

Rat Anti-SRBC IgM ELISA Kit

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^{1,2}. 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 ghosts² 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^A (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)

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.

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 μ l of diluent into separate tubes.
 2. Pipette and mix 1.5 μ l of the serum/plasma sample into a tube containing 298.5 μ l 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 μ 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 μ 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 μ 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 μ 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 μ 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

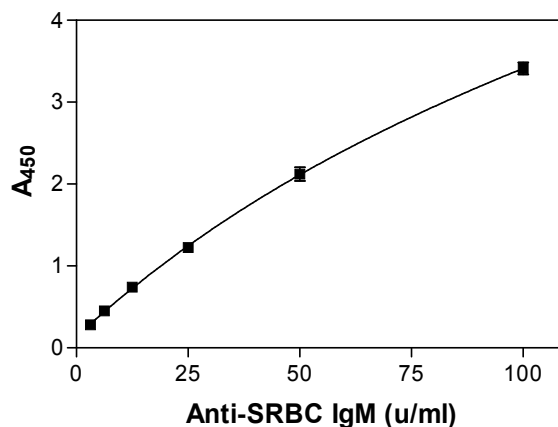
1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in u/ml on linear graph paper, with absorbance values on the vertical or 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 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)