



Human Transferrin Assay

Immunoenzymetric Assay for the Measurement of Human Transferrin Catalog # F035N

Intended Use

This kit is intended for use in quantitating human transferrin. The kit is for Research and Manufacturing use only and is not intended for diagnostic use in humans or animals.

Summary and Explanation Of the Test

The sensitive and specific quantitation of very low levels of human transferrin 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 human transferrin and thus the assay will provide accurate results even in the presence of very high concentrations of other proteins. This kit was designed to detect trace contamination of human transferrin in pharmaceutical and other biological products. The kit may be useful in quantitating human transferrin 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 contamination 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 contaminants but it may still be necessary to determine trace contamination 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 contaminants. Efforts to reduce trace media contamination to the lowest levels practical through optimal process design, validation, and final product testing require a highly sensitive and reliable analytical method. The *Cygnus Technologies* human transferrin assay is designed to provide a simple to

use, precise, and highly sensitive method to detect human transferrin to less than 100pg/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 transferrin assay is a two-site immunoenzymetric assay. Samples containing human transferrin are reacted in microtiter strips coated with an affinity purified capture antibody. A second HRP labeled anti-human transferrin antibody is reacted, forming a sandwich complex of solid phase antibody-transferrin-HRP labeled antibody. After a wash step to remove any unbound reactants the strips are then reacted with TMB substrate, followed by the addition of a stop solution changing the color from blue to yellow. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of human transferrin present in the sample. Accurate quantitation is achieved by comparing the signal of unknowns human transferrin standards assayed at the same time.

Reagents & Materials Provided

Component	Product #
Anti-human Transferrin labeled with HRP Affinity purified goat antibody in a protein matrix with preservative, 1x12mL	F039
Goat anti-transferrin coated microtiter strips 12x8 well strips in a bag with desiccant	F059
Human Transferrin Standards human transferrin in a protein matrix with preservative. Standards at 0, 0.125, 0.5, 2, and 8 ng/mL. 1mL/vial	F037
TMB Substrate 3,3',5,5'-Tetramethyl benzidine, 1x12mL	F005
Stop Solution 0.5N Sulfuric Acid, 1x12mL	F006
Wash Concentrate (20X) Tris buffered saline with preservative, 1x50mL	F004

Storage & Stability

- * All reagents should be stored a 2°C to 8°C for stability until the expiration date printed.
- * The substrate reagent should not be used if its absorbance at 450nm after adding stop solution is greater than 0.1.
- * 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 -50µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Sample Diluent, (recommend *Cygnus* Cat. # I028)
Distilled water
1 liter wash bottle for diluted wash solution

Precautions

For research or manufacturing use only.

The human transferrin 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.

This is an extremely sensitive assay for human transferrin. Many laboratories contain very concentrated sources of transferrin such as culture media or serum in which the transferrin concentration may be in the mg/mL range. These concentrated sources can contaminate work surfaces, pipettes, and other equipment as well as become airborne and then contaminate the reagents used in this kit resulting in falsely high values, poor precision or high zero standard blanks. Do not use equipment that has come into contact with concentrated sources of transferrin, clean all work surfaces and equipment prior to performing this assay, cover microtiter strips during incubation steps, and reseal all kit reagents immediately after use.

Preparation of Reagents

- * Bring all reagents to room temperature.
- * Dilute wash concentrate to 1 liter in distilled water, and 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 minus a substrate blank is greater than 0.2, evaluate plate washing procedure for proper performance.
2. Dilution of samples will be required for samples >8ng/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 validated 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 albumin.
3. High Dose Hook Effect may be observed in samples with very high concentrations of transferrin. Samples greater than 200µg/mL may give absorbances less than the 8ng/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 human transferrin, it is possible to inadvertently contaminate the kit reagents with various external sources of human transferrin. Such contamination can arise from use of pipetting or other laboratory equipment or surfaces that have come into contact with more concentrated forms of human transferrin such as serum or culture media. Airborne contamination 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 contamination.

Limitations

* This kit does not cross react with bovine transferrin. Cross reactivity of antibodies used in this kit with transferrin from other species has not been extensively investigated. Use of this kit to measure human transferrin in samples containing significant quantities of transferrin from another species should be appropriately validated. See “Specificity Section.”

* 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 (<6.0 and >8.5) or very high protein concentrations may give erroneous results. It is recommended to test the sample matrix for interference by diluting the 8ng/mL standard 1 part to 3 parts of the matrix containing no or very low levels of human transferrin. This diluted standard when assayed as an unknown should give an added value of 1.5 to 2.5ng/mL. In cases where transferrin 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.

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 microtiter plate shaker or rotator for the immunological step. 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 by about 60 minutes to achieve comparable results to the 2 hour 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 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 insure 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 this appears to be the case read 100µL of substrate + 100µL stop solution against a water blank. If the absorbance is greater than 0.1 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.

Protocol

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

2. Pipette 100µL of anti-transferrin HRP (# F039) into each well.

3. Transfer to rotator and incubate at 180rpm for 2 hours at room temperature, 24°C ± 4°.

4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding wells from a squirt bottle or by pipeting in ~350 µL. Dump and bang again. Repeat for a total of 4 washes.

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

6. Incubate for 30 minutes.

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

8. Read absorbance at 450/650nm blanking on the Zero standard.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL transferrin
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	0.125 ng/mL	0.042		
1D	0.125 ng/mL	0.041	0.042	
1E	0.5 ng/mL	0.167		
1F	0.5 ng/mL	0.163	0.165	
1G	2 ng/mL	0.665		
1H	2 ng/mL	0.675	0.670	
2A	8 ng/mL	2.188		
2B	8 ng/mL	2.341	2.267	
2C	sample 1	0.015		
2D	sample 1	0.010	0.013	<0.06
2E	sample 2	0.170		
2F	sample 2	0.158	0.164	0.5

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 greater than 0.5ng/mL. CVs for samples < 0.5ng/mL may be greater than 10%.
- For optimal performance the absorbance of the substrate when blanked against water should be < 0.1.
- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Performance Characteristics/

Cygnus Technologies has validated this assay by conventional criteria as summarized below. A more detailed copy of this "Validation Summary" report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and sample specific validation 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 albumin within or above the analytical range of this assay should be evaluated for dilutional linearity to insure 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 validation 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 ~50 pg/mL.

The lower limit of quantitation (LOQ) is defined as the lowest concentration where concentration coefficients of variation (CVs) are <20%.

LOQ is ~0.125pg/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 2 pools with low (~0.5 ng/mL) and high concentrations (~4ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	7.2%	8.5%
High	3.3%	5.6%

Specificity/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 laboratory test their particular sample matrix material for cross reactivity in a similar experiment. Sera from the following animal species was diluted 1:10 and 1:1000 and tested for cross reactivity. None of these sera gave evidence of cross reactivity with antibodies used in this kit. Sera tested: Bovine, Mouse, Canine, Chicken, Horse and Sheep. This data suggests that transferrin from these species and their other serum proteins do not cross react. The human apo and holo forms of transferrin cross react 100% on a molar basis indicating that the degree of iron saturation has no effect on the assay.

Recovery/ Interference Studies

Various buffer matrices have been evaluated by adding known amounts of the human transferrin 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 (<6.0 and >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 human transferrin. Each user should validate 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 8ng/mL standard provided with this kit into 3 parts of the sample matrix in question. Recovery should be on the order of 1.5 to 2.5 ng/mL human transferrin. Consult *Cygnus Technologies* Technical Services if you have recovery problems in your matrix.

Hook Capacity

Increasing concentrations of human transferrin >8 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration that gives an absorbance reading less than the 8 ng/mL standard was ~200 µg/mL.

Ordering Information/ Customer Service

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

Tel: 910-454-9442 Fax: 910-454-9443
Email: cygnustec@aol.com
Web site: www.cygnustechnologies.com
4701 Southport Supply Road, Suite 7
Southport, NC 28461 USA

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

Assay	Catalog #
hIgA	F165
hIgM	F170
hIgG	F160
human albumin (HSA)	F035N
h Insulin	F040