CYGNUS TECHNOLOGIES
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Human Serum Albumin Assay

Immunoenzymetric Assay for the Measurement of Human Serum Albumin Catalog # F055

Intended Use

This kit is intended for use in quantitating human serum albumin (HSA). 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 HSA can be accomplished by the use of a two-site immunoenzymetric assay, also termed ELISA. The antibodies used in this kit are very specific for HSA and thus the assay will provide accurate results for HSA even in the presence of very high concentrations of other proteins. This kit was designed to detect trace impurities of HSA in pharmaceutical and other biological products. The kit may be useful in quantitating HSA in human serum and other samples such as urine however, because these samples typically contain concentrations above the analytical range of this assay it may be necessary to significantly dilute such samples to get them within the analytical range of this very sensitive assay. The manufacture of products by various biotechnological processes such as cell or tissue culture can result in residual impurities of the desired product by components used in the culture media. The use of so called serum free defined media greatly reduces the number of potential impurities but it may still be necessary to determine trace impurity levels of the proteins and growth factors used in these media. Many commercial formulations of so-called serum free media contain significant amounts of albumin and transferrin usually of either human or bovine origin, and insulin from various species. When the intended product may be used as a therapeutic agent in humans or animals the product should be highly purified to avoid potential health risks or other problems that might result from trace impurities. Efforts to reduce trace media impurities to the lowest levels practical through optimal process design, qualification, and final product testing require a highly sensitive and reliable analytical method. The *Cygnus Technologies* Human Serum Albumin assay is designed to provide a simple to use, precise, and highly sensitive method to detect human Albumin to less than 200pg/mL. As such, this kit can be used as a tool to aid in optimal purification process development and in routine quality control of in-process streams as well as final product.

Principle of the Procedure

The human serum albumin assay is a two-site immunoenzymetric assay using an amplified biotin/streptavidin detection system for maximum sensitivity. Samples containing human serum albumin are reacted in microtiter strips coated with an affinity purified capture antibody. A second biotinylated anti-HSA antibody is reacted forming a sandwich complex of solid phase antibody- albumin-biotinylated antibody. After a wash step to remove any unbound reactants the strips are then reacted with streptavidin labeled with alkaline phosphatase. Another wash sequence is performed followed by the addition of p-nitrophenyl phosphate (PNPP) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of human serum albumin present. Accurate quantitation is achieved by comparing the signal of unknowns to HSA standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-human, albumin, biotinylated Affinity purified goat antibody conjugated to biotin in a protein matrix with preservative. 1x12mL	**F056**
Anti-albumin coated microtiter strips 12x8 well strips in a bag with desiccant	**F058***
Human Serum Albumin Standards Human Serum Albumin in a protein matrix with preservative. Standards at 0, 0.5, 2, 5, and 20ng/mL. 1 mL/vial	**F057**
Streptavidin:Alkaline Phosphatase In a protein matrix with preservative. 1x12mL	**F009A**
PNPP Substrate p-nitrophenyl phosphate in a Diethanolamine buffer with preservative. 1x12mL	**F008**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**

*All components can be purchased separately except # F058.

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.
- The substrate reagent should not be used if its absorbance at 405nm is greater than 0.4.
- 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 405 & 492nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.)*

- Pipettors - 50µL 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.
- The human serum albumin 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.
- Avoid impurities of kit reagents with equipment or work areas that have been in contact with concentrated sources of human serum albumin containing products such as human serum or cell culture media. If possible use dedicated pipettes and tips with aerosol barrier filters to minimize chances for impurities from these sources of HSA.

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 0.300, evaluate plate washing procedure for proper performance.

2. Dilution of samples will be required for samples greater than 20ng/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 human serum albumin.

3. High Dose Hook Effect may be observed in samples with very high concentrations of albumin. Samples greater than 20µg/mL may give absorbances less than the 20ng/mL standard. 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 HSA, it is possible to inadvertently contaminate the kit reagents with various external sources of HSA. Such HSA impurities can arise from use of pipetting or other laboratory equipment or surfaces that have come into contact with more concentrated forms of HSA. For example, human serum contains on the order of 40 mg/mL of HSA or about two million fold greater than the highest standard 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

- Cross reactivity of these antibodies with albumin from other species has not been extensively investigated. Use of this kit to measure human serum albumin in samples containing significant quantities of albumin from another species should be appropriately qualified.

- 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 20ng/mL standard 1 part to 3 parts of the matrix containing no or very low levels of HSA. This diluted standard when assayed as an unknown should give an added value of 4 to 6ng/mL. In cases where HSA concentrations are high enough to allow for 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.

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.

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 the 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 and streptavidin incubations steps by about 30 minutes to achieve comparable results to the 1 hour shaking protocol. **Do not shake during the 1 hour 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 405nm for the test wavelength and 492nm for the reference wavelength. Blank the instrument using the zero standard wells after assay completion.

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

- Maintain a repetitive timing sequence from well to well for all assay steps to ensure 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 yellow color prior to the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.5 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

- 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.

Assay Protocol

1. Pipette 50µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of biotinylated anti-human albumin (#F056) into each well.

3. Cover & incubate on orbital shaker at 400 – 600 rpm for 1 hour 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 Streptavidin:Alkaline Phosphatase.

5. Pipette 100µL of Streptavidin:Alkaline Phosphatase (#F009A) into each well.

6. Cover & incubate on orbital shaker at 400 – 600 rpm for 1 hour at room temperature, 24°C ± 4°C.

7. 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 PNPP substrate.

8. Pipette 100µL of PNPP substrate (#F008).

9. Incubate at room temperature for 1 hour. DO NOT SHAKE.

10. Read absorbance at 405/492nm.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 1ng/mL. CVs for samples less than 1ng/mL may be greater than 10%.
- For optimal performance the absorbance of the substrate when blanked against water should be less than 0.4.
- 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 405-490nm	Mean Abs.
A1	Zero Std	0.000	0.001
B1	Zero Std	0.002	
C1	0.5ng/mL	0.032	0.032
D1	0.5ng/mL	0.031	
E1	2ng/mL	0.127	0.125
F1	2ng/mL	0.123	
G1	5ng/mL	0.485	0.490
H1	5ng/mL	0.495	
A2	20ng/mL	1.937	1.967
B2	20ng/mL	1.997	

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 and 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 human serum albumin 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 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 ~0.15 ng/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 ~0.28 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 2 pools with low (~2 ng/mL) and high concentrations (~20ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	7.0%	7.6%
High	4.4%	5.1%

Specificity/Cross-Reactivity

Cross reactivity in two site ELISAs can manifest itself as either a false increase in HSA levels (positive cross reactivity) or as a false decrease in true HSA (negative cross reactivity). Positive cross reactivity was evaluated by assaying the sample as an unknown. Negative cross reactivity was evaluated by spiking 5 ng/mL of HSA into each of the potential cross reactants and dividing the recovered value by 5 ng/mL. None of the materials below showed either type of cross reactivity. The antibodies used in this kit have been affinity purified to minimize cross reactivity. However cross reactivity has not been extensively evaluated in this kit. It is strongly recommended that each user test their particular sample matrix material for cross reactivity in a similar experiment.

Substance	% Cross-Reactivity
Bovine albumin	Not detectable
Mouse serum	Not detectable

Recovery/ Interference Studies

Various buffer matrices have been evaluated by adding known amounts of the HSA 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 HSA. 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 20ng/mL standard provided with this kit into 3 parts of the sample matrix in question. Recovery should be on the order of 4 to 6 ng/mL HSA. Consult *Cygnus Technologies* Technical Services if you have recovery problems in your matrix.

Hook Capacity

Increasing concentrations of HSA greater than 20 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration that gives an absorbance reading less that the 20 ng/mL standard was ~20 µg/mL.

Ordering Information / Customer Service

Cygnus Technologies also sells the following related microtiter plate ELISA kits:

Assay	Catalog #
hIgA	F165
hIgM	F170
hIgG	F160
h Transferrin	F035N
h Insulin	F040

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

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