ELISA Kit Components	Amount	Part No.
KLH-coated Microwell Strip Plate	8-well strips (12)	700-101
Goat Anti-KLH IgG Calibrator 20 U/ml	0.65 ml	700-113B
Goat Anti-KLH IgG Calibrator 50 U/ml	0.65 ml	700-113C
Goat Anti-KLH IgG Calibrator 100 U/ml	0.65 ml	700-113D
Goat Anti-KLH IgG Calibrator 250 U/ml	0.65 ml	700-113E
Anti-Goat IgG HRP Conjugate (100X)	0.15 ml	GtH-G
Sample Diluent Concentrate (10X)	10 ml	SD-20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	700-100-KLG

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Anti-Goat IgG-HRP contain ProClin 300 (0.05%, v/v). Stop Solution contains diluted 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 ProClin 300, if not already on file, can be requested or obtained from the ADI website.

PRODUCT SPECIFICATIONS

Specificity
Purified KLH is used to coat the microwells; thus the assay is specific for antibodies directed to KLH. The anti-goat IgG HRP conjugate is specific for goat IgG and does not react with IgM, IgA or IgE.

Assay Sensitivity
The diluted anti-goat IgG HRP produces a 1.0 OD signal with 30ng of goat IgG coated on a microwell (30 min anti-goat IgG HRP incubation). The KLH antigen coating level is optimized to differentiate anti-KLH from background (non-antibody) signal with goat serum samples diluted 1:100.

Calibrator Values
The Calibrators are composed of dilutions of antisera from KLH immunized animals. Values are assigned as arbitrary anti- KLH activity units (see **Limits of the Assay**).

Instruction Manual No. M-700-100-KLG

Goat Anti-Keyhole Limpet Hemocyanin (KLH) IgG

ELISA Kit # 700-100-KLG

For Semi-Quantitative Determination of Anti-KLH IgG in Biological Fluids

ALPHA DIAGNOSTIC INTERNATIONAL

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INTENDED USE

The Goat Anti-KLH IgG ELISA Kit is an immunoassay suitable for quantifying or titering IgG antibodies specific for KLH in serum, plasma or other biological fluids, including tissue culture medium.

INTRODUCTION

Keyhole Limpet Hemocyanin (KLH), a copper-containing, high molecular weight glycoprotein of the marine gastropod Megathura crenulata, is a potent immunoactivator of TH-2 like humoral and cellular immune responses. Thus, KLH has widespread use in research and clinical studies, including use as a highly immunogenic antigen for assessment of immune competence. KLH exhibits several carbohydrate determinants, including one found in the Schistosoma parasite, and others that are likely targets for natural antibodies of innate immunity, which have been measured in chickens and primates. In humans, KLH is an immunotherapeutic agent for bladder cancer, and has been assessed for a role in treatment of other immune based pathologies.

KLH is frequently used as a carrier of low molecular mass peptides and haptens, such as oligosaccharides, gangliosides or (glyco)peptides, designed to facilitate antibody production, often with the scope of developing anti-cancer therapy. In these cases, where antibodies are produced to both KLH and the peptide/hapten, and 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.

PRINCIPLE OF THE TEST

The Goat KLH IgG ELISA kit is based on the binding of goat anti- KLH in samples to KLH immobilized on the microwells, and anti- KLH IgG antibody is detected by anti-goat IgG-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 goat IgG in samples is calculated relative to Anti-KLH reference calibrators.

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 specific IgG concentrations may display similar anti-KLH activities, due to differences in avidity. The quantitation or potency of the samples is, therefore, appropriately expressed in Activity Units (titer),rather than mass units of IgG (e.g., ug/ml).

Calibrator Curve Quantitation

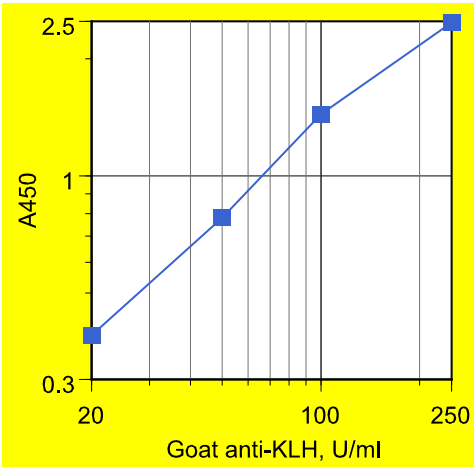
To quantitate antibody activity from a Calibrator curve (such as provided with the kit), the dilution curve of the samples must be parallel to the Calibrator curve, to avoid different values being obtained from different regions of the curve. Antibodies that are not matched in anti-KLH avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as a titer relative to a reference positive such as the 100 U/ml Calibrator, or another Calibrator in the kit.

CALCULATION OF RESULTS (continued)

Method III. 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 and multiplying the value by the sample dilution.

Typical Calibrator Curve



Typical Results

Positive Samples from KLH-immunized animals, assayed as 5-fold dilutions, produced the OD values in the following table and plotted, see Method II (p.5).

III. Values were read from the Calibrator curve:

Sample	Dilution	A280	Activity	Total Activity	Agreement
A	1:10k	1.922	7.3 U	73 kU	**96%**
	1:50k	0.718	1.52 U	76 kU	Good
B	1:10k	1.576	5.1 U	51 kU	**78%**
	1:50k	0.403	0.8 U	40 kU	Borderline
C	1:10k	1.483	4.6 U	46 kU	**96%**
	1:50k	0.504	0.96 U	48 kU	Good
D	1:10k	1.908	7.2 U	72 kU	**52%**
	1:50k	1.08	2.75 U	138 kU	Poor

When sample dilutions read from the upper & lower regions of the Calibrator curve are concordant (agree > 90%) use of the curve is validated. In the above example, samples B and D demonstrate the common occurrence of non-parallel curves. Method II becomes a more accurate representation of the data in this case.

CALCULATION OF RESULTS

Several data reduction methods may be considered to optimize precision and to best represent the relationships among experimental and control groups.

Method I. Single Dilution or Low Titer Samples

With samples tested at a common dilution, report values in OD units, or a Calibrator value may be used as an index to normalize between-assay variation:

1. Calculate the mean net ODs for replicate samples and selected Calibrator.
2. Divide each sample OD value by the Calibrator OD value [may also multiply by sample dilution and/or Calibrator (U/ml) value] = **Activity Units.**

Typical Results:

1.61 [Serum sample, net OD] ÷ **1.35** [100 U/ml Calibrator, net OD]
X **100** U/ml X 200 dilution = **23.8** kU/ml.

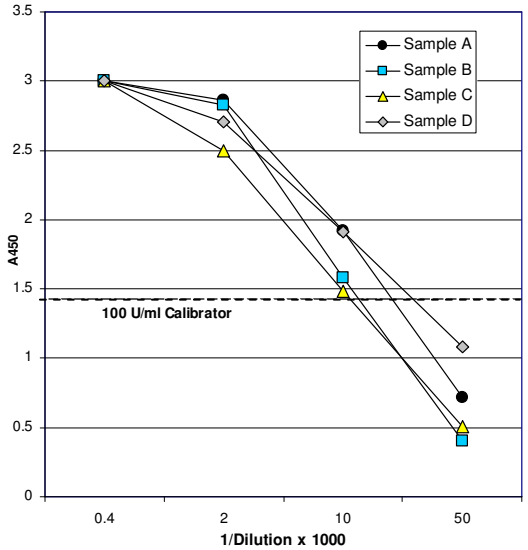
Method II. High Titer, Multiple Sample Dilutions

When the dilution curves of samples are not parallel to the Calibrator curve, antibody potency can be expressed in semi-quantitative activity units, using an arbitrary value, e.g., 1.0 or 0.5 OD, as Index, or by using one of the Calibrators as the Index to normalize between-assay variation:

1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the pair of 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 IgG Antibody Activity Units**

Typical Results: see Data Table under Method III, p.6

II. The **100 U/ml Calibrator**, OD=1.35, was used as the Index to determine titer.



Titer Values

Sample A = 19,800 units
Sample B = 12,700 units
Sample C = 11,500 units
Sample D = 26,000 units

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	Instructions for Use
Sample Diluent Concentrate (10x) Cat. No. SD-20T, 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-Goat IgG - HRP Conjugate Concentrate (100x) Part No. GtH-G, 0.15ml	Peroxidase conjugated anti-Goat IgG in buffer with protein, detergents and ProClin 300 as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate 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.
Goat Anti-KLH IgG Calibrators			
20 U/ml 50 U/ml 100 U/ml 250 U/ml	700-113B 700-113C 700-113D 700-113E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-KLH levels in arbitrary Activity Units; diluted in buffer with protein, detergents and ProClin 300 as stabilizers.
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 pipetter is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Goat 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, stored refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

Samples, Calibrators and Controls

Dilute **Samples** in Working Sample Diluent according to expected anti-KLH activity levels; for serum: dilute at least 200-fold (e.g., 5 ul sample + 955 ul Diluent) for reduced nonspecific signals. At least 2 dilutions of each sample is recommended in order to determine if reading values from the Calibrator curve is valid (see Limits of the Assay).

Do not dilute the **Calibrators**. Include Working Sample Diluent as a Negative Control to determine proper assay performance (signal should be < 0.3 OD) and to subtract from sample and Calibrator values to obtain net OD. Internal **Controls** that represent the lab's expected results should also be included in each assay run.

Plate Set-up

Bring all reagents to room temperature (18-30^{o} 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.

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.

- Add 200-300ul Working Wash Solution before sample addition to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel.

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 by adding 250-300ul Working Wash Solution; aspirate or dump the liquid 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-Goat 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.