Human Immunoglobulin A

Immunoenzymetric Assay for the Measurement of Human Immunoglobulin A
Catalog # F165

Intended Use

This kit is intended for use in quantifying very low concentrations of human immunoglobulin A (hIgA). The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

The sensitive and specific quantitation of very low levels of hIgA can be accomplished by the use of a two-site immunoenzymetric assay, also termed ELISA. This technology allows for detection of hIgA down to a few picograms/mL. The antibodies used in this kit are very specific for hIgA, and thus the assay will provide accurate results for hIgA even in the presence of other human immunoglobulins. Because of the very high sensitivity of this assay it is useful in detecting trace impurities by hIgA in a variety of sample types. This kit can be used to quantitate hIgA in human serum samples however it will be necessary to significantly dilute most serum samples to get them within the analytical range of this very sensitive assay.

Reagents & Materials Provided

Component	Product #
Anti-hIgA:HRP Affinity purified goat antibody to hIgA conjugated to HRP in a protein matrix with preservative. 1x12mL	F168
Anti-hIgA coated microtiter strips 12x8 well strips in a bag with desiccant	F167*
hIgA Standards Highly purified hIgA in a bovine serum albumin matrix with preservative. Standards at 0, 0.5, 1.5, 5, 15, and 50ng/mL. 1mL/vial	F166
Stop Solution 0.5M sulfuric acid. 1x12mL	F006
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004

*All components can be purchased separately except # F167.

Principle of the Procedure

The hIgA assay is a two-site immunoenzymetric assay. Samples containing hIgA are reacted in microtiter strips coated with an affinity purified capture antibody. A second, horseradish peroxidase (HRP) enzyme labeled anti-hIgA antibody, is reacted simultaneously resulting in the formation of a sandwich complex of solid phase antibody-hIgA-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate, tetramethylbenzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of hIgA present.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.

- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section.

- Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

- Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)*
- Pipettors - 25μL and 100μL
- Repeating or multichannel pipettor - 100μL
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # I028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.

- Stop reagent is 0.5M H_2SO_4. Avoid contact with eyes, skin, and clothing.

- The source serum used in preparation of the kit standards has been tested by FDA approved methods and has been found negative for antibody to human immunodeficiency virus (HIV-I & HIV-II), antibody to Hepatitis C virus, and for Hepatitis B surface antigen. No known test method can offer total assurance that HIV, Hepatitis B and C, or other infectious agents are absent. *Handle these reagents as if they were potentially infectious.*

- This kit should only be used by qualified technicians.

Preparation of Reagents

- Bring all reagents to room temperature.

- Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the "0" standard is greater than 0.2, evaluate plate washing procedure for proper performance.

2. Dilution of samples will be required for samples greater than 50ng/mL. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat # I028 available in 100mL, 500mL, or 1 liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in I028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents should be qualified in the assay to demonstrate that they do not give elevated background and are not contaminated with human serum proteins. The diluent should also give acceptable recovery when spiked with known quantities of hIgA.

3. High Dose Hook Effect may be observed in samples with very high concentrations of hIgA. Samples greater than 50,000ng/mL may give absorbances less than the 50ng/mL standard. High Dose Hook samples are most likely to be encountered in samples derived from human serum or blood. If a hook effect is possible samples should also be assayed diluted. If the absorbance of the undiluted sample is less than the diluted samples this may be indicative of the hook effect. Such samples should be diluted until the dilution adjusted value remains constant.

4. Because this is an extremely sensitive assay for hIgA, it is possible to inadvertently contaminate the kit reagents with various external sources of hIgA. Such hIgA impurities can arise from use of pipettors or other laboratory equipment, or surfaces that have come into contact with more concentrated forms of hIgA. For example, human serum contains on the order of 1mg/mL of IgA or on the order of a million fold greater than the standards used in this kit. Airborne impurities from these same concentrated sources or from technician mucosal aerosols or dander will also easily contaminate the kit reagents and potentially give false values and or poor assay reproducibility. Take precautions to minimize impurities.

Limitations

- Certain sample matrices may interfere in this assay. Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, high salt concentration, extremes of pH (less than 6.0 and greater than 8.5) or very high protein concentrations may give erroneous results. It is recommended to test the sample matrix for interference by diluting the 50ng/mL standard 1 part to 4 parts of the matrix containing no or very low levels of hIgA. This diluted standard when assayed as an unknown should give an added value of 8 to 12ng/mL. In cases where hIgA in the sample will allow for sample dilution, such dilution will often overcome sample matrix interference. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

- Avoid the assay of samples containing sodium azide (NaN_3), which will destroy the HRP activity of the conjugate and could result in the under-estimation of hIgA levels.

Assay Protocol

- The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Before modifying the protocol from what is recommended, users are advised to contact our technical services for input on the best way to achieve your desired goals.

- The protocol specifies use of an approved orbital microtiter plate shaker for the immunological step. These can be purchased from most laboratory supply companies. If you do not have such a device, it is possible to incubate the plate without shaking however it will be necessary to extend the immunological incubation step in the plate by about 1 hour in order to achieve comparable results to the shaking protocol. **Do not shake during the 30-minute substrate incubation step as this may result in higher backgrounds and worse precision.**

- Bring all reagents to room temperature. Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and 650nm for the reference.

- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.

- All standards, controls, and samples should be assayed in duplicate.

- Maintain a repetitive timing sequence from well to well for all assay steps to that all incubation times are the same for each well.

- Make a work list for each assay to identify the location of each standard, control, and sample.

- If the substrate has a distinct blue color prior to the assay it may have been contaminated. If the absorbance of 100µL of substrate plus 100µL of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.

2 Pipette 100µL of anti-hIgA:HRP (#F168) into each well.

3. Cover & incubate on orbital shaker at 400-600 rpm for 2 hours at room temperature, 24°C ± 4°C.

4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.

5. Pipette 100µL of TMB substrate (#F005).

6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.

7. Pipette 100µL of Stop Solution (#F006).

8. Read absorbance at 450/650nm.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL. This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 0.5 ng/mL and less than 50 ng/mL. CVs for samples less than 0.5 ng/mL may be greater than 10%.

- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.000	0.001
B1	Zero Std	0.002	
C1	0.5ng/mL	0.024	0.022
D1	0.5ng/mL	0.020	
E1	1.5ng/mL	0.072	0.074
F1	1.5ng/mL	0.076	
G1	5ng/mL	0.240	0.245
H1	5ng/mL	0.250	
A2	15ng/mL	0.810	0.791
B2	15ng/mL	0.772	
C2	50ng/mL	2.152	2.101
D2	50ng/mL	2.050	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A more detailed copy of this "Qualification Summary" report can be obtained by request. This qualification is generic in nature and is intended to supplement but not replace certain user and sample specific qualification that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing hIgA within or above the analytical range of this assay should be evaluated for dilutional linearity to ensure that the assay is accurate and has sufficient antibody excess. Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user qualification protocols can be obtained by contacting our Technical Services Department or on-line at our web site.

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is less than 150 pg/mL.

The lower limit of quantitation (**LOQ**) is defined as the lowest concentration where concentration coefficients of variation (CVs) are less than 20%. LOQ is ~250 pg/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (1.45ng/mL), medium (4.95ng/mL), and high concentrations (14.89ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	4.1%	7.2%
Medium	2.5%	3.6%
High	3.2%	2.2%

Specificity/Cross-Reactivity

The antibodies used in this kit are essentially human alpha chain specific and have been affinity purified against hIgA. Because it is difficult to obtain hIgA for either immunogen, antigen affinity purification, or assay calibrator purposes that does not have trace impurities of other human immunoglobulin classes like IgG or IgM, nor is it possible to obtain IgG and IgM that does not have trace impurities with IgA it is problematic to accurately determine cross reactivity. With these limitations considered, it is estimated that cross reactivity for human IgG and human IgM is negligible (less than 0.1%) for most assay applications. It is recommended that each user consider testing their particular sample types and sample matrices for cross reactivity, or other types of interferences. Sera from various other animals were diluted 1:1000 and tested for apparent reactivity in this assay. As indicated in the table below, certain sera showed very minimal positive reactivity on a weight to weight basis, however at these very low levels it cannot be conclusively determined if this is true immunological cross reactivity or some non-specific increase in assay signal.

Animal Species	% Cross-Reactivity
Cat	Not detectable
Chicken	Not detectable
Cow	Not detectable
Dog	Not detectable
Goat	Not detectable
Hamster	Not detectable
Horse	Not detectable
Mouse	Not detectable
Pig	Not detectable
Rabbit	~ 0.0049%
Rat	Not detectable
Sheep	~0.001%

Recovery/ Interference Studies

Various buffer matrices have been evaluated by adding known amounts of the hIgA preparation used to make the standards in this kit. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery defined as between 80-120%. In general, extremes in pH (less than 5.0 and greater than 8.5) as well as some detergents like SDS

and Tween can cause under-recovery. Very high concentrations of certain proteins can also interfere in accurate detection of hIgA. Each user should qualify that their sample matrices yield accurate recovery by performing a similar experiment. For example, this experiment can be performed by diluting one part of the 50ng/mL standard provided with this kit into 4 parts of the sample matrix in question. Recovery should be on the order of 8 to 12 ng/mL hIgA. Consult *Cygnus Technologies* Technical Services if you have recovery problems in your matrix.

Hook Capacity

Increasing concentrations of hIgA greater than 50 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less that the 50 ng/mL standard was ~50 μg/mL.

Ordering Information/ Customer Service

To place an order or to obtain additional product information contact *Cygnus Technologies*:

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