

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

INTRODUCTION

Keyhole Limpet Hemocyanin (KLH) is a large oxygen-carrying, copper-containing glycoprotein from the marine mollusk *Megathura crenulata*. KLH is well known as a potent stimulator of humoral and cellular immune responses. It is widely used in research and clinical studies including, for example, as a carrier of low molecular weight haptens used in vaccines and as an antigen for assessing immune function in the screening of drug candidates.

Stellar KLH is manufactured by Stellar Biotechnologies, Inc. directly from controlled, land-based aquaculture. Stellar has developed industry-leading sustainable practices that protect the source species *Megathura crenulata* and ensure quality, consistent KLH.

In drug screening applications, determination of a drug candidate's effects on anti-KLH antibody levels allows easy assessment of immune system regulation.¹ 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 IgM or IgG levels in drug-treated vs. control groups reveals effects on immune response.

This Monkey Anti-KLH IgM ELISA Kit is made using Stellar KLH and is suitable for rapid and quantitative measurement of anti-KLH IgM levels in serum or plasma.^{2,3}

PRINCIPLE OF THE TEST

This Monkey anti-KLH IgM ELISA is a solid phase enzyme-linked immunosorbent assay. It uses Stellar KLH for solid phase (microtiter wells) immobilization, and a horseradish peroxidase (HRP) conjugated mouse monoclonal anti-monkey IgM antibody for detection^A. The use of Stellar KLH improves performance of the assay. Importantly, Stellar KLH coated plates can be used to detect anti-KLH IgM in animals immunized with either subunit or whole molecule KLH.

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 450nm. The concentration of anti-KLH IgM is proportional to the optical density.

^A Specificity of the monoclonal antibody was determined in competitive ELISA's at Life Diagnostics using recombinant monkey IgM as reference materials. All reference IgM's were kindly provided by The NIH Nonhuman Primate Reagent Resource center.

Monkey Anti-KLH IgM ELISA Kit

Stellar Biotechnologies, Inc., Product Code ELI-03M

KIT COMPONENTS

Materials provided with the kit:

- Stellar KLH coated 96-well plate (12 strips of 8 wells)
- Anti Monkey IgM HRP Conjugate, 11 ml
- Anti-KLH IgM Stock^B (lyophilized)
- 20x Wash Solution, 50 ml
- 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 IgM 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 removed from storage conditions and 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.

5. Working 1000-15.62 ng/ml anti-KLH IgM standards should be used within 1 hour of preparation.
6. 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*).

^B The reference standard provided with the kit was calibrated using affinity purified rhesus monkey anti-KLH IgM prepared at Life Diagnostics, Inc.

7. Label 6 polypropylene or glass tubes as 1000, 500, 250, 125, 62.5, 31.25, 15.62 ng/ml.
8. Into the tube labeled 1000 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 1000 ng/ml standard.
9. Dispense 250 μ l of diluent into the tubes labeled 500, 250, 125, 62.5, 31.25, and 15.62 ng/ml.
10. Prepare a 500 ng/ml standard by diluting and mixing 250 μ l of the 1000 ng/ml standard with 250 μ l of diluent in the tube labeled 500 ng/ml.
11. Similarly prepare the 250, 125, 62.5, 31.25, and 15.62 ng/ml standards by serial dilution.

SAMPLE PREPARATION

The optimal sample dilution should be determined empirically. However, studies at Stellar Biotechnologies, 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.

12. Dispense 48 μ l and 237.5 μ l of diluent into separate tubes.
13. Pipette and mix 2 μ l of the serum/plasma sample into the tube containing 48 μ l of diluent. This provides a 25 fold diluted sample.
14. Mix 12.5 μ l of the 25 fold diluted sample with the 237.5 μ l of diluent in the second tube. This provides a 500 fold dilution of the sample.
15. 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

16. Secure the desired number of coated wells in the holder.
17. Dispense 100 μ l of standards and diluted samples into the wells (we recommend that samples be tested in triplicate).
18. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
19. 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.
20. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
21. Add 100 μ l of HRP conjugate into each well.
22. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
23. Wash as detailed in 4 to 5 above.
24. Dispense 100 μ l of TMB Reagent into each well.
25. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
26. Stop the reaction by adding 100 μ l of Stop Solution to each well.
27. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
28. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

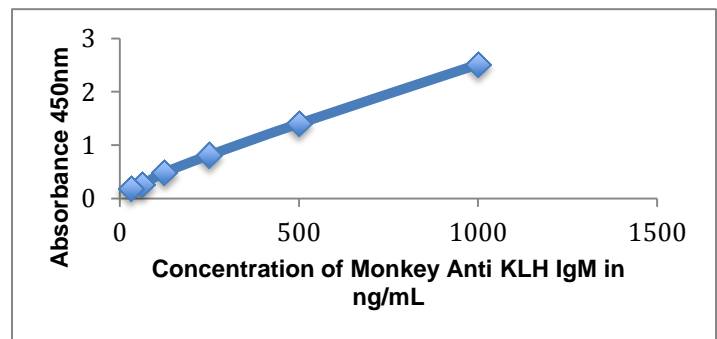
29. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
30. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
31. Using the mean absorbance value for each sample, determine the corresponding concentration of anti-KLH IgM in ng/ml from the standard curve.
32. Multiply the derived concentrations by the dilution factor to determine the actual concentration of anti-KLH IgM in the serum/plasma sample.
33. PC graphing software may be used for the above steps.
34. 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

Anti-KLH IgM (ng/ml)	Absorbance (450 nm)
1000	2.513
500	1.403
250	0.810
125	0.486
62.5	0.262
31.25	0.187
15.62	0.119

should obtain his or her data and standard curve in each experiment.



REFERENCES

1. JR Picotti et.al. T-cell-dependent antibody response: Assay development in cynomolgus monkeys. *Journal of Immunotoxicology*, 2:191-196 (2005)
2. ED Williamson et.al. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. *Infection and Immunity* 73:5978-5987 (2005)
3. 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 IgM Standard (component of kit ELI-03M)

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. Stellar Biotechnologies, 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.

Stellar Biotechnologies, Inc.
332 East Scott Street, Port Hueneme, CA 93041
+1 (805) 488-2800

KLHinfo@stellariotech.com • www.stellariotech.com
www.klbsite.org

PI-006, Rev. d