference IgG<sup>§</sup> were kindly provided by The NIH Nonhuman Primate Reagent Resource center.

<sup>B</sup> The reference standard provided with the kit was calibrated using affinity purified rhesus monkey anti-KLH IgG prepared at Life Diagnostics, Inc. IgG1 content was measured using a monkey IgG1 ELISA developed at Life Diagnostics Inc.

- Diluent (50 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 THE TEST KIT

On receipt, the anti-KLH IgG1 standard stock should be stored frozen at -20°C or lower. The remainder of 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. **DO NOT FREEZE THE HRP CONJUGATE OR TMB SOLUTIONS.** Test kits will remain stable for six months from the date of purchase provided that the components are stored as described.

#### GENERAL INSTRUCTIONS

1. Please read the instructions thoroughly before using the kit.
2. All reagents should be allowed to reach room temperature (18-25°C) before use.
3. The optimal sample dilution should be determined empirically. Do not use dilutions less than 100-fold (i.e., do not use dilutions of 50-fold).
4. 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.

#### STANDARD PREPARATION

##### **PLEASE READ ATTACHED MSDS FOR BIOHAZARD INFORMATION**

1. Working 30 – 0.94 ng/ml anti-KLH IgG1 standards should be used within 1 hour of preparation.
2. The anti-KLH IgG1 stock is provided in lyophilized form. Reconstitute as directed on the vial label (**the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if additional use is intended**).
3. Label 6 polypropylene or glass tubes as 30, 15, 7.5, 3.75, 1.88 and 0.94 ng/ml.
4. Into the tube labeled 30 ng/ml, pipette the volume of diluent detailed on the stock vial label. Then add the indicated

- volume of anti-KLH IgG1 stock (also detailed on the vial label) and mix gently. This provides the 30 ng/ml standard.
- Dispense 250  $\mu$ l of diluent into the tubes labeled 15, 7.5, 3.75, 1.88 and 0.94 ng/ml.
  - Prepare a 15 ng/ml standard by diluting and mixing 250  $\mu$ l of the 30 ng/ml standard with 250  $\mu$ l of diluent in the tube labeled 30 ng/ml.
  - Similarly prepare the 7.5, 3.75, 1.88 and 0.94 ng/ml standards by serial dilution.

### SAMPLE PREPARATION

The optimal sample dilution should be determined empirically. However, studies at Life Diagnostics, Inc., suggest that a 500-fold dilution is a reasonable starting point. In order to achieve high dilutions we suggest that a serial dilution strategy be used. If, for example, a 500-fold sample dilution is desired the following procedure should be used. This approach minimizes diluent usage and favors accurate and precise sample dilution.

- Dispense 48  $\mu$ l and 237.5  $\mu$ l of diluent into separate tubes.
- Pipette and mix 2  $\mu$ l of the serum/plasma sample into the tube containing 48  $\mu$ l of diluent. This provides a 25 fold diluted sample.
- Mix 12.5  $\mu$ l of the 25 fold diluted sample with the 237.5  $\mu$ l of diluent in the second tube. This provides a 500 fold dilution of the sample.
- Repeat this procedure for each sample to be tested.

**Do not use dilutions less than 100-fold (i.e., do not use dilutions of 50-fold).**

### ASSAY PROCEDURE

- Secure the desired number of coated wells in the holder.
- Dispense 100  $\mu$ l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- 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.
- Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
- Add 100  $\mu$ l of HRP conjugate into each well.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Wash as detailed in 4 to 5 above.
- Dispense 100  $\mu$ l of TMB Reagent into each well.
- Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
- Stop the reaction by adding 100  $\mu$ l of Stop Solution to each well.
- Gently mix. *It is important to make sure that all the blue color changes to yellow.*
- Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

### CALCULATION OF RESULTS

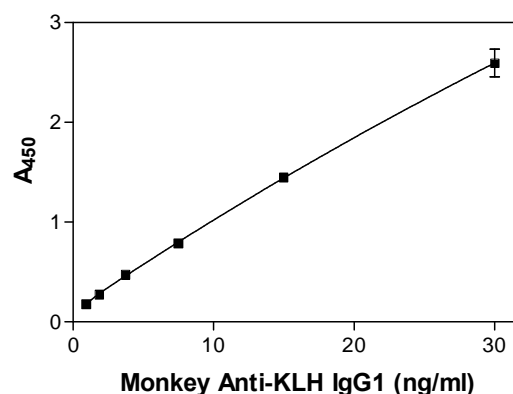
- Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.

- 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.
- Using the mean absorbance value for each sample, determine the corresponding concentration of anti-KLH IgG1 in ng/ml from the standard curve.
- Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgG1 in the serum/plasma sample.
- PC graphing software may be used for the above steps.
- 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-KLH IgG1 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 IgG1 (ng/ml)	Absorbance (450 nm)
30	2.729
15	1.448
7.5	0.783
3.75	0.447
1.88	0.275
0.94	0.177



### REFERENCES

- JR Picotti et.al. T-cell-dependent antibody response: Assay development in cynomolgus monkeys. *Journal of Immunotoxicology*, 2:191-196 (2005)
- ED Williamson et.al. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. *Infection and Immunity* 73:5978-5987 (2005)
- P Procell-Wilkins et.al. Isotypic analysis of humoral immune responses in rhesus monkeys to an adult microsomal antigen of *Schistosoma Mansoni*: an indicator of successful treatment. *Am. J. Trop. Med. Hyg.* 45:629-635 (1991)

## MATERIAL SAFETY DATA SHEET

### Monkey Anti-KLH IgG Standard (component of kit 4010-4-N)

**DESCRIPTION:** The monkey anti-KLH IgG standard is comprised of rhesus monkey serum diluted in a proprietary matrix. It is provided in a sealed vial in lyophilized format.

#### CUSTOMER INFORMATION

Please forward this abbreviated MSDS to your coordinator for review and filing. Please assure that this MSDS reaches the intended user of this material.

#### HAZARD INFORMATION

##### **HANDLE THIS MATERIAL AND ITS DERIVATIVES AS A BIOHAZARD**

Nonhuman primates can carry a variety of zoonotic diseases including B virus (*Cercopithecine Herpes Virus 1* or *Herpesvirus simae*), Measles, Influenza, Pox viruses (Monkeypox and Yaba virus), filoviruses such as Ebola virus, Gastrointestinal disease (*Salmonella*, *Shigella*, *Giardia*, *Entamoeba histolytica*, *Balantidium coli*), Bacterial pneumonia (*Streptococcus pneumoniae*), and Tuberculosis (*Mycobacterium tuberculosis*). Zoonotic diseases are those that can be transmitted between species. It is important to note that a disease that does not cause serious health effects in one species may cause severe, life-threatening illness in another species.

Care must be taken by all personnel who handle this material to prevent potential exposure to zoonotic pathogens. Contact with this material may irritate the eyes, skin, or mucous membranes and potentially result in infection. In order to limit exposure, exercise all due caution and wear appropriate personal protective equipment when handling this material. Good laboratory and manufacturing procedures are essential for safe use. If eye exposure occurs, flush product from eyes with water for at least 15 minutes, see a physician. If skin exposure occurs, wash and scrub the exposed area thoroughly with soap, concentrated solution of detergent, povidone-iodine, or chlorhexidine and water, irrigate the area with running water for 15-20 minutes, see a physician.

#### FIRE AND SPILL INFORMATION

In case of fire use suitable extinguishing agent such as water, carbon dioxide, foam or dry chemical to suppress the surrounding fire. In case of spill collect material in a leak proof container and decontaminate the spilled material with a freshly made 1% bleach solution (a 1:5 dilution of household bleach) or similar disinfectant with virucidal properties, and dispose of according to Federal, State, and local regulations. Decontaminate the area of the spill with a freshly made 1% bleach solution (a 1:5 dilution of commercial bleach) or similar disinfectant with virucidal properties. Allow sufficient contact time (30 minutes) before final clean up of surfaces.

#### PERSONAL PROTECTIVE EQUIPMENT

Protective gloves, safety goggles, face shield, long sleeved lab coat or gown and access to a safety eyewash station are recommended. Protective clothing should be replaced if it is contaminated. Protective clothing should be removed on leaving the work area. Wash hands after removing gloves.

*The information, data, and recommendations contained herein have been compiled from sources believed to be reliable and are believed to be accurate. Life Diagnostics, Inc. makes no warranty of any kind whatsoever with respect thereto and disclaims all liability from reliance thereon. This information is offered solely to you in advisement for the safe use and handling of this material. We reserve the right to revise this information periodically as new information becomes available.*

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Revision Date: 08/19/11

# Monkey Anti-KLH IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4000-4-N

### ELISA for the Quantitative Determination of Monkey Anti-KLH IgM in Serum or Plasma

#### INTRODUCTION

Drug candidates are routinely screened for evidence of immune system regulation during the discovery process. It is important that the immune response is not enhanced or decreased since such effects may lead to hypersensitivity or increased susceptibility to infection. Determination of a drug candidate's effects on anti-KLH antibody levels allows easy assessment of immune system regulation<sup>1</sup>. Animals are immunized with KLH while undergoing drug treatment and serum is collected at appropriate times post immunization. Typically, serum collected 5-7 days after immunization is used for measurement of anti-KLH IgM levels, and serum collected 14+ days post immunization is used to measure anti-KLH IgG levels. Comparison of anti-KLH IgG or IgM levels in drug treated, versus control groups reveals effects on the immune response.

This ELISA allows rapid and quantitative measurement of anti-KLH IgM levels in serum or plasma.

#### PRINCIPLE OF THE TEST

The monkey anti-KLH IgM ELISA is a solid phase enzyme-linked immunosorbent assay. It uses KLH for solid phase (microtiter wells) immobilization and a horseradish peroxidase (HRP) conjugated goat anti-monkey IgM antibody for detection. 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.

#### MATERIALS AND COMPONENTS

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

- KLH coated 96-well plate (provided as 12 strips of 8 wells)
- Anti Monkey IgM HRP Conjugate, 11 ml
- Anti-KLH IgM Stock<sup>A</sup> (lyophilized)
- 20x Wash Solution, 50 ml
- Diluent (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

<sup>A</sup> The reference standard provided with the kit was calibrated using affinity purified rhesus monkey anti-KLH IgM prepared at Life Diagnostics, Inc.

##### **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 THE TEST KIT

On receipt, the anti-KLH IgM stock should be stored frozen at -20°C or lower. The remainder of 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. **DO NOT FREEZE THE HRP CONJUGATE OR TMB SOLUTIONS.** Test kits will remain stable for six months from the date of purchase provided that the components are stored as described.

#### GENERAL INSTRUCTIONS

1. Please read the instructions thoroughly before using the kit.
2. All reagents should be allowed to reach room temperature (18-25°C) before use.
3. The optimal sample dilution should be determined empirically. Do not use dilutions less than 100-fold (i.e., do not use dilutions of 50-fold).
4. 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.

#### STANDARD PREPARATION

##### **PLEASE READ ATTACHED MSDS FOR BIOHAZARD INFORMATION**

1. Working 400 – 12.5 ng/ml anti-KLH IgM standards should be used within 1 hour of preparation.
2. The anti-KLH IgM stock is provided in lyophilized form. Reconstitute as directed on the vial label (***the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if additional use is intended***).
3. Label 6 polypropylene or glass tubes as 400, 200, 100, 50, 25 and 12.5 ng/ml.
4. Into the tube labeled 400 ng/ml, pipette the volume of diluent detailed on the stock vial label. Then add the indicated volume of anti-KLH IgM stock (also detailed on the vial label) and mix gently. This provides the 400 ng/ml standard.
5. Dispense 250 µl of diluent into the tubes labeled 200, 100, 50, 25 and 12.5 ng/ml.

6. Prepare a 200 ng/ml standard by diluting and mixing 250  $\mu$ l of the 400 ng/ml standard with 250  $\mu$ l of diluent in the tube labeled 200 ng/ml.
7. Similarly prepare the 100, 50, 25 and 12.5 ng/ml standards by serial dilution.

### SAMPLE PREPARATION

The optimal sample dilution should be determined empirically. However, studies at Life Diagnostics, Inc., suggest that a 500-fold dilution is a reasonable starting point. In order to achieve high dilutions we suggest that a serial dilution strategy be used. If, for example, a 500-fold sample dilution is desired the following procedure should be used. This approach minimizes diluent usage and favors accurate and precise sample dilution.

1. Dispense 48  $\mu$ l and 237.5  $\mu$ l of diluent into separate tubes.
2. Pipette and mix 2  $\mu$ l of the serum/plasma sample into the tube containing 48  $\mu$ l of diluent. This provides a 25 fold diluted sample.
3. Mix 12.5  $\mu$ l of the 25 fold diluted sample with the 237.5  $\mu$ l of diluent in the second tube. This provides a 500 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

**Do not use dilutions less than 100-fold (i.e., do not use dilutions of 50-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 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 ng/ml on linear graph paper, with absorbance

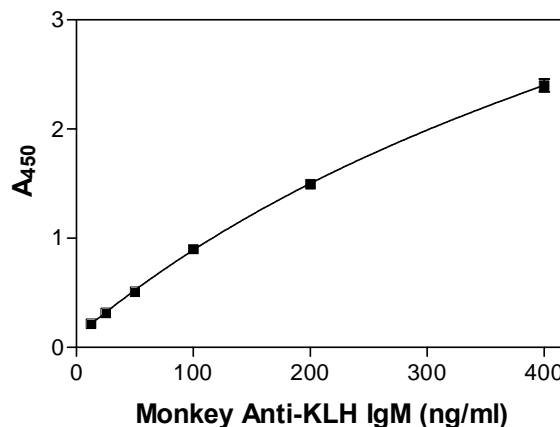
values on the vertical or Y-axis and concentrations on the horizontal or X-axis.

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, 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-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)
400	2.729
200	1.448
100	0.788
50	0.474
25	0.277
12.5	0.179



### REFERENCES

1. JR Picotti et.al. T-cell-dependent antibody response: Assay development in cynomolgus monkeys. *Journal of Immunotoxicology*, 2:191-196 (2005)

## 2. MATERIAL SAFETY DATA SHEET

### Monkey Anti-KLH IgM Standard (component of kit 4010-4-N)

**DESCRIPTION:** The monkey anti-KLH IgM standard is comprised of rhesus monkey serum diluted in a proprietary matrix. It is provided in a sealed vial in lyophilized format.

#### CUSTOMER INFORMATION

Please forward this abbreviated MSDS to your coordinator for review and filing. Please assure that this MSDS reaches the intended user of this material.

#### HAZARD INFORMATION

##### **HANDLE THIS MATERIAL AND ITS DERIVATIVES AS A BIOHAZARD**

Nonhuman primates can carry a variety of zoonotic diseases including B virus (*Cercopithecine Herpes Virus 1* or *Herpesvirus simae*), Measles, Influenza, Pox viruses (Monkeypox and Yaba virus), filoviruses such as Ebola virus, Gastrointestinal disease (*Salmonella*, *Shigella*, *Giardia*, *Entamoeba histolytica*, *Balantidium coli*), Bacterial pneumonia (*Streptococcus pneumoniae*), and Tuberculosis (*Mycobacterium tuberculosis*). Zoonotic diseases are those that can be transmitted between species. It is important to note that a disease that does not cause serious health effects in one species may cause severe, life-threatening illness in another species.

Care must be taken by all personnel who handle this material to prevent potential exposure to zoonotic pathogens. Contact with this material may irritate the eyes, skin, or mucous membranes and potentially result in infection. In order to limit exposure, exercise all due caution and wear appropriate personal protective equipment when handling this material. Good laboratory and manufacturing procedures are essential for safe use. If eye exposure occurs, flush product from eyes with water for at least 15 minutes, see a physician. If skin exposure occurs, wash and scrub the exposed area thoroughly with soap, concentrated solution of detergent, povidone-iodine, or chlorhexidine and water, irrigate the area with running water for 15-20 minutes, see a physician.

#### FIRE AND SPILL INFORMATION

In case of fire use suitable extinguishing agent such as water, carbon dioxide, foam or dry chemical to suppress the surrounding fire. In case of spill collect material in a leak proof container and decontaminate the spilled material with a freshly made 1% bleach solution (a 1:5 dilution of household bleach) or similar disinfectant with virucidal properties, and dispose of according to Federal, State, and local regulations. Decontaminate the area of the spill with a freshly made 1% bleach solution (a 1:5 dilution of commercial bleach) or similar disinfectant with virucidal properties. Allow sufficient contact time (30 minutes) before final clean up of surfaces.

#### PERSONAL PROTECTIVE EQUIPMENT

Protective gloves, safety goggles, face shield, long sleeved lab coat or gown and access to a safety eyewash station are recommended. Protective clothing should be replaced if it is contaminated. Protective clothing should be removed on leaving the work area. Wash hands after removing gloves.

*The information, data, and recommendations contained herein have been compiled from sources believed to be reliable and are believed to be accurate. Life Diagnostics, Inc. makes no warranty of any kind whatsoever with respect thereto and disclaims all liability from reliance thereon. This information is offered solely to you in advisement for the safe use and handling of this material. We reserve the right to revise this information periodically as new information becomes available.*

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Revision Date: 08/19/11

# Rat Anti-KLH IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4010-2C

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

#### INTRODUCTION

Recent studies have demonstrated that suppression of anti-KLH IgG levels by therapeutic agents provides a useful indicator of immunosuppression (ref. 1). The rat anti-KLH IgG ELISA developed by Life Diagnostics, Inc., allows rapid and quantitative measurement of rat anti-KLH IgG levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The rat anti-KLH IgG 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 IgG 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 30 minutes. Anti-KLH IgG 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 IgG 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) **Store  $\leq -20^{\circ}\text{C}$**
- Rat KLH IgG control (lyophilized) **Store  $\leq -20^{\circ}\text{C}$**
- 20x Wash Solution, 50 ml
- Diluent (30 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  $\sim 150$  rpm
- Plate washer
- Plate reader with an optical density range of 0-4 at 450nm

<sup>1</sup> The levels of rat anti-KLH IgG in the reference standard were determined relative to purified anti-KLH IgG prepared at Life Diagnostics. Actual IgG concentration of the purified anti-KLH IgG was determined using a rat IgG ELISA from an independent laboratory.

- Graph paper (PC graphing software is optional)

#### STORAGE OF TEST KIT AND INSTRUMENTATION

The reference standard and control vials should be stored at or below  $-20^{\circ}\text{C}$ . All other kit components should be stored at  $2-8^{\circ}\text{C}$  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.

#### GENERAL INSTRUCTIONS

1. Please read and understand the instructions thoroughly before using the kit.
2. All reagents should be allowed to reach room temperature ( $18-25^{\circ}\text{C}$ ) before use.
3. The assay was designed for use with serum or plasma obtained from rats 14 days after i.v. immunization with KLH, at which point the immune response originates primarily from IgG.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., using serum obtained from rats immunized intravenously with KLH, indicate that an initial sample dilution of 100 fold is a good starting point.
5. 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.

#### CONTROL PREPARATION

Reconstitute the lyophilized rat anti-KLH IgG control with the volume of diluent indicated on the vial label. The concentration range of rat anti-KLH IgG after reconstitution is shown on the vial label. The assay value of the control should be within the specified range. **Discard any remaining control after use.**

#### STANDARD PREPARATION

1. The rat anti-KLH IgG standard is provided as a lyophilized stock. Reconstitute with the volume of diluent indicated on the vial label to give a 500 ng/ml solution of rat anti-KLH IgG (**the reconstituted standard remains stable for at least one week at  $2-8^{\circ}\text{C}$  but should be aliquoted and frozen at or below  $-20^{\circ}\text{C}$  after reconstitution if use beyond this time is intended**).
2. Label 5 polypropylene or glass tubes as 250, 125, 62.5, 31.2 and 15.6 ng/ml and pipette 250  $\mu\text{l}$  of diluent into each tube.
3. Into the tube labeled 250 ng/ml, pipette and mix 250  $\mu\text{l}$  of the reconstituted 500 ng/ml anti-KLH IgG. This provides the 250 ng/ml standard.

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

### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-KLH IgG is present in rat serum at concentrations up to approximately 20  $\mu$ g/ml 14 days after i.v. immunization with KLH. Levels are likely higher after 14 days. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 100 fold using the following procedure for each sample to be tested. Optimum dilutions may need to be determined empirically.

1. Dispense 297  $\mu$ l of diluent into a polypropylene or glass tube.
2. Pipette and mix 3  $\mu$ l of the serum/plasma sample into the tube containing 297  $\mu$ l of diluent. This provides a 100 fold diluted sample.
3. 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 (500 – 15.6 ng/ml), controls and diluted samples into the wells (standards, controls and samples should 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 30 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.

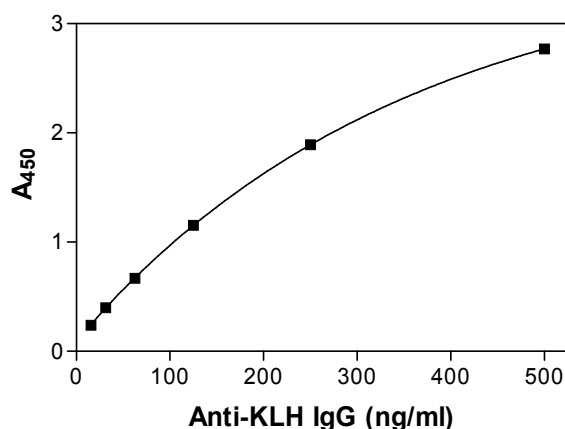
3. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-KLH IgG in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the  $OD_{450}$  values of samples fall outside the standard curve when tested at a dilution of 100, 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-KLH 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. Each user should obtain his or her data and standard curve in each experiment.

Anti-KLH IgG (ng/ml)	Absorbance (450 nm)
500	2.769
250	1.892
125	1.153
62.5	0.670
31.25	0.399
15.63	0.238

### Representative Rat Anti-KLH IgG 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)

# Rat Anti-KLH IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4010-2

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

#### INTRODUCTION

Recent studies have demonstrated that suppression of anti-KLH IgG levels by therapeutic agents provides a useful indicator of immunosuppression<sup>1</sup>. The rat anti-KLH IgG ELISA developed by Life Diagnostics, Inc., allows rapid and quantitative measurement of rat anti-KLH IgG levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The rat anti-KLH IgG 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 IgG 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 30 minutes. Anti-KLH IgG 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 IgG 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) **Store ≤ -20°C**
- 20x Wash Solution, 50 ml
- Diluent (30 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)

<sup>1</sup> The levels of rat anti-KLH IgG in the reference standard were determined relative to purified anti-KLH IgG prepared at Life Diagnostics. Actual IgG concentration of the purified anti-KLH IgG was determined using a rat IgG ELISA from an independent laboratory.

#### STORAGE OF TEST KIT AND INSTRUMENTATION

The reference standard should be stored at or below -20°C. All other kit components 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.

#### 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 14 days after i.v. immunization with KLH, at which point the immune response originates primarily from IgG.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., using serum obtained from rats immunized intravenously with KLH, indicate that an initial sample dilution of 100 fold is a good starting point.
5. 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.

#### STANDARD PREPARATION

1. The rat anti-KLH IgG standard is provided as a lyophilized stock. Reconstitute with the volume of diluent indicated on the vial label to give a 500 ng/ml solution of rat anti-KLH IgG (***the reconstituted standard remains stable for at least one week at 2-8°C but should be aliquoted and frozen at or below -20°C after reconstitution if use beyond this time is intended***).
2. Label 5 polypropylene or glass tubes as 250, 125, 62.5, 31.2 and 15.6 ng/ml and pipette 250 µl of diluent into each tube.
3. Into the tube labeled 250 ng/ml, pipette and mix 250 µl of the reconstituted 500 ng/ml anti-KLH IgG. This provides the 250 ng/ml standard.
4. Prepare a 125 ng/ml standard by diluting and mixing 250 µl of the 250 ng/ml standard with 250 µl of diluent in the tube labeled 125 ng/ml.
5. Similarly prepare the 62.5, 31.25 and 15.6 ng/ml standards by serial dilution.

## SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-KLH IgG is present in rat serum at concentrations up to approximately 20 µg/ml 14 days after i.v. immunization with KLH. Levels are likely higher after 14 days. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 100 fold using the following procedure for each sample to be tested. Optimum dilutions may need to be determined empirically.

1. Dispense 297 µl of diluent into a polypropylene or glass tube.
2. Pipette and mix 3 µl of the serum/plasma sample into the tube containing 297 µl of diluent. This provides a 100 fold diluted sample.
3. 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 standards (500 – 15.6 ng/ml) and diluted samples into the wells (standards and samples should 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 µl/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 µl 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 30 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 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 IgG in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.

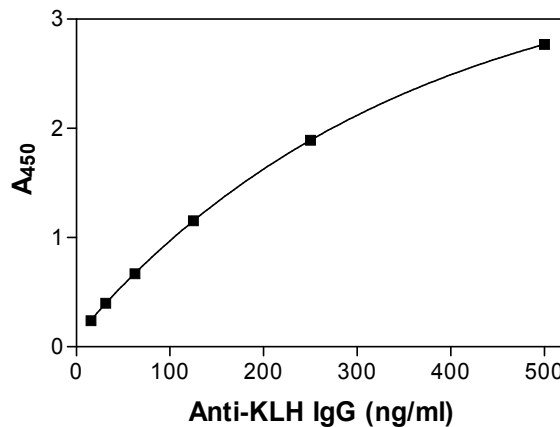
6. If the  $OD_{450}$  values of samples fall outside the standard curve when tested at a dilution of 100, samples should be diluted appropriately and re-tested.

## TYPICAL STANDARD CURVE

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

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## 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)

# Rat Anti-KLH IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4000-2C

### 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)
- Rat KLH IgM control (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

<sup>1</sup> The levels of rat anti-KLH IgM in the reference standard were determined relative to purified anti-KLH IgM prepared at Life Diagnostics. The IgM concentration of the purified anti-KLH IgM was determined using a rat IgM ELISA from an independent laboratory.

- 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 water required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

#### CONTROL PREPARATION

Reconstitute the lyophilized rat anti-KLH IgM control with the volume of distilled or deionized water indicated on the vial label. The concentration range of rat anti-KLH IgM after reconstitution is shown on the vial label. The assay value of the control should be within the specified range. **Discard any remaining control after use.**

#### 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  $\mu$ l 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

**General Note:** Studies at Life Diagnostics, Inc., indicate that 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 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, controls and diluted samples into the wells (we recommend that standards, controls and 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.

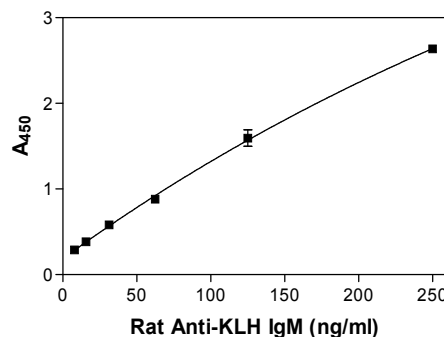
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, 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)

# Rat Anti-KLH IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4000-2

### 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)

<sup>1</sup> The levels of rat anti-KLH IgM in the reference standard were determined relative to purified anti-KLH IgM prepared at Life Diagnostics. The IgM concentration of the purified anti-KLH IgM was determined using a rat IgM ELISA from an independent laboratory.

#### 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.

- 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.
- Similarly prepare the 62.5, 31.25, 15.6, and 7.8 ng/ml standards by serial dilution.

### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that 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:

- Dispense 196  $\mu$ l and 285  $\mu$ l of 1x diluent into separate tubes.
  - 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.
  - 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.
  - Repeat this procedure for each sample to be tested
- Important: Do not use dilutions lower than 500 fold.**

### ASSAY PROCEDURE

- Secure the desired number of coated wells in the holder.
- Dispense 100  $\mu$ l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- 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.
- Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
- Add 100  $\mu$ l of enzyme conjugate reagent into each well.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Wash as detailed in 4 to 5 above.
- Dispense 100  $\mu$ l of TMB Reagent into each well.
- Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
- Stop the reaction by adding 100  $\mu$ l of Stop Solution to each well.
- Gently mix. *It is important to make sure that all the blue color changes to yellow.*
- Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

### CALCULATION OF RESULTS

- Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
- 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.

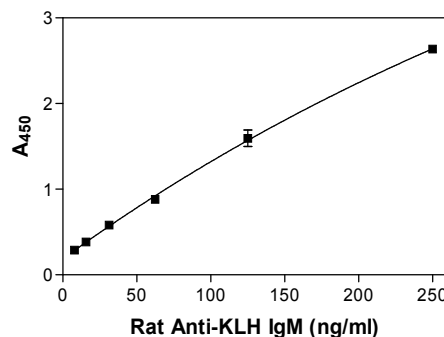
- Using the mean absorbance value for each sample, determine the corresponding concentration of anti-KLH IgM in ng/ml from the standard curve.
- Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgM in the serum/plasma sample.
- PC graphing software may be used for the above steps.
- 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

- 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.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

### REFERENCES

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

# Mouse Anti-KLH IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4010-1

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

#### INTRODUCTION

Measurement of KLH induced anti-KLH antibody levels allows quantitative evaluation of the immune response (ref 1). This ELISA is designed for the rapid and quantitative measurement of mouse anti-KLH IgG levels in serum or plasma. A companion ELISA, catalog number 4000-1, is designed for measurement of mouse anti-KLH IgM levels.

#### PRINCIPLE OF THE TEST

The mouse anti-KLH IgG 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-mouse IgG antibodies for detection. Test serum or plasma samples are diluted and incubated in the microtiter wells for 1 hour. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. Anti-KLH IgG 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 IgG 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
- Diluent (60 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)

<sup>1</sup> Mouse anti-KLH IgG levels are measured in nominal units and are calibrated with reference anti-KLH mouse serum at Life Diagnostics, Inc.

#### STORAGE OF THE TEST KIT

- The reference standard should be stored at -20°C for optimal stability
- All remaining kit components 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 kit 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. This kit is intended for measurement of anti-KLH IgG levels in mouse serum or plasma obtained 14-days after immunization with KLH.
3. All reagents should be allowed to reach room temperature (18-25°C) before use.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., indicate that an initial sample dilution of 20,000 fold works well for most 14-day post-immunization samples. Please do not use dilutions less than 25-fold.
5. 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.

#### STANDARD PREPARATION

1. The mouse anti-KLH IgG standard is provided as a lyophilized stock. Reconstitute with 100 µl of distilled or deionized water (*the reconstituted standard is stable at 4°C for one week but should be aliquoted and frozen at -20°C after reconstitution if future use is intended*).
2. Label 5 polypropylene or glass tubes as 100, 50, 25, 12.5 and 6.25 units/ml (u/ml).
3. Into the tube labeled 100 u/ml, pipette the volume of diluent detailed on the anti-KLH IgG standard vial label. Then add the indicated volume of anti-KLH IgG standard (shown on the anti-KLH IgG standard vial label) and mix gently. This provides the 100 u/ml standard.
4. Dispense 250 µl of diluent into the tubes labeled 50, 25, 12.5, and 6.25 u/ml.
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, and 6.25 u/ml standards by serial dilution.

## SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-KLH IgG is present in serum from KLH immunized mice at concentrations of ~750,000 u/ml. In order to obtain values within range of the standard curve, we suggest that samples initially be diluted 20,000 fold using the following procedure for each sample to be tested:

1. Dispense 248  $\mu$ l and 318  $\mu$ l of diluent into separate tubes.
2. Pipette and mix 2  $\mu$ l of the serum sample into the tube containing 248  $\mu$ l of diluent. This provides a 125 fold diluted sample.
3. Mix 2  $\mu$ l of the 125 fold diluted sample with the 318  $\mu$ l of diluent in the second tube. This provides a 20,000 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  $\mu$ 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 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 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 IgG in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.

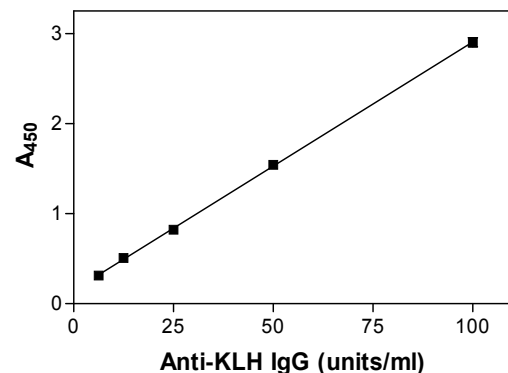
6. If the  $OD_{450}$  values of samples fall outside the standard curve when tested at a dilution of 20,000, samples should be diluted appropriately and re-tested.

## TYPICAL STANDARD CURVE

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

Anti-KLH IgG (u/ml)	Absorbance (450 nm)
100	2.903
50	1.542
25	0.821
12.5	0.509
6.25	0.312

## Representative Mouse Anti-KLH IgG 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. Wada H, Noguchi Y, Marino MW, Dunn AR and Old LJ. T-cell functions in granulocyte/macrophage colony-stimulating factor deficient mice. Proc Natl Acad Sci. 94:12557-61 (1997)

# Rat Anti-SRBC IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4210-2

### ELISA for the Quantitative Determination of Rat Anti-Sheep Red Blood Cell (SRBC) IgG in Serum and Plasma

#### INTRODUCTION

Recent studies have demonstrated that suppression of anti-SRBC immunoglobulin levels by therapeutic agents serves as a useful indicator of immunosuppression<sup>1,2</sup>. This ELISA allows rapid and quantitative measurement of rat anti-SRBC IgG levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The rat anti-SRBC IgG ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses detergent solubilized SRBC ghosts<sup>2</sup> for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat IgG 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-SRBC IgG molecules are thus sandwiched between immobilized SRBC antigens 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-SRBC IgG in a test sample is proportional to the optical density and is derived by reference to a standard curve.

#### MATERIALS AND COMPONENTS

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

- SRBC coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard stock<sup>A</sup> (lyophilized)
- 20x Wash Solution, 50 ml
- Diluent (30 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)

<sup>A</sup> The levels of rat anti-SRBC IgG are measured in nominal units and are calibrated with reference rat anti-SRBC serum at Life Diagnostics, Inc.

#### STORAGE OF TEST KIT

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 AND PRECAUTIONS

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  $\geq 14$  days after immunization with SRBC. Samples obtained prior to 14 days after immunization may contain high levels of anti-SRBC IgM that compete with anti-SRBC IgG for the immobilized SRBC antigens.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 100-fold.
5. Serum or plasma samples must be diluted **at least** 25-fold in diluent.
6. Optimum results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.
7. Unlike other ELISA kits manufactured by Life Diagnostics, Inc., this assay typically has an elevated background signal as evidenced by an OD in the range of 0.4 – 1 OD units for the 6.25 u/ml standard. This does not detract from the performance of the assay.
8. Ant-SRBC IgG levels are undetectable in serum from naïve animals.

#### 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. Reconstitute one vial of the lyophilized rat anti-SRBC IgG standard stock with distilled or deionized water as described on the standard vial label (**the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if additional use is intended**).
2. Label 5 polypropylene or glass tubes as 100, 50, 25, 12.5 and 6.25 u/ml.
3. In the tube labeled 100 u/ml, prepare a 100 u/ml stock by mixing the volume of reconstituted standard stock with the volume of diluent detailed on the reference standard stock vial label.
4. Dispense 250  $\mu$ l of diluent into the tubes labeled 50, 25, 12.5 and 6.25 u/ml.

5. Prepare a 50 u/ml standard by diluting and mixing 250  $\mu$ l of the 100 u/ml standard with 250  $\mu$ l of diluent in the tube labeled 50 u/ml.
6. Similarly prepare the 25, 12.5 and 6.25 u/ml standards by serial dilution.

### SAMPLE PREPARATION

Studies at Life Diagnostics, Inc., indicate that anti-SRBC IgG is present in rat serum or plasma from SRBC immunized animals at concentrations of ~2000 u/ml (14 days after immunization). In order to obtain values within the range of the standard curve, we suggest that samples initially be diluted 100 fold using the following procedure for each sample to be tested:

1. For each test sample dispense 247.5  $\mu$ l of diluent into separate tubes.
2. Pipette and mix 2.5  $\mu$ l of the serum/plasma sample into a tube containing 247.5  $\mu$ l of diluent. This provides a 100 fold diluted sample.
3. Repeat this procedure for each sample to be tested

**Important: Do not use dilutions lower than 25 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 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-SRBC IgG in u/ml from the standard curve.

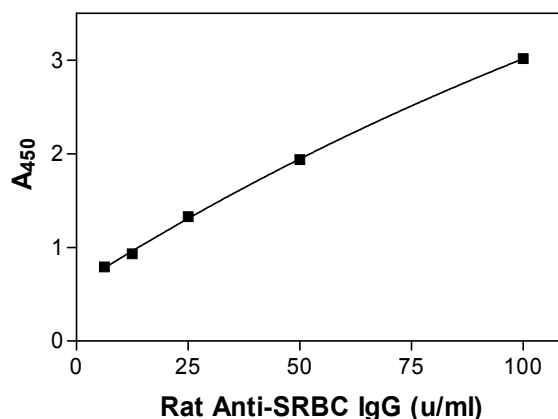
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-SRBC IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the  $OD_{450}$  values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

### TYPICAL STANDARD CURVE

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

Anti-SRBC IgG (u/ml)	Absorbance (450 nm)
100	3.016
50	1.939
25	1.330
12.5	0.933
6.25	0.793

### Representative Rat Anti-SRBC IgG 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. GS Ladics. Use of SRBC antibody responses for immunotoxicity testing. *Methods* 41:9-19 (2007)
2. L. Temple, T. T. Kawabata, A. E. Munson and K. L. White. Comparison of ELISA and Plaque-Forming Cell Assays for Measuring the Humoral Immune Response to SRBC in Rats and Mice Treated with Benzo[a]pyrene or Cyclophosphamide *Fundamental and Applied Toxicology* 21(4):412-419 (1993)

# Monkey Anti-Tetanus Toxoid IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4300-4-N

### ELISA for the Quantitative Determination of Monkey Anti-Tetanus Toxoid IgM

#### INTRODUCTION

Drug candidates are routinely screened for evidence of immune system regulation during the discovery process. It is important that the immune response is not enhanced or decreased since such effects may lead to hypersensitivity or increased susceptibility to infection. Determination of a drug candidate's effects on anti-tetanus toxoid antibody levels allows easy assessment of immune system regulation. Animals are immunized with tetanus toxoid while undergoing drug treatment and serum is collected at appropriate times post immunization. Serum collected 5-7 days after immunization is used for measurement of anti-KLH IgM levels, and serum collected 14+ days post immunization is used to measure anti-KLH IgG levels. Comparison of anti-KLH IgG or IgM levels in drug treated, versus control groups reveals effects on the immune response.

This ELISA allows rapid and quantitative measurement of anti-tetanus toxoid IgM levels in Rhesus or Cynomolgus monkey serum or plasma.

#### PRINCIPLE OF THE TEST

The monkey anti-tetanus toxoid IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses tetanus toxoid for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-monkey IgM antibodies for detection. Standards and diluted serum or plasma samples are incubated in the microtiter wells for 60 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. Anti-tetanus toxoid IgM molecules are thus sandwiched between immobilized tetanus toxoid 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-tetanus toxoid IgM is proportional to the optical density. Anti-tetanus toxoid IgM levels in the samples are derived by reference to a standard curve.

#### MATERIALS AND COMPONENTS

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

- Tetanus toxoid coated 96-well plate (12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Standard stock (lyophilized)<sup>1</sup>, **Store ≤ -20°C**
- 20x Wash Solution, 50 ml

<sup>1</sup> The standard was calibrated with affinity purified anti-tetanus toxoid IgM prepared at Life Diagnostics, Inc.

- Diluent, 30 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

The reference standard stock should be stored at or below -20°C. All other kit components 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 shipment provided that the components are stored as described.

#### 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 monkeys 5 days after immunization with tetanus toxoid, at which point the immune response originates predominantly from IgM.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc. indicate that an initial sample dilution of 1000 fold is a good starting point. **It is recommended that samples not be tested at dilutions below 400 fold.**
5. 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.

#### STANDARD PREPARATION

1. The monkey anti-tetanus toxoid IgM reference standard is provided as a lyophilized stock. Reconstitute with 200 µL of distilled or deionized water (**the reconstituted standard is stable for one day at 4°C, but should be aliquoted and frozen at -20°C if future use is intended**).

- Label 5 polypropylene or glass tubes as 1000, 500, 250, 125, and 62.5 ng/ml.
- Into the tube labeled 1000 ng/ml, pipette the volume of diluent detailed on the reference standard vial label. Then add the indicated volume of the reference standard and mix gently. This provides the 1000 ng/ml standard.
- Dispense 250  $\mu$ l of diluent into the tubes labeled 500, 250, 125, and 62.5 ng/ml.
- Prepare the 500 ng/ml standard by diluting and mixing 250  $\mu$ l of the 1000 ng/ml standard with 250  $\mu$ l of diluent in the tube labeled 500 ng/ml.
- Similarly prepare the 250, 125, and 62.5 ng/ml standards by serial dilution.

### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-tetanus toxoid IgM is present in monkey serum at concentrations of 75 ug/ml or greater<sup>2</sup>. We suggest that samples be diluted 1000-fold using the following procedure for each sample to be tested. Dilutions lower than 400-fold should not be tested.

- Dilute 5  $\mu$ l of serum with 95  $\mu$ l of diluent to give a 20-fold dilution.
- Dilute 10  $\mu$ l of the 20-fold diluted sample with 490  $\mu$ l of diluent to give a 1000-fold dilution.

### ASSAY PROCEDURE

- Secure the desired number of coated wells in the holder (we suggest that standards and samples be tested in duplicate).
- Dispense 100  $\mu$ l of standards and diluted samples into appropriate wells.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 60 minutes.
- 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.
- Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
- Add 100  $\mu$ l of enzyme conjugate reagent into each well.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Wash as detailed in 4 to 5 above.
- Dispense 100  $\mu$ l of TMB Reagent into each well.
- Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
- Stop the reaction by adding 100  $\mu$ l of Stop Solution to each well.
- Gently mix. *It is important to make sure that all the blue color changes to yellow.*
- Read the optical density at 450 nm with a microtiter plate reader *within 5 minutes*.

### CALCULATION OF RESULTS

<sup>2</sup> Please note that the levels of anti-tetanus toxoid IgM in a particular study can vary significantly depending on the source of tetanus toxoid used for immunization. Optimal sample dilutions should therefore be determined empirically.

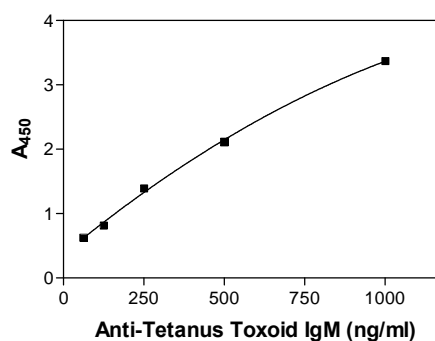
- Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
- Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of anti-tetanus toxoid IgM in ng/ml from the standard curve.
- Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-tetanus toxoid IgM in the serum/plasma sample.
- PC graphing software may be used for the above steps.
- If the  $OD_{450}$  values of 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-tetanus toxoid 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-tetanus toxoid IgM (ng/ml)	Absorbance (450 nm)
1000	3.3695
500	2.1145
250	1.3945
125	0.82
62.5	0.626

**Typical Monkey Anti-Tetanus Toxoid IgM Standard Curve**



### LIMITATIONS OF THE PROCEDURE

- 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.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

# Rat Anti-Tetanus Toxoid IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4310-2

### ELISA for the Quantitative Determination of Rat Anti-Tetanus Toxoid IgG

#### INTRODUCTION

Evaluation of the levels of anti-tetanus toxoid IgG following immunization with tetanus toxoid provides a useful indicator of aspects of the immune response. The rat anti-tetanus toxoid IgG ELISA developed by Life Diagnostics, Inc., facilitates rapid and quantitative measurement of rat anti-tetanus toxoid IgG levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The rat anti-tetanus toxoid IgG ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses tetanus toxoid for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat IgG 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-tetanus toxoid IgG molecules are thus sandwiched between immobilized tetanus toxoid 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-tetanus toxoid IgG is proportional to the optical density of the test sample.

#### MATERIALS AND COMPONENTS

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

- Tetanus toxoid coated 96-well plate (12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard<sup>1</sup> (lyophilized), 2 vials **Store ≤ -20°C**
- 20x Wash Solution, 50 ml
- Diluent (30 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)

<sup>1</sup> The levels of rat anti-tetanus toxoid IgG are measured in nominal units and are calibrated with reference anti-tetanus toxoid rat serum at Life Diagnostics, Inc.

#### STORAGE OF TEST KIT

The reference standard should be stored at or below -20°C. All other kit components 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.

#### 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 14 days or more after immunization with tetanus toxoid, at which point the immune response originates predominantly from IgG.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., using serum obtained from rats immunized intraperitoneally with tetanus toxoid, indicate that an initial sample dilution of 2000 fold is a good starting point. **Do not test samples at dilutions below 200 fold.**
5. 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.

#### STANDARD PREPARATION

1. The rat anti-tetanus toxoid IgG standard is provided as a lyophilized stock. Reconstitute with the volume of diluent indicated on the vial label to give a 100 unit/ml (u/ml) solution of rat anti-tetanus toxoid IgG (**the reconstituted standard should be aliquoted and frozen at or below -20°C after reconstitution if additional use is intended**).
2. Label 5 polypropylene or glass tubes as 50, 25, 12.5, 6.25 and 3.125 u/ml and pipette 250 µl of diluent into each tube.
3. Into the tube labeled 50 u/ml, pipette and mix 250 µl of the reconstituted 100 u/ml anti-tetanus toxoid IgG. This provides the 50 u/ml standard.
4. Similarly prepare the 25, 12.5, 6.25 and 3.125 u/ml standards by serial dilution.

#### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-tetanus toxoid IgG is present in rat serum at concentrations of 50,000 u/ml or greater. We suggest that samples be diluted 2000 fold using the following procedure for

each sample to be tested. Optimum dilutions may need to be determined empirically.

1. Dispense 98  $\mu\text{l}$  and 243.75  $\mu\text{l}$  of diluent into separate polypropylene or glass tubes.
2. Pipette and mix 2  $\mu\text{l}$  of the serum/plasma sample into the tube containing 98  $\mu\text{l}$  of diluent. This provides a 50 fold diluted sample.
3. Dilute 6.25  $\mu\text{l}$  of the 50 fold diluted sample into the tube containing 243.75  $\mu\text{l}$  of diluent and mix. This provides a 2000 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  $\mu\text{l}$  of standards (100 – 3.125 u/ml) and diluted samples into the wells (standards and samples should 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  $\mu\text{l}$ /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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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

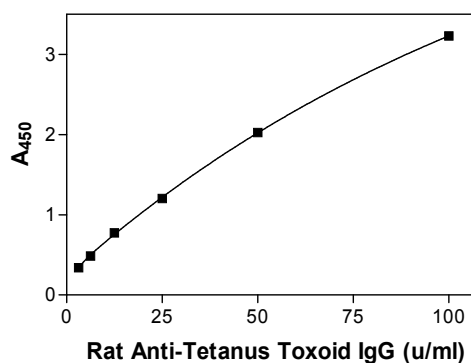
1. Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in u/ml on linear graph paper, with absorbance values on the vertical 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-tetanus toxoid IgG in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-tetanus toxoid 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 when tested at a dilution of 2000, 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-tetanus toxoid 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. Each user should obtain his or her data and standard curve in each experiment.

Anti-tetanus toxoid IgG (u/ml)	Absorbance (450 nm)
100	3.229
50	2.026
25	1.204
12.5	0.776
6.25	0.484
3.125	0.341

**Representative Rat Anti-Tetanus Toxoid IgG 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.

# Rat Anti-Tetanus Toxoid IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4300-2

### ELISA for the Quantitative Determination of Rat Anti-Tetanus Toxoid IgM

#### INTRODUCTION

Evaluation of the levels of anti-tetanus toxoid IgM after immunization with tetanus toxoid provides a useful indicator of aspects of the immune response. The rat anti-tetanus toxoid IgM ELISA developed by Life diagnostics, Inc., facilitates rapid and quantitative measurement of rat anti-tetanus toxoid IgM levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The rat anti-tetanus toxoid IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses tetanus toxoid for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat IgM antibodies for detection. Standards and diluted serum or plasma samples are 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-tetanus toxoid IgM molecules are thus sandwiched between immobilized tetanus toxoid 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-tetanus toxoid IgM is proportional to the optical density. Anti-tetanus toxoid IgM levels in the samples are derived by reference to a standard curve.

#### MATERIALS AND COMPONENTS

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

- Tetanus toxoid coated 96-well plate (12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Standard stock<sup>1</sup> (lyophilized), 3 vials **Store  $\leq$  -20°C**
- 20x Wash Solution, 50 ml
- Diluent (30 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)

<sup>1</sup> The levels of rat anti-tetanus toxoid IgM are measured in nominal units and are calibrated with reference anti-tetanus toxoid rat serum at Life Diagnostics, Inc.

#### STORAGE OF TEST KIT

The reference standard stocks should be stored at or below -20°C. All other kit components 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.

#### 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 5 days after immunization with tetanus toxoid, at which point the immune response originates predominantly from IgM.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., using serum obtained from rats immunized intraperitoneally with tetanus toxoid, indicate that an initial sample dilution of 50 fold is a good starting point. **It is recommended that samples not be tested at dilutions below 20 fold.**
5. 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.

#### STANDARD PREPARATION

1. The rat anti-tetanus toxoid IgM standard is provided as a lyophilized stock. Reconstitute one vial with distilled or deionized water as described on the vial label and mix gently until dissolved (**the reconstituted standard is stable for at least 1 day if stored at 4°C**).
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, pipette the volume of diluent indicated on the stock vial label. Then add the volume of reconstituted standard stock detailed on the vial label and mix. This provides the working 100 u/ml standard.
4. Pipette 250  $\mu$ l of diluent into the tubes labeled 50, 25, 12.5, 6.25 and 3.125 u/ml.
5. Into the tube labeled 50 u/ml, pipette and mix 250  $\mu$ l of the reconstituted 100 u/ml anti-tetanus toxoid IgM standard. This provides the 50 u/ml standard.
6. Similarly prepare the 25, 12.5, 6.25 and 3.125 u/ml standards by serial dilution.

## SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-tetanus toxoid IgM is present in rat serum at concentrations of ~2500 u/ml 5-days after i.p. immunization with tetanus toxoid<sup>2</sup>. We suggest that samples be diluted 50 fold using the following procedure for each sample to be tested.

1. Dispense 343  $\mu$ l of diluent into polypropylene or glass tubes.
2. Pipette and mix 7  $\mu$ l of the serum/plasma sample into the tube containing 343  $\mu$ l of diluent. This provides a 50 fold diluted sample.
3. Repeat this procedure for each sample to be tested

## ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder (standards should be tested in duplicate and we recommend that samples be tested in triplicate).
2. Dispense 100  $\mu$ l of standards (100 – 6.25 u/ml) and diluted samples into appropriate wells.
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 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-tetanus toxoid IgM in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-tetanus toxoid IgM in the serum/plasma sample.

<sup>2</sup> Please note that the levels of anti-tetanus toxoid IgM in a particular study can vary significantly depending on the source of tetanus toxoid used for immunization. Optimal sample dilutions should therefore be determined empirically.

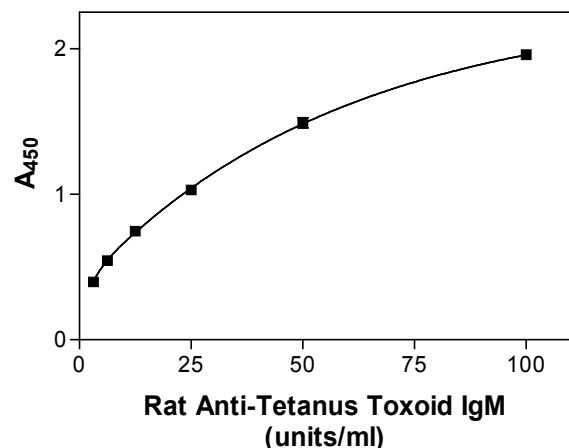
5. PC graphing software may be used for the above steps.
6. If the  $OD_{450}$  values of fall outside the standard curve samples should be diluted appropriately and re-tested.

## TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against anti-tetanus toxoid 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-tetanus toxoid IgM (u/ml)	Absorbance (450 nm)
100	1.956
50	1.491
25	1.029
12.5	0.745
6.25	0.543
3.125	0.396

## Representative Rat Anti-Tetanus Toxoid 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.

# Chicken Anti-SRBC IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4210-5

### ELISA for the Quantitative Determination of Chicken Anti-Sheep Red Blood Cell (SRBC) IgG in Serum and Plasma

#### INTRODUCTION

Measurement of anti-SRBC immunoglobulin levels is used to assess immune function in chickens. To date, a somewhat qualitative hemagglutination assay has often been used to measure antibody titers. Disadvantageously, the hemagglutination assay does not readily differentiate between IgG (IgY) and IgM responses. To address these issues, we at Life Diagnostics, Inc. have developed an enzyme linked immunosorbant assay (ELISA) that allows rapid and quantitative measurement of chicken anti-SRBC IgG levels in serum or plasma. We also manufacture a companion ELISA (catalog number 4200-5) that allows quantitative measurement of chicken anti-SRBC IgM levels.

Studies at Life Diagnostics, Inc., have demonstrated that i.v. immunization of chickens with 0.5 ml of a 10% solution of SRBC caused an elevation of anti-SRBC IgG levels from undetectable levels on day zero to ~11,000 units/ml on day seven.

#### PRINCIPLE OF THE TEST

The chicken anti-SRBC IgG ELISA uses detergent solubilized SRBC ghosts for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-chicken IgG 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-SRBC IgG molecules are thus sandwiched between immobilized SRBC antigens 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-SRBC IgG is proportional to the optical density of the test sample and is derived from a standard curve.

#### MATERIALS AND COMPONENTS

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

- SRBC coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard stock<sup>A</sup> (lyophilized)
- 20x Wash Solution, 50 ml
- Diluent (50 ml)

<sup>A</sup> Chicken anti-SRBC IgG levels are measured in nominal units and are calibrated with reference chicken anti-SRBC serum prepared at Life Diagnostics, Inc.

- 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

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 chickens 5-7 days after immunization with SRBC.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 500 fold.
5. 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.

#### STANDARD PREPARATION

1. Reconstitute the vial of the lyophilized chicken anti-SRBC IgG standard stock with 200 µl of diluent and then prepare the 100 u/ml standard in a polypropylene or glass tube as described on the vial label.
2. Label 4 polypropylene or glass tubes as 50, 25, 12.5, and 6.25 u/ml and dispense 250 µl of diluent into each tube.
3. 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.
4. Similarly prepare the 25, 12.5 and 6.25 u/ml standards by serial dilution.

**After reconstitution the standard stock solution is stable for at least one week if stored at 4°C. It should be frozen at or below -20°C if use beyond this time is intended. Avoid multiple freeze-thaws.**

### SAMPLE PREPARATION

**General Note: Studies at Life Diagnostics, Inc., revealed anti-SRBC IgG levels of approximately 10,000 u/ml 7 days after immunization with SRBC. In order to obtain values within the range of the standard curve, we suggest that samples initially be diluted 500 fold using the following procedure for each sample to be tested:**

1. For each test sample dispense 90 µl and 200 µl of diluent into separate tubes.
2. Pipette and mix 10 µl of the serum/plasma sample into the tube containing 90 µl of diluent. This provides a 10 fold diluted sample.
3. Mix 5 µl of the 10-fold diluted sample with 245 µl of diluent in the second tube to give a 500-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 standards and diluted samples into the wells (we recommend that samples and standards 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 µl/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 µl 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 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 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.

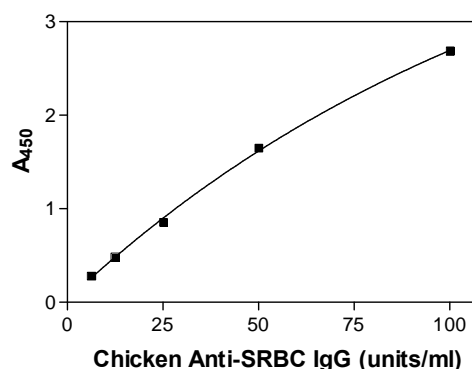
3. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-SRBC IgG in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-SRBC IgG in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the  $OD_{450}$  values of samples fall outside the standard curve when tested at a dilution of 500, 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-SRBC 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. Each user should obtain his or her data and standard curve in each experiment.

Anti-SRBC IgG (u/ml)	Absorbance (450 nm)
100	2.686
50	1.649
25	0.855
12.5	0.481
6.25	0.279

### Representative Chicken Anti-SRBC IgG 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.

# Chicken Anti-SRBC IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4200-5

### ELISA for the Quantitative Determination of Chicken Anti-Sheep Red Blood Cell (SRBC) IgM in Serum and Plasma

#### INTRODUCTION

Measurement of anti-SRBC immunoglobulin levels is used to assess immune function in chickens. To date, a somewhat qualitative hemagglutination assay has often been used to measure antibody titers. Disadvantageously, the hemagglutination assay does not readily differentiate between IgM and IgG (IgY) responses. To address these issues, we at Life Diagnostics, Inc. have developed an enzyme linked immunosorbent assay (ELISA) that allows rapid and quantitative measurement of chicken anti-SRBC IgM levels in serum or plasma. We also manufacture a companion ELISA (catalog number 4210-5) that allows quantitative measurement of chicken anti-SRBC IgG levels.

Studies at Life Diagnostics, Inc., have demonstrated that i.v. immunization of chickens with 0.5 ml of a 10% solution of SRBC caused an elevation of anti-SRBC IgM levels from ~200 units/ml on day zero to ~11,000 units/ml on day seven.

#### PRINCIPLE OF THE TEST

The chicken anti-SRBC IgM ELISA uses detergent solubilized SRBC ghosts for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-chicken 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-SRBC IgM molecules are thus sandwiched between immobilized SRBC antigens 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-SRBC IgM is proportional to the optical density of the test sample and is derived from a standard curve.

#### MATERIALS AND COMPONENTS

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

- SRBC coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard stock<sup>A</sup> (lyophilized)
- 20x Wash Solution, 50 ml
- Diluent (50 ml)

<sup>A</sup> Chicken anti-SRBC IgM levels are measured in nominal units and are calibrated with reference chicken anti-SRBC serum prepared at Life Diagnostics, Inc.

- 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

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 chickens 5-7 days after immunization with SRBC.
4. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 500 fold.
5. 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.

#### STANDARD PREPARATION

1. Reconstitute the vial of the lyophilized chicken anti-SRBC IgM standard stock with 200 µl of diluent and then prepare the 100 u/ml standard in a polypropylene or glass tube as described on the vial label.
2. Label 6 polypropylene or glass tubes as 50, 25, 12.5, 6.25, 3.13, and 1.56 u/ml and dispense 250 µl of diluent into each tube.
3. 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.
4. Similarly prepare the 25, 12.5, 6.25, 3.13 and 1.56 u/ml standards by serial dilution.

**After reconstitution the standard stock solution is stable for at least one week if stored at 4°C. It should be frozen at or below -20°C if use beyond this time is intended. Avoid multiple freeze-thaws.**

### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., revealed anti-SRBC IgM levels of approximately 10,000 u/ml 7 days after immunization with SRBC. In order to obtain values within the range of the standard curve, we **suggest** that samples initially be diluted 500 fold using the following procedure for each sample to be tested:

1. For each test sample dispense 90 µl and 200 µl of diluent into separate tubes.
2. Pipette and mix 10 µl of the serum/plasma sample into the tube containing 90 µl of diluent. This provides a 10 fold diluted sample.
3. Mix 5 µl of the 10-fold diluted sample with 245 µl of diluent in the second tube to give a 500-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 standards and diluted samples into the wells (we recommend that samples and standards 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 µl/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 µl 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 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 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.

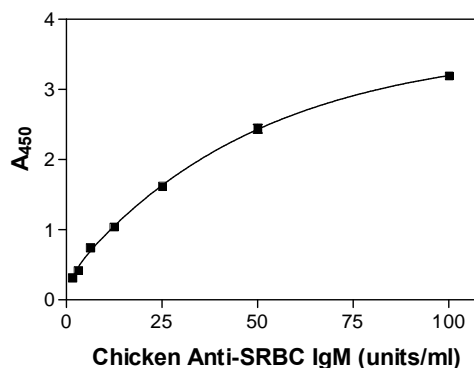
3. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-SRBC IgM in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-SRBC IgM in the serum/plasma sample.
5. PC graphing 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 500, 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-SRBC 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-SRBC IgM (u/ml)	Absorbance (450 nm)
100	3.196
50	2.440
25	1.620
12.5	1.042
6.25	0.744
3.13	0.421
1.56	0.316

**Representative Chicken Anti-SRBC 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.

# Mouse Anti-SRBC IgG ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4210-1

### ELISA for the Quantitative Determination of Mouse Anti-Sheep Red Blood Cell (SRBC) IgG in Serum and Plasma

#### INTRODUCTION

Recent studies have demonstrated that suppression of anti-SRBC IgG levels by therapeutic agents serves as a useful indicator of immunosuppression<sup>1,2</sup>. This ELISA allows rapid and quantitative measurement of mouse anti-SRBC IgG levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The mouse anti-SRBC IgG ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses detergent solubilized SRBC ghosts<sup>2</sup> for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-mouse IgG 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-SRBC IgG molecules are thus sandwiched between immobilized SRBC antigens 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-SRBC IgG is proportional to the optical density of the test sample.

#### MATERIALS AND COMPONENTS

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

- SRBC coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard stock<sup>A</sup> (lyophilized)
- 20x Wash Solution, 50 ml
- Diluent (30 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

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 mice fourteen days after immunization with SRBC, at which point the immune response originates almost exclusively from IgG.
4. Serum or plasma samples must be diluted **at least** 15-fold in diluent.
5. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 50-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.

#### STANDARD PREPARATION

1. Reconstitute one vial of the lyophilized mouse anti-SRBC IgG standard stock with diluent as described on the standard vial label (***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 100, 50, 25, 12.5, 6.25, and 3.125 u/ml.
3. In the tube labeled 100 u/ml, prepare a 100 u/ml stock by mixing the volume of reconstituted standard stock with the volume of diluent detailed on the reference standard stock vial label.
4. Dispense 250 µl of diluent into the tubes labeled 50, 25, 12.5, 6.25, and 3.125 u/ml.
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.

<sup>A</sup> The levels of mouse anti-SRBC IgG are measured in nominal units and are calibrated with reference mouse anti-SRBC serum at Life Diagnostics, Inc.

## SAMPLE PREPARATION

General Note: Studies at Life Diagnostics, Inc., indicate that anti-SRBC IgG is present in mouse serum or plasma from SRBC immunized animals at concentrations in excess of 500 u/ml. In order to obtain values within the range of the standard curve, we suggest that samples initially be diluted 50 fold using the following procedure for each sample to be tested:

1. For each test sample dispense 294  $\mu$ l of diluent into separate tubes.
  2. Pipette and mix 6  $\mu$ l of the serum/plasma sample into a tube containing 294  $\mu$ l of diluent. This provides a 50 fold diluted sample.
  3. Repeat this procedure for each sample to be tested
- Important: Do not use dilutions lower than 15 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 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-SRBC IgG in u/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-SRBC 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 when tested at a dilution of 50, samples should be diluted

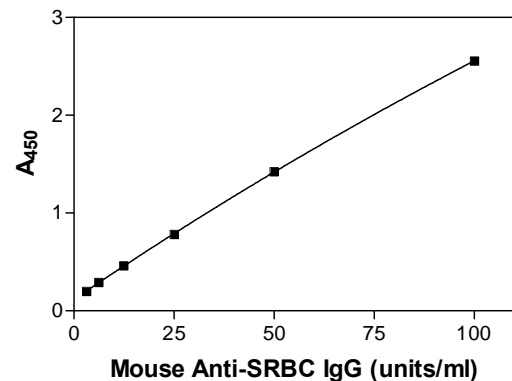
appropriately and re-tested (do not use dilutions lower than 15 fold).

## TYPICAL STANDARD CURVE

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

Anti-SRBC IgG (u/ml)	Absorbance (450 nm)
100	2.556
50	1.423
25	0.782
12.5	0.461
6.25	0.292
3.125	0.201

## Typical Mouse Anti-SRBC IgG 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. GS Ladics. Use of SRBC antibody responses for immunotoxicity testing. *Methods* 41:9-19 (2007)
2. L. Temple, T. T. Kawabata, A. E. Munson and K. L. White. Comparison of ELISA and Plaque-Forming Cell Assays for Measuring the Humoral Immune Response to SRBC in Rats and Mice Treated with Benzo[a]pyrene or Cyclophosphamide *Fundamental and Applied Toxicology* 21(4):412-419 (1993)

# Mouse Anti-SRBC IgM ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4200-1

### ELISA for the Quantitative Determination of Mouse Anti-Sheep Red Blood Cell (SRBC) IgM in Serum and Plasma

#### INTRODUCTION

Recent studies have demonstrated that suppression of anti-SRBC IgM levels by therapeutic agents serves as a useful indicator of immunosuppression<sup>1,2</sup>. This ELISA allows rapid and quantitative measurement of mouse anti-SRBC IgM levels in serum or plasma samples.

#### PRINCIPLE OF THE TEST

The mouse anti-SRBC IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses detergent solubilized SRBC ghosts<sup>2</sup> for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-mouse 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-SRBC IgM molecules are thus sandwiched between immobilized SRBC antigens 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-SRBC IgM is proportional to the optical density of the test sample.

#### MATERIALS AND COMPONENTS

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

- SRBC coated 96-well plate (provided as 12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard stock<sup>A</sup> (lyophilized), 2 vials
- 20x Wash Solution, 50 ml
- Diluent (30 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)

<sup>A</sup> The levels of mouse anti-SRBC IgM are measured in nominal units and are calibrated with reference mouse anti-SRBC serum at Life Diagnostics, Inc.

#### STORAGE OF TEST KIT

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 mice five days after immunization with SRBC, at which point the immune response originates almost exclusively from IgM.
4. Serum or plasma samples must be diluted **at least** 15-fold in diluent.
5. The optimal sample dilution should be determined empirically. However, studies performed at Life Diagnostics, Inc., suggest an initial sample dilution of 50 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.

#### STANDARD PREPARATION

1. Reconstitute one vial of the lyophilized mouse anti-SRBC IgM standard stock with diluent as described on the standard vial label to give the 100 u/ml standard.
2. Label 5 polypropylene or glass tubes as 50, 25, 12.5, 6.25, and 3.125 u/ml and dispense 250 µl of diluent into each tube.
3. 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.
4. Similarly prepare the 25, 12.5, 6.25, and 3.125 u/ml standards by serial dilution.

#### SAMPLE PREPARATION

**General Note:** Studies at Life Diagnostics, Inc., indicate that anti-SRBC IgM is present in mouse serum or plasma from SRBC immunized animals at concentrations in excess of 2000 u/ml. In order to obtain values within the range of the standard curve, we **suggest** that samples initially be diluted 50 fold using the following procedure for each sample to be tested:

1. For each test sample dispense 294 µl of diluent into separate tubes.
2. Pipette and mix 6 µl of the serum/plasma sample into a tube containing 294 µl of diluent. This provides a 50 fold diluted sample.

- Repeat this procedure for each sample to be tested  
**Important: Do not use dilutions lower than 15 fold.**

### ASSAY PROCEDURE

- Secure the desired number of coated wells in the holder.
- Dispense 100  $\mu$ l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- 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.
- Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
- Add 100  $\mu$ l of enzyme conjugate reagent into each well.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Wash as detailed in 4 to 5 above.
- Dispense 100  $\mu$ l of TMB Reagent into each well.
- Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
- Stop the reaction by adding 100  $\mu$ l of Stop Solution to each well.
- Gently mix. *It is important to make sure that all the blue color changes to yellow.*
- Read the optical density at 450 nm with a microtiter plate reader *within 5 minutes*.

### CALCULATION OF RESULTS

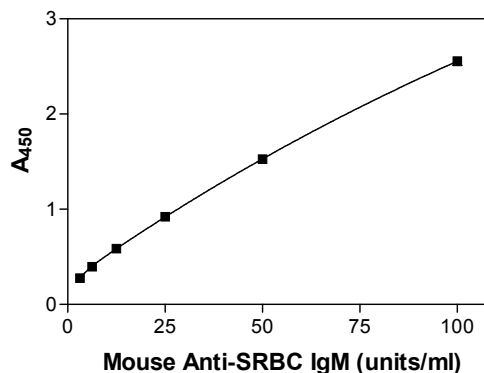
- Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
- Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in u/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of anti-SRBC IgM in u/ml from the standard curve.
- Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-SRBC IgM in the serum/plasma sample.
- PC graphing software may be used for the above steps.
- If the  $OD_{450}$  values of samples fall outside the standard curve when tested at a dilution of 50, samples should be diluted appropriately and re-tested (do not use dilutions lower than 15 fold).

### TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against anti-SRBC 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-SRBC IgM (u/ml)	Absorbance (450 nm)
100	2.555
50	1.526
25	0.922
12.5	0.586
6.25	0.396
3.125	0.277

### Representative Mouse Anti-SRBC IgM Standard Curve



### LIMITATIONS OF THE PROCEDURE

- 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.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

### REFERENCES

- GS Ladics. Use of SRBC antibody responses for immunotoxicity testing. *Methods* 41:9-19 (2007)
- L. Temple, T. T. Kawabata, A. E. Munson and K. L. White. Comparison of ELISA and Plaque-Forming Cell Assays for Measuring the Humoral Immune Response to SRBC in Rats and Mice Treated with Benzo[a]pyrene or Cyclophosphamide *Fundamental and Applied Toxicology* 21(4):412-419 (1993)

# Monkey Anti-Tetanus Toxoid IgG1 ELISA Kit

## Life Diagnostics, Inc., Catalog Number: 4310-4-1

### ELISA for the Quantitative Determination of Monkey Anti-Tetanus Toxoid IgG1

#### INTRODUCTION

Drug candidates are routinely screened for evidence of immune system regulation during the discovery process. It is important that the immune response is not enhanced or decreased since such effects may lead to hypersensitivity or increased susceptibility to infection. Determination of a drug candidate's effects on anti-tetanus toxoid antibody levels allows easy assessment of immune system regulation. Animals are immunized with tetanus toxoid while undergoing drug treatment and serum is collected at appropriate times post immunization. Serum collected 5-7 days after immunization is used for measurement of anti-KLH IgM levels, and serum collected 14+ days post immunization is used to measure anti-KLH IgG levels. Comparison of anti-KLH IgG or IgM levels in drug treated, versus control groups reveals effects on the immune response.

This ELISA allows rapid and quantitative measurement of anti-tetanus toxoid IgG1 levels in serum or plasma. IgG1 is the major IgG subclass in monkeys<sup>1,2</sup>.

#### PRINCIPLE OF THE TEST

The monkey anti-tetanus toxoid IgG ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses tetanus toxoid for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated mouse monoclonal anti-monkey IgG1 antibody for detection. Serum or plasma samples are diluted and incubated in the microtiter wells for 60 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. Anti-tetanus toxoid IgG1 molecules are thus sandwiched between immobilized tetanus toxoid 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-tetanus toxoid IgG1 is proportional to the optical density of the test sample.

#### MATERIALS AND COMPONENTS

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

- Tetanus toxoid coated 96-well plate (12 strips of 8 wells)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard<sup>1</sup> (lyophilized), 2 vials. **Store ≤ -20°C**
- 20x Wash Solution, 50 ml
- Diluent (50 ml)

<sup>1</sup>The standard was calibrated with affinity purified anti-tetanus toxoid IgG1 prepared at Life Diagnostics, Inc.

- 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

The reference standard should be stored at or below -20°C. All other kit components 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.

#### 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 monkeys 14 days or more after immunization with tetanus toxoid, at which point the immune response originates predominantly from IgG.
4. The optimal sample dilution should be determined empirically. **Do not test samples at dilutions below 50 fold.**
5. 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.

#### STANDARD PREPARATION

1. The monkey anti-tetanus toxoid IgG1 reference standard is provided as a lyophilized stock. Reconstitute with the volume of diluent indicated on the vial label.
2. Prepare a 2.5 µg/ml working standard of monkey anti-tetanus toxoid IgG1 as further directed on the reference standard vial label (**the reconstituted standard should be frozen at or below -20°C after reconstitution if additional use is intended**).
3. Label 5 polypropylene or glass tubes as 1.25, 0.625, 0.313 and 0.156 and 0.078 µg/ml and pipette 250 µl of diluent into each tube.

4. Into the tube labeled 1.25  $\mu\text{g/ml}$ , pipette and mix 250  $\mu\text{l}$  of the 2.5  $\mu\text{g/ml}$  anti-tetanus toxoid IgG with 250  $\mu\text{l}$  of diluent. This provides the 1.25  $\mu\text{g/ml}$  standard.
5. Similarly prepare the 0.625, 0.313 and 0.156 and 0.078  $\mu\text{g/ml}$  standards by serial dilution.

### SAMPLE PREPARATION

**General Note:** The level of anti-tetanus toxoid IgG1 will depend on dose, route of immunization and time of sample collection. We found that anti-tetanus toxoid IgG1 is present in monkey serum at concentrations of 50  $\mu\text{g/ml}$  or greater. We suggest that samples initially be diluted 200 fold using the following procedure for each sample to be tested. Optimum dilutions must be determined empirically. Dilutions of 50 fold or lower should not be used.

1. Dispense 298.5  $\mu\text{l}$  of diluent into separate polypropylene or glass tubes.
2. Pipette and mix 1.5  $\mu\text{l}$  of the serum/plasma sample into the tube containing 298.5  $\mu\text{l}$  of diluent. This provides a 200 fold diluted sample.
3. 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  $\mu\text{l}$  of standards (2.5 – 0.078  $\mu\text{g/ml}$ ) and diluted samples into the wells (standards and samples should be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 60 minutes.
4. Aspirate the contents of the microtiter wells and wash the wells 5-6 times with 1x wash solution using a plate washer (400  $\mu\text{l/well}$ ). 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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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

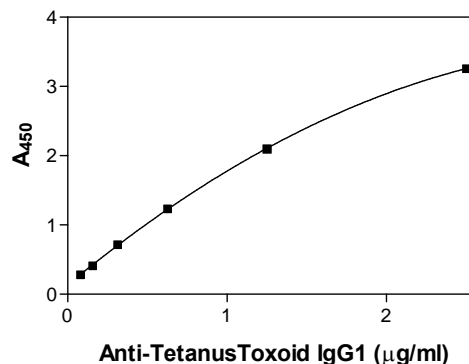
1. Calculate the average absorbance values ( $A_{450}$ ) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in  $\mu\text{g/ml}$  on linear graph paper, with absorbance values on 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-tetanus toxoid IgG1 in  $\mu\text{g/ml}$  from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-tetanus toxoid IgG1 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 200, samples should be diluted appropriately and re-tested.

### TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450 nm on the Y axis against anti-tetanus toxoid IgG1 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-tetanus toxoid IgG ( $\mu\text{g/ml}$ )	Absorbance (450 nm)
2.500	3.259
1.250	2.099
0.625	1.232
0.313	0.714
0.156	0.410
0.078	0.282



### REFERENCES

1. ED Williamson et.al. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. *Infection and Immunity* 73:5978-5987 (2005)
2. P Procell-Wilkins et.al. Isotypic analysis of humoral immune responses in rhesus monkeys to an adult microsomal antigen of *Schistosoma Mansoni*: an indicator of successful treatment. *Am. J. Trop. Med. Hyg.* 45:629-635 (1991)