



# 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 Of the Test

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. 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 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* Bovine Transferrin ELISA assay is designed to provide a simple to use, precise, and highly sensitive method to detect bovine transferrin contamination 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 Tetramethyl benzidine (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

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

### 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 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. # 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 and skin. Flush exposed areas immediately with distilled water.**
- **At the concentrations used in this kit none of the other reagents are believed to be hazardous.**

## 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. 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. This kit is a very sensitive assay for bovine transferrin (< 250pg/mL). Since bovine serum and transferrin are a 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 contamination will manifest itself as either high assay background, poor precision, or unexpected results.
3. Dilution of samples will be required for samples >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 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 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 40µg/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 (<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 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.

## Specificity

Antibodies in this kit were tested with human and mouse transferrin and were found to cross react <0.0001 % on a weight for weight basis.

## 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 microtiter plate shaker 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 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 Procedure Note #3).
- \* 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 plus 100µL 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.

## 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 1ng/mL. CVs for samples < 1ng/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.
- A validation summary detailing other assay performance parameters is available on request from *Cygnus Technologies*.

## Protocol

- 1. Pipette 50µL of standards, controls and samples into wells indicated on work list.**
- 2. Pipette 100µL of anti-bovine transferrin:HRP (#F123).**
- 3. Transfer to rotator and incubate at 180rpm for 1 hour 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 substrate.**
- 6. Incubate for 30 minutes.**
- 7. Pipette 100µL of Stop Solution.**
- 8. Read absorbance at 450/650nm, blanking on the Zero standard.**

## Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL bovine transferrin
<b>1A</b>	Zero Std	<b>0.002</b>		
<b>1B</b>	Zero Std	<b>-0.002</b>	<b>0.000</b>	
<b>1C</b>	.5ng/mL	<b>0.035</b>		
<b>1D</b>	.5 ng/mL	<b>0.038</b>	<b>0.037</b>	
<b>1E</b>	2ng/mL	<b>0.162</b>		
<b>1F</b>	2ng/mL	<b>0.170</b>	<b>0.166</b>	
<b>1G</b>	8ng/mL	<b>0.650</b>		
<b>1H</b>	8ng/mL	<b>0.672</b>	<b>0.661</b>	
<b>2A</b>	32ng/mL	<b>1.952</b>		
<b>2B</b>	32ng/mL	<b>1.928</b>	<b>1.940</b>	
<b>2C</b>	sample 1	<b>0.005</b>		
<b>2D</b>	sample 1	<b>0.010</b>	<b>0.008</b>	<b>&lt;0.2ng/mL</b>
<b>2E</b>	sample 2	<b>0.164</b>		
<b>2F</b>	sample 2	<b>0.169</b>	<b>0.167</b>	<b>2ng/mL</b>

## Performance Characteristics

*Cygnus Technologies* has validated this assay by conventional criteria as indicated 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 bovine transferrin 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 ~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 <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 & 1µg/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 (<6.0 and >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 validate 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 HCPs > 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* Customer Support:**

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