## INTENDED USE

The Mouse Anti-KLH Total IgG ELISA Kit detects and quantifies KLH (keyhole limpet hemocyanin) -specific IgG in mouse serum or plasma of vaccinated, immunized and/or infected hosts. This immunoassay is suitable for:

- Determining **immune status** relative to controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols.

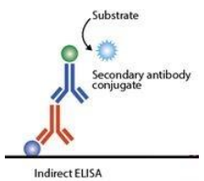
For research use only (RUO), not for diagnosis, cure or prevention of the disease.

## GENERAL INFORMATION

Keyhole Limpet Hemocyanin (KLH), an oxygen-transporting protein of the marine gastropod *Megathura crenulata*, is recognized as a potent immunoactivator, and therefore is widely used in research and clinical studies. Present applications of KLH include: (a) use as a highly immunogenic antigen for assessment of immune competence of an organism, and (b) frequent use as a carrier of low molecular mass peptides and haptens, such as oligosaccharides, gangliosides, designed to facilitate antibody production. In these cases, antibodies made to small peptides/haptens are generally raised by coupling to a large carrier protein like KLH. Antibodies are produced to both KLH and the peptide/hapten; because anti-KLH may give non-specific signals in various immunoassays, removal by solid phase immunoaffinity chromatography is common. The ELISA is useful for determining levels of anti-KLH in sera and for monitoring anti-KLH removal from purified samples after antibody production.

The ADI anti-KLH ELISA is designed with high sensitivity for discriminating the lower level antibodies, with specially formulated diluents to minimize interfering background signals.

## PRINCIPLE OF THE TEST




The Mouse Anti-KLH IgG ELISA kit is based on the binding of antibody in samples to KLH antigen immobilized on the microwells, and bound antibody is detected by anti-mouse IgG-specific HRP conjugate. After a washing step, TMB is added and color (blue) is developed which is directly proportional to the amount of antibody present in the sample. Stop Solution is added (converts blue to yellow) to terminate the reaction, and A450nm is then measured using an ELISA reader. The activity of mouse antibody in samples is determined relative to Calibrators.

## PRODUCT SPECIFICATIONS

**Specificity**

Pharma grade keyhole limpet hemocyanin (KLH) is used to coat the microwells; stabilizing postcoat contains BSA; thus, no other antibody specificity is detectable in the assay. The anti-Mouse IgG-HRP conjugate reacts with mouse IgG; IgM or IgA antibodies would not be measured above background.



## KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8oC until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

**To Be Reconstituted:** Store as indicated.

| Component | Preparation Instructions |
|---|---|
| **Wash Solution Concentrate (100x)** Cat. No. WB-100, 10ml | Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely. |
| **Sample Diluent Concentrate (20x)** Cat. No. SD-20T, 10ml | Dilute 0.5ml + 9.5ml with distilled or deionized water as needed for HRP Conjugate and Sample Dilution. Label as **Working Sample Diluent** and store at 2-8°C until the kit lot expires or is used up. |
| **Anti-Mouse Ig - HRP Conjugate Concentrate (100x)** Part No. H-MsGAM.211, 0.15ml | in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **HRP Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8°C storage. |

**Ready For Use:** Store as indicated on labels.

| Component | Part | Amt | Contents |
|---|---|---|---|
| **KLH Microwell Strip Plate** | 700-101 | 8-well strips (12) | Coated with KLH, and post-coated with stabilizers. |
| **Anti-KLH Calibrators** | | | |
| 10 U/ml<br>25 U/ml<br>50 U/ml<br>100 U/ml | 700-112B<br>700-112C<br>700-112D<br>700-112E | 0.65 ml<br>0.65 ml<br>0.65 ml<br>0.65 ml | Four (4) vials, each containing anti-KLH levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers. |
| **Mouse Anti-KLH Positive Control** | 700-130PC | 0.65 ml | Mouse IgG reactive with KLH. ELISA values should be greater than **0.5** net OD. |
| **Low NSB Sample Diluent** | TBTm | 30 ml | Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution |
| **TMB Substrate** | 80091 | 12 ml | Chromogenic substrate for HRP containing TMB and peroxide. |
| **Stop Solution** | 80101 | 12 ml | Dilute sulfuric acid. |

**Materials Required But Not Provided:**

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Mouse Ig HRP Concentrate.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.



## ASSAY DESIGN AND SET-UP

**Sample Collection and Handling**

For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, including **tissue culture media**, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent.

**Antibody Stability & Sample Dilution**

Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 10 times the initial dilution and performed the same day as the assay.

Example: Initial (1/5): **10**ul serum + **40**ul WSD [or 0.1ml + 0.4ml]
Further (1/50): **10**ul initial (1/5) + **90**ul LNSD (1/50)

**Assay Design**

Review Calculation of Results (p5-7) and Limits of the Assay (above) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be <0.5 OD. This is usually 1/100 or greater dilution for mouse sera with normal levels of IgG and IgM.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required (**See Method A)**.
- Run a set of Calibrators. Calibrators validate that the assay was performed to specifications; results can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve, **Method B**, has limitations. See Limits of the Assay (above).
- Run the Mouse Anti-KLH IgM **Positive Control**. A net OD value greater than **0.5** indicates proper assay performance.
- Run a range of sample dilutions for expected higher positives that allows calculation of antibody **Titer** (when specific titer is at least 4-fold higher than non-immune)**. See Method C.**
- Run samples in duplicate if used for quantitation; non-immunes that are significantly lower than immunes may be run in singlicate. The Calibrators that are used for quantitation, e.g., for between-assay normalization, should be run in duplicate. When determining titer from a dilution curve, singlicates can be run if more than two dilution points are used for titer calculations.

## LIMITATIONS OF THE ASSAY

**Quantitation of Antibody in a Sample**

The ELISA measures anti-KLH activity, a combination of antibody concentration and avidity for the KLH antigens. Antibodies with substantially different total Ig concentrations may display similar anti-KLH activities, due to differences in avidity. The quantitation or activity of the samples is, therefore, appropriately expressed in activity Units (titer), rather than mass units of IgG (e.g., ug/ml).

**Calibrator Curve Quantitation**

To quantitate antibody activity from a calibrator curve (such as provided with the kit), the dilution curve of the samples must be parallel to the calibrator curve, to avoid different values being obtained from different regions of the curve. Antibodies that are not matched in KLH avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as a titer relative to a reference positive such as the 8 U/ml Calibrator, or another Calibrator in the kit (see Calculation of Results).

## PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10



**Plate Set-up**

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

## Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

**1. 1st Incubation [100ul – 60 min; 4 washes]**

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

**2. 2nd Incubation [100ul – 30 min; 5 washes]**

- Add 100ul of diluted Anti-Mouse IgG-HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

**3. Substrate Incubation [100ul – 15 min]**

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

**4. Stop Step [Stop: 100ul]**

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

**5. Absorbance Reading**

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

## INTERPRETATION OF RESULTS

**Quality Control & Expected Results**
Sample Diluent Blank: < 0.3 OD; higher ODs suggest inadequate washing. If the OD of the Blank is greater than the lowest Calibrator, re-run the assay.

Non-immune/ Non-vaccinated Serum: Pre-immune/ non-immune sera at 1/100 dilutions should produce ODs lower than the low Calibrator. Very low antibody levels, including **innate antibodies**, may be assayed at dilutions less than 1/100; because net ODs will be elevated, compare the results with pre-immune controls at the same dilutions.

Calibrators: ODs should be in ascending order with low-to-high range > 1.0 OD. Variance of replicate values >25% indicates invalid precision for the run.

**Calculation of Results**
Several data reduction methods may be considered to best represent the relationships among experimental and control groups, to determine **Positive Immune** and **Negative Non-immune**, and to **Quantitate** positive antibody levels.

**Method A. Positive Index**
Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

A sample value would be determined as **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution. This calculation **quantifies** the positive Antibody Activity level.

**Example:**

| | Assay Net OD | | Calculated Antibody Activity | |
|---|---|---|---|---|
| Sample | Control | Exptl | Control | Exptl |
| 1 | 0.243 | 2.358 | 0.49 | **4.79** |
| 2 | 0.351 | 0.597 | 0.71 | **1.21** |
| 3 | 0.286 | 1.421 | 0.58 | **2.89** |
| 4 | 0.357 | 1.268 | 0.73 | **2.58** |
| 5 | 0.512 | 0.857 | **1.04** | **1.74** |
| 6 | 0.342 | 1.296 | 0.70 | **2.63** |
| 7 | 0.298 | 0.608 | 0.61 | **1.24** |
| 8 | 0.285 | 0.369 | 0.58 | 0.75 |
| 9 | 0.157 | 0.864 | 0.32 | **1.76** |
| 10 | 0.187 | 0.543 | 0.38 | **1.10** |
| Mean | 0.302 | | | |
| SD | 0.095 | | | |
| Mean +2 SD | **0.492** | = **Positive Index** | | |



## CALCULATION OF RESULTS (continued)

**Method B. Use of a Calibrator Curve**

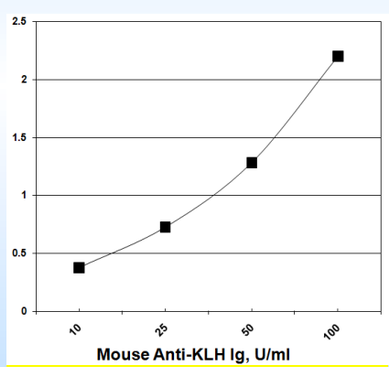
When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the anti-KLH activity units may be determined by interpolation from the Calibrator curve, as follows:

1. The results may be calculated using any immunoassay software package. If software is not available, anti-KLH activity concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the calibrators (y-axis) against the concentration (U/ml) of anti-KLH (x-axis). Draw the best fit curve through these points to construct the calibrator curve. A point-to-point construction is most common and reliable.
4. The anti-KLH activity concentrations in unknown samples and controls can be determined by interpolation from the calibrator curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 16 U/ml calibrator should be further diluted and re-assayed.

**Typical Results:**

| Wells | Calibrators | | A450 nm |
|---|---|---|---|
| A1,2 | **Negative Diluent Blank** | | 0.08 |
| B1,2 | 10 U/ml | **Calibrator** | 0.38 |
| C1,2 | 25 U/ml | **Calibrator** | 0.73 |
| D1,2 | 50 U/ml | **Calibrator** | 1.28 |
| E1,2 | 100 U/ml | **Calibrator** | 2.20 |
| F1,2 | Sample | **1:100** | 1.36 |

Sample Result: **53** U/ml x 100 dilution = **5.3** kU/ml






## CALCULATION OF RESULTS (continued)

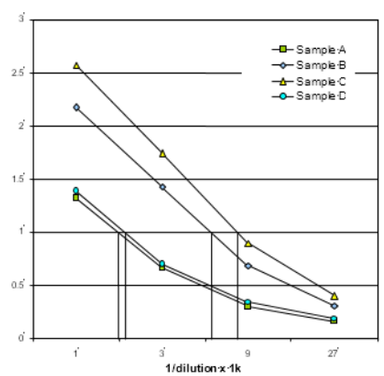
**Method C. Titers from Sample Dilution Curves**
The titer of antibody activity calculated from a dilution curve of each sample is recommended as the most accurate quantitative method. Best precision can be obtained using the following guidelines:

1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals higher and lower than the selected index. With accurate diluting, duplicates may not be required if at least 4 dilutions are run per sample.
3. A 5-fold dilution scheme is useful to efficiently cover a wide range which produces ODs both above and below 1.0 OD. The dilution scheme can be tightened to 3-fold or 2-fold for more precise comparative data.
4. A Calibrator value in the mid-OD range (e.g., 4 U/ml) can be used to normalize inter-assay values.

Calculations
1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the two dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary or selected Calibrator).
2. From a point-to-point line drawn between the two sample ODs, read the dilution value (x-axis) corresponding to the OD of the selected Index
   = **Total Ig Antibody Activity Units**

**Example:**

**II.** A **1.0 OD** Index was used to determine titer of 4 antibodies.




**Titer Values**
**Sample A = 1.72 kU** **Sample B = 5.70 kU**
**Sample C = 1.85 kU** **Sample D = 7.90 kU**



Instruction Manual No. M-700-150-VAG

# Mouse Anti-KLH IgG ELISA Kit

## Cat. No. 700-150-VAG

For Quantitation of Total Anti-Keyhole Limpet Hemocyanin (KLH) IgG(IgG+IgM+IgA) in Serum, plasma or other biological fluids

*For research use only, not for diagnostic or therapeutic use.*

ALPHA DIAGNOSTIC INTERNATIONAL

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| ELISA Kit Components | Amount | Part |
|---|---|---|
| KLH Coated Microwell Strip Plate | 8-well strips (12) | 700-101 |
| Mouse Anti-KLH Positive Control | 0.65 ml | 700-130PC |
| Anti-KLH Calibrator 10 U/ml | 0.65 ml | 700-112B |
| Anti-KLH Calibrator 25 U/ml | 0.65 ml | 700-112C |
| Anti-KLH Calibrator 50 U/ml | 0.65 ml | 700-112D |
| Anti-KLH Calibrator 100 U/ml | 0.65 ml | 700-112E |
| Anti-Mouse Ig HRP Conjugate (100X) | 0.15 ml | H-MsGAM.211 |
| Sample Diluent (20X) | 10 ml | SD20T |
| Low NSB Sample Diluent | 30 ml | TBTm |
| Wash Solution Concentrate (100X) | 10 ml | WB-100 |
| TMB Substrate | 12 ml | 80091 |
| Stop Solution | 12 ml | 80101 |
| Product Manual | 1 ea | M-700-150 |