

Rat Anti-KLH IgG ELISA

Life Diagnostics, Inc., Catalog Number: KLHG-2

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INTRODUCTION

Recent studies have demonstrated that suppression of anti-KLH (keyhole limpet hemocyanin) IgG levels by therapeutic agents provides a useful indicator of immunosuppression.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 ASSAY

The rat anti-KLH IgG test kit 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. 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* (lyophilized) **Store \leq -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD30-1, 30 ml
- TMB Reagent (One-Step): TMB11-1, 11 ml
- Stop Solution (1N HCl): SS11-1, 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)

* The level of rat anti-KLH IgG in the reference standard was 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

The reference standard should be stored at or below -20°C . All other kit components should be stored at 4°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 (25°C) before use.
3. The assay was designed for use with serum or plasma obtained from rats 14 days after IV 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. Optimal results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

WASH SOLUTION PREPARATION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water.

STANDARD PREPARATION

1. The 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 4°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, 31.25 and 15.63 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 15.63 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 $\mu\text{g/ml}$ 14 days after IV immunization with KLH. Levels are likely higher after 14 days. 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. Optimal dilutions may need to be determined empirically.

1. Dispense 297 μ l of diluent into a polypropylene or glass tube.
2. Pipette and mix 3 μ l of the serum/plasma sample into the tube containing 297 μ 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 μ l of standards (500 – 15.63 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 (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 (25°C) for 30 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 (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

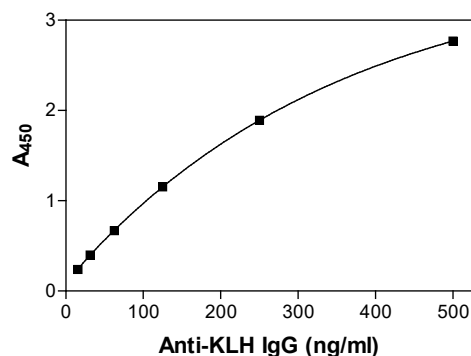
1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or 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 concentration 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 100-fold dilution, 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)	A_{450}
500	2.769
250	1.892
125	1.153
62.5	0.670
31.25	0.399
15.63	0.238

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)

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