

# Bovine Transferrin Assay

## Immunoenzymetric Assay for the Measurement of Bovine Transferrin Catalog # F120

### Intended Use

This kit is intended for use in quantitating bovine transferrin. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

### Summary and Explanation

The manufacture of products by various biotechnological processes such as cell or tissue culture can result in residual pollutants 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 pollutant levels of the proteins and growth factors used in these media. Most commercial formulations of serum free media contain significant amounts of albumin and transferrin either of bovine or human 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 pollutants 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* Bovine Transferrin ELISA assay is designed to provide a simple to use, precise, and highly sensitive method to detect bovine transferrin impurities to less than 250 pg/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

This assay is a two-site immunoenzymetric assay. Samples containing bovine transferrin are reacted in microtiter strips coated with an affinity purified capture antibody. A second anti-bovine transferrin antibody labeled with the enzyme horseradish peroxidase (HRP) is reacted, forming a sandwich complex of solid phase antibody-bovine transferrin-HRP labeled antibody. After a wash step to remove any unbound reactants the strips are then reacted with Tetramethylbenzidine (TMB) substrate. The amount of hydrolyzed substrate is read

on a microtiter plate reader and will be directly proportional to the concentration of bovine transferrin present. Accurate quantitation is achieved by comparing the signal of unknowns to bovine transferrin standards assayed at the same time.

### Reagents & Materials Provided

Component	Product #
<b>Anti-bovine transferrin:HRP</b> Sheep polyclonal antibody conjugated to HRP in a protein matrix preservative. 1x12mL	<b>F123</b>
<b>Anti-bovine transferrin coated microtiter strips</b> 12x8 well strips in a bag with desiccant	<b>F122*</b>
<b>Bovine Transferrin Standards</b> Bovine transferrin in a protein matrix with preservative. Standards at 0, 0.5, 2, 8, and 32ng/mL. 1 mL/vial	<b>F121</b>
<b>Stop Solution</b> 0.5M sulfuric acid. 1x12mL	<b>F006</b>
<b>TMB Substrate</b> 3,3',5,5' Tetramethylbenzidine. 1x12mL	<b>F005</b>
<b>Wash Concentrate (20X)</b> Tris buffered saline with preservative. 1x50mL	<b>F004</b>

\*All components can be purchased separately except # F122.

### Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- Reconstituted wash solution is stable until the expiration date of 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.

## 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 - 50 $\mu$ L and 100 $\mu$ L
- Repeating or multichannel pipettor - 100 $\mu$ L
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # F031A)
- Distilled water
- 1 liter wash bottle for diluted wash solution

## Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H<sub>2</sub>SO<sub>4</sub>. Avoid contact with eyes, skin, and clothing.
- 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 0.250, evaluate plate washing procedure for proper performance.

2. This kit is a very sensitive assay for bovine transferrin (less than 250pg/mL). Since bovine serum and transferrin are common reagents in many laboratories and are often used at relatively high concentrations it is very important to use extreme care to avoid contamination of any of the reagents in this kit with external sources of bovine transferrin. Bovine transferrin

pollutants will manifest itself as either high assay background, poor precision, or unexpected results.

3. Dilution of samples will be required for samples greater than 32ng/mL. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat. # F031A 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 F031A its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilution artifacts. Other prospective diluents should be qualified in the assay to demonstrate that they do not give elevated background and are not contaminated with BSA. The diluent should also give acceptable recovery when spiked with known quantities of BSA.

4. High Dose Hook Effect may be observed in samples with very high concentrations of bovine transferrin. Samples greater than 40 $\mu$ g/mL may give absorbances less than the 32ng/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.

## 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 32ng/mL standard 1 part to 3 parts of the matrix, which does not contain any bovine transferrin. This diluted standard when assayed as an unknown should give an added value of 6 to 10ng/mL. In cases where bovine transferrin levels 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.

## 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 30 minutes 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.**
- Avoid the assay of samples containing Sodium Azide, (NaN<sub>3</sub>) which will destroy the HRP activity of the conjugate and could result in the under-estimation of bovine transferrin levels in that sample.
- 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. Samples that could contain very high levels of bovine transferrin above the 32ng/mL standard and in the "Hook" region of this assay should also be assayed diluted. Avoid the use of diluents which contain NaN<sub>3</sub> or could be contaminated with trace levels of bovine transferrin (See Procedural Note #3).
- 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 blue color prior to the assay it may have been contaminated. If this

appears to be the case, read 100 $\mu$ L of substrate plus 100 $\mu$ L of 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.

- Plates should be read within 30 min. after adding stop solution since color will fade over time.
- 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 50 $\mu$ L of standards, controls and samples into wells indicated on work list.
2. Pipette 100 $\mu$ L of anti:bovine transferrin:HRP (#F123) into each well.
3. Cover & incubate on orbital shaker at 400-600 rpm for 1 hour at room temperature, 24°C  $\pm$  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 ~350 $\mu$ L. 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 100 $\mu$ L of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100 $\mu$ L 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 – 32ng/mL. CVs for samples less than 1ng/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.
- A qualification summary detailing other assay performance parameters is available on request from Cygnus Technologies.

## Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.002	0.000
B1	Zero Std	-0.002	
C1	0.5ng/mL	0.035	
D1	0.5ng/mL	0.038	0.037
E1	2ng/mL	0.162	
F1	2ng/mL	0.170	0.166
G1	8ng/mL	0.650	
H1	8ng/mL	0.672	
A2	32ng/mL	1.952	1.940
B2	32ng/mL	1.928	

## 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 bovine transferrin 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 ~200 pg/mL in the recommended protocol. The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are less than 20%. The LOQ is ~250 pg/mL.

### Precision

Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the standard deviation by the mean value for a number of replicate determinations of two different control samples in the low and high concentration range of the assay. Both intra and inter-assay (n=5 assays) precision were determined on 2 pools with low (~0.5ng/mL) and high concentrations (~8ng/mL).

Intra-assay			Inter-assay		
# of tests	Mean ng/mL	%CV	# of assays	Mean ng/mL	%CV
20	0.52	6.2	5	0.51	6.9
20	8.1	4.1	5	7.9	5.6

## Specificity/Cross-Reactivity

In sandwich ELISA cross reactivity can manifest itself either as a false increase in bovine transferrin (positive cross reactivity) or as a false decrease in bovine transferrin (negative cross reactivity) when bovine transferrin present in the sample competes with the cross reactant for the kit antibodies. No cross reactivity was detected to human and mouse transferrins at the concentrations indicated both in the absence of bovine transferrin and in the presence of 15 ng/mL bovine transferrin. The holo and apo forms of bovine transferrin cross react at 100% and thus the degree of iron saturation appears to have no effect in quantitation of the transferrin itself. It is recommended that each user test known materials in their sample matrices for cross reactivity in a similar experiment.

Materials Tested for Cross-Reactivity to Bovine Transferrin		
Substance	Concentration Tested	% Cross-Reactivity
Human transferrin	1 mg/mL & 1 µg/mL	0
Mouse transferrin	1 mg/mL	0
Holo bovine transferrin	8ng/mL	100
Apo bovine transferrin	8 ng/mL	100

## Recovery/ Interference Studies

Various buffer matrices have been evaluated by adding known amounts of bovine transferrin. 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 6.0 and greater than 8.5), high salt concentrations, as well as certain detergents can cause under-recovery. Some product proteins in high concentration may also interfere in the accurate measurement of bovine transferrin. Each user should qualify that their sample matrices yield accurate recovery. Such an experiment can be easily performed by diluting the 32ng/mL standard provided with this kit into the sample matrix in question. For example, add 1 part of the 32ng/mL standard to 3 parts of the matrix containing no or very low bovine transferrin contaminants. This diluted standard when assayed as an unknown should give an added bovine transferrin value of 6 to 10 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

## Hook Capacity

Increasing concentrations of bovine transferrin greater than 32 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 32 ng/mL standard, was 40 µg/mL.

## Ordering Information/ Customer Service

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

[www.cygnustechnologies.com](http://www.cygnustechnologies.com)

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