ELISA Kit Components	Amount	Part No.
KLH Coated Microwell Strip Plate	8-well strips (12)	700-101
Mouse Anti-KLH IgG Calibrator 2 U/ml	0.65 ml	700-132B
Mouse Anti-KLH IgG Calibrator 4 U/ml	0.65 ml	700-132C
Mouse Anti-KLH IgG Calibrator 8 U/ml	0.65 ml	700-132D
Mouse Anti-KLH IgG Calibrator 16 U/ml	0.65 ml	700-132E
Anti-Mouse Ig HRP Conjugate (100X)	0.15 ml	MsH-GAM
Sample Diluent (20x) Concentrate	10 ml	SD20TG
Low NSB Sample Diluent	60 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-700-130-KLM

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Anti-Mouse Ig-HRP contain Bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water.

MSDS for TMB, sulfuric acid and BND, if not already on file, can be requested or obtained.

LIMITS 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 antigen. Antibodies with substantially different 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 4 U/ml Calibrator, or another Calibrator in the kit (see Calculation of Results).

Non-specific Binding and Blocking of Antibody Activity
Antigen blocking is a strategy to verify that signal in the assay is due to specific Anti-KLH antibody activity, rather than non-specific binding of non-Anti-KLH IgG to the microwell. It is observed that antigen blocking of higher avidity antibodies is often more complete than with lower avidity antibodies, due to both antigen composition and antibody affinities. Therefore, the percent blocking of samples may vary from that of the Positive Control, and will rarely be essentially complete in an ELISA. For positive samples that display low % blocking, it is recommended they be comparatively tested on non-antigen coated microwells.

Instruction Manual No. M-700-130-KLM

Mouse Anti-Keyhole Limpet Hemocyanin (KLH) Ig

ELISA Kit Cat. No. 700-130-KLM

For Quantitative Determination of Total Anti-KLH Ig (IgG+IgA+IgM) in Serum

ALPHA DIAGNOSTIC INTERNATIONAL

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INTENDED USE

The Mouse Anti-Keyhole Limpet Hemocyanin (KLH) ELISA Kit is an immunoassay suitable for quantifying or titering total antibody activity (IgG, IgA and IgM) specific for KLH in serum, plasma or other biological fluids.

RESEARCH USE OF THE TEST

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 or (glyco)peptides, 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 KLH ELISA kit is based on the binding of Mouse anti- KLH in samples to KLH immobilized on the microwells, and anti- KLH Ig antibody is detected by anti-mouse IgG+IgA+IgM-specific antibody conjugated to HRP (horseradish peroxidase) enzyme. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate, which is directly proportional to the amount of anti-KLH present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microwell reader. The amount of Mouse Ig in samples is calculated relative to mouse anti-KLH reference calibrators.

PRODUCT SPECIFICATIONS

Specificity
Purified KLH is used to coat the microwells; thus the assay is specific for antibodies directed to KLH. The anti-Mouse IgG+IgA+IgM (H+L) HRP conjugate reacts with mouse IgG, IgA and IgM class antibodies that bind to KLH on the plate. IgE antibody would not be measured above background signals.

Assay Sensitivity
The KLH coating level and HRP conjugate concentration are optimized to differentiate anti-KLH Ig from background (non-antibody) signal with mouse serum samples diluted 1:100.

Calibrator Values
The calibrators are dilutions of mouse IgG antibody reactive to KLH. Values are assigned as arbitrary Anti-KLH activity units (see Limits of the Assay).

CALCULATION OF RESULTS (continued)

***High Titer/Multiple Dilutions*:** Samples, especially expected **Positives**, are diluted to produce signals at a selected signal level which is used as an Index to determine the **Titer** of the samples.

Method D. Titers from Sample Dilution Curves

The titer of antibody activity calculated from a dilution curve of each sample is recommended when all sample curves are not parallel to each other and/or the Calibrator curve. Best precision can be obtained using the following guidelines:

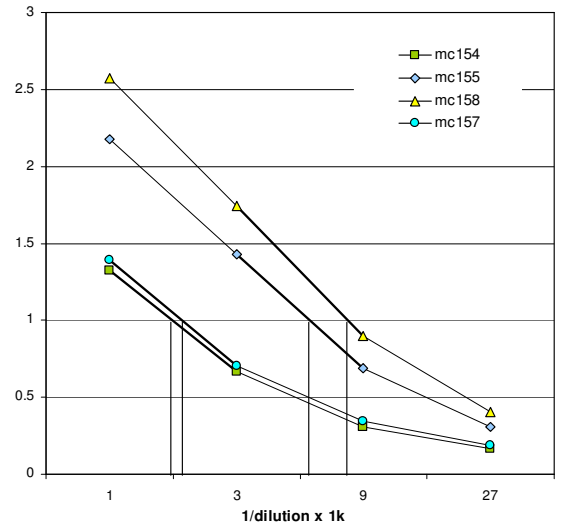
1. Use an OD value **Index** in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 0.5 or 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce 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 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 Antibody Activity Units**

Typical Results:

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



Titer Values

mc154 = 1.72 kU
mc155 = 5.70 kU
mc157 = 1.85 kU
mc158 = 7.90 kU

CALCULATION OF RESULTS

***Single Dilution/Low Titer*:** Expected **Positive** samples are assayed at the same dilution as **non-immune** or **Negative** samples.

Method A. Antibody Activity:
Represent as net OD units (A450 signal; blank subtracted). Signals can be normalized for assay variation by dividing each sample signal by the signal of a selected Calibrator. Units can also contain the sample dilution and the Calibrator U/ml as shown below:

- Calculate the mean net ODs for replicate samples and the selected Calibrator.
- Divide each sample OD value by the Calibrator OD value, and multiply by the sample dilution and the Calibrator (U/ml) value = **Total Activity Units**

Typical Results:

1.05 [Sample, net OD] ÷ **0.88** [4 U/ml Calibrator, net OD]
x **500** dilution x **4** U/ml = **2.4 k** Activity Units in serum.

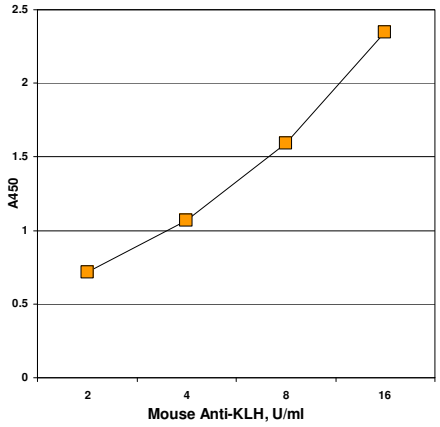
Method B. Positive Index
Values of **Experimental** samples may be expressed relative to the values of **Control** samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the mean + 2 SD of the Control samples = **Positive Index.**
2. Divide each sample value by the Positive Index. Values above 1.0 are a measure of **Positive** antibody activity; below 1.0 are **Negative** for antibody.

***Low to High Titer*:**

Method C. Use of a Calibrator Curve
When the dilution curves of samples are parallel to the Calibrator curve, the antibody activity units may be determined by interpolating from the curve and multiplying the value by the sample dilution (blank OD does not need to be subtracted). See Limits of the Assay, page 7.

Typical Results:



Wells	Calibrators	A450 nm
A1,2	**Negative Diluent Blank**	0.19
B1,2	2 U/ml **Calibrator**	0.72
C1,2	4 U/ml **Calibrator**	1.07
D1,2	8 U/ml **Calibrator**	1.60
E1,2	16 U/ml **Calibrator**	2.35
F1,2	Sample **1:500**	1.24
Result: **4.4** U/ml x500 dilution = **2.2** KU/ml		

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8° C 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	Reagent Preparation
Sample Diluent Concentrate (20x) Cat. No. SD-20TG, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8°C until the kit lot expires or is used up.
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.
Anti-Mouse IgG- HRP Conjugate Concentrate (100x) Part MsH-GAM, 0.15ml	Peroxidase conjugated anti-Mouse Ig in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. **Do not use TBTm.** Use within the working day and discard. Return 100X to 2-8° C storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
KLH Microwell Strip Plate	700-101	8-well strips (12)	Coated with KLH, and post-coated with stabilizers.
Anti-KLH IgG Calibrators			
2 U/ml 4 U/ml 8 U/ml 16 U/ml	700-132B 700-132C 700-132D 700-132E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing mouse anti-KLH IgG levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
Low NSB Sample Diluent Reduces non-specific binding	TBTm	60 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution. See **Assay Design**, page 3. **Not** for HRP Conjugate dilution.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	1% 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 IgG-HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate and Sample Diluent concentrate; 200ml to 1L.
- 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

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. 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 Working Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Assay Design

Review Calculation of Results (p5,6) and Limits of the Assay (p7) 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, when non-immune samples give net signal <0.5 OD. This is usually 1/100 or greater dilution for mouse sera with normal levels of IgG and IgM. Dilute samples in **Working Sample Diluent (1xSD20TG)** or in **Low NSB Sample Diluent (TBTm); TBTm** is recommended when lower dilutions are required for detection of low titer positives and NSB is elevated. Note: **all samples** must be diluted in the same diluent for proper comparison – either TBTm **or** 1xSD20T.
- 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. Blank OD should be <0.3. **See Method A** and **B**.
- Run a set of Calibrators. Calibrators validate that the assay was performed to specifications, and can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve, **Method C**, has limitations. See page 7, Limits of the Assay.
- 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 D.**
- 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.

ASSAY PROCEDURE

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.

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 Conjugate 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.