Title: Quantitation of Residual Bovine Transferring Using the Cygnus Inc. ELISA Kit

SOP Number: 23105

Revision Number: 02

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Effective Date: JUN 14 2017

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

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1.0 Purpose

1.1 This SOP describes the use of the Cygnus, Inc. ELISA kit for residual Bovine Transferrin quantitation.

2.0 Scope

2.1 This SOP applies to PA/QC personnel who will perform the Bovine Transferrin ELISA.

3.0 Authority and Responsibility

3.1 The Director, Biopharmaceutical Process Analytics/ Quality Control (PA/QC) has the authority to define this procedure.

3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC personnel are responsible for performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

4.1 Bovine Transferrin ELISA kit, BDP PN 30678, Cygnus Technologies, Catalog Number F120.

4.2 BSA Sample Diluent, BDP PN 30403 Cygnus Technologies, Catalog Number F031A.

4.3 Calibrated multi-channel pipettor.

4.4 Calibrated pipettors, 2-200 µL and 100-1000 µL.

4.5 Aerosol Barrier Pipet tips, 2-200 µL, BDP PN 20673 and 1-1000 µL, BDP PN 20769, VWR or BDP approved equivalent.

4.6 Microtiter plate shaker, VWR Catalog Number 57019-600, or BDP approved equivalent.

4.7 Ziploc™ Bag, BDP PN 20339, or equivalent

4.8 Reagent reservoirs, Lonza, BDP PN 20270, or BDP approved equivalent.

4.9 Microcentrifuge tubes 0.5 mL, BDP PN 21369, or BDP approved equivalent.

4.10 Ultrapure water or equivalent.

4.11 1 Liter Graduated Cylinder to prepare 1x wash solution.

4.12 Squirt/wash bottle with the tip cut off.

4.13 Low-lint or lint-free absorbent paper, BDP PN 21493 or BDP approved equivalent.

4.14 Labsystems iEMS Microtiter Plate Reader MF with Ascent software version 2.4.2, Model Number 1401, BDP MEF 66160, or BDP approved equivalent.

5.0 General Comments

5.1 Pipetting accuracy and reproducibility are critical for the success of this assay.

5.2 Use a new pipette tip for each pipetting procedure (between dilutions).

5.3 Avoid contamination of the workspace when handling Bovine Transferrin Standards, ejecting pipettes, etc. Pipette tips possessing an aerosol barrier must be used.

5.4 Good organization and attention to detail are essential to avoid confusion of sample identities and data.

5.5 Although the assay is designed to minimize matrix interference, materials such as detergents in high concentration, extremes of pH (< 6.0 and > 8.0) or very high protein concentrations may give erroneous results. A buffer blank is required to be submitted with the sample.

6.0 Preparation

NOTE: The reagents are stored at 2-8°C. Bring all reagents to ambient temperature (up to one hour). All standards, controls, and samples will be assayed in duplicate. The total volume of the samples must take this into account

6.1 Sample Preparation: (Linear range of assay = 0-32 ng/mL)

6.1.1 Prepare the test article immediately before use, undiluted and at 1:5 and 1:50 dilution using the BSA sample diluent (BDP PN 30403). If the absorbance results indicate that the value for the 1:50 dilution is greater than 32 ng/mL, then repeat the ELISA assay with appropriately-diluted samples. An example for making dilutions is the following:

- For duplicate measurements of spiked and unspiked diluted test article (a total of four 50 µL aliquots), add 100 µL test article to 400 µL diluent in a microcentrifuge tube to prepare a 1:5 dilution.
- A 1:50 dilution can be made up in microcentrifuge tubes by adding 100 µL of the 1:5 diluted test article to 900 µL diluent.

6.1.2 Prepare the spiked sample of the test article. Spiked samples can be prepared by the following.

- A spiked sample can be made up in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 8 ng/mL Bovine Transferrin, prepare a 4x dilution using the 32 ng/mL Cygnus, Inc. standard. For example, add 50 µL of the 32 ng/mL standard to 150 µL of the sample test article prepared in step 6.1.1. Vortex well.

6.2 Standard Preparation

6.2.1 Prepared standards at 0, 0.5, 2, 8, and 32 ng/mL are included in the Cygnus Bovine Transferrin kit. To increase the number of points on the standard curve, one more standard, either a 4 ng/mL and a 16 ng/mL standard, are prepared fresh immediately before use. For example:

- To make a 4 ng/mL standard, prepare a 1:1 dilution with the 8 ng/mL standard. Add 25 µL of the 8 ng/mL standard to 25 µL of the diluent directly into the well. This will need to be done in duplicate. Draw the sample and the spike up and down in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.
- To make a 16 ng/mL standard, prepare a 1:1 dilution with the 32 ng/mL standard. Add 25 µL of the 32 ng/mL standard to 25 µL of the diluent directly into the microtiter strip well. This will need to be done in duplicate. Draw in the pipette tip 2 or 3 times for thorough mixing. Use a new pipette tip with each well.

6.3 Positive, Negative, and Spike Control Preparation

6.3.1 The positive control will be either the 4 ng/mL or 16 ng/mL standard that was prepared in section 6.2.1.

6.3.2 The negative control is the BSA sample diluent (BDP PN 30403) used to make up all the dilutions.

6.3.3 A spiked negative control can be made up in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 8 ng/mL Bovine Transferrin, prepare a 4x dilution using the 32 ng/mL Cygnus, Inc. standard. For example, add 50 µL of the 32 ng/mL standard to 150 µL of the BSA Sample Diluent prepared in step 6.3.2. Vortex well.

6.3.4 Wash Solution Preparation

6.3.5 Empty the entire contents of the wash solution bottle into a 1 L graduated cylinder. Bring to 1 L with Ultrapure water. Log the wash solution in the BQC Solutions logbook. Label the solution bottle with the QCB number, initials, date prepared, and expiration date. Refer ***to SOP 22702, Solutions Used in PA***.

7.0 Procedure: The procedure is taken from the Cygnus Kit Insert (Attachment V).

Prepare an ELISA Worklist (Attachment I) by labeling the wells where the samples and standards will be placed. Count the microtiter strips needed.

7.1 Remove the required number of microtiter strips from the kit and place them in the provided frame.

7.2 Pipette 50 µL of standards, controls and samples into wells indicated on the ELISA Worklist (Attachment I). All standards, controls and samples must be assayed in duplicate.

7.3 Pipette 100 µL of anti-Bovine Transferrin:HRP into each well.

7.3.1 Pour contents of anti-Bovine Transferrin:HRP bottle into a new reagent reservoir.

7.3.2 Use a multi-pipettor to fill rows of wells. Get fresh pipette tips with each row that is filled.

7.4 Carefully place the plate into a Ziploc™ bag.

7.5 Transfer to the microtiter plate shaker and incubate for 1 hour ± 2 minutes at a setting of "2" (180 rpm).

7.6 Using a manual microtiter plate procedure, wash the plate with at least 350 µL of diluted wash solution from step 4.4 and dump. Wash a total of 4 times

7.6.1 Remove the liquid from the plate. Grab the plate from the bottom with the thumb in the middle of one side and the fingers on the other side. If the thumb and fingers slightly overlap the tabs on the ends of the middle strip or strips, the operator will usually be able to avoid having any strips fall out of the strip holder. Holding the plate over the sink, turn the plate upside down just as the operator rapidly accelerates the arm and hand downward. Abruptly stop the arm movement, causing the liquid to be forced from the strips into the sink. When done properly, there should not be any liquid on the fingers or on the outside of the strip wells or plate holder. Repeat the removal motion a second time.

7.6.2 Blot and strike the plate. Immediately blot the upside down plate onto the low-lint absorbent paper. Move the plate to an unused section of the blotting paper and allow it to drain upside down for at least 30 seconds. Strike the plate hard 4 times over the unused areas of the paper. Do not be afraid to strike vigorously. Anything short of breaking the strip holder or strips is not too hard. Wash the plate. Use the squirt bottle with the narrow portion of the tip cut off to give the largest possible orifice so that the flow will be generous and gentle. Fill all wells to overflowing with 1x wash solution. Do not worry about overflowing the wells because the bottom of the wells will be wiped off before adding the substrate. Remove the wash solution and blot and strike the plate as described in step 7.6.1 and 7.6.2. Repeat the washing procedure 3 more times for a total of 4 washes. After the last wash let the plate rest upside down for at least 60 seconds to drain. Strike the plate again for about 4-6 times rotating the plate 180° between each bang. This rotation ensures that the ends of the plate receive on average the same energy and impact.

7.6.3 Wipe the bottom outside of the wells with clean absorbent paper to remove any liquid from the washing. If the washing technique has been performed correctly the center of each well should have a small film of liquid (< 1 microliter). If the film is not uniform in terms of the area between the wells, then strike the plate more forcefully. Wells are now ready to have the substrate added to them.

Do not add substrate near the sink location where the removal and striking has taken place since the washing procedure can generate aerosols that could re-contaminate the wells or substrate.

7.7 Pipette 100 μL of the substrate as in steps 7.3.1 - 7.3.2.

NOTE: 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 μL of substrate plus 100 μL of stop solution against a water blank. If the absorbance is greater than 0.2, it may be necessary to obtain new substrate (substrates are not kit specific) or the sensitivity of the assay may be compromised.

7.8 Place the microtiter plate into a new Ziploc™ bag. Incubate at room temperature for 30 minutes ± 1 minute.

7.9 Remove the plate from the Ziploc™ bag and pipette 100 μL of the stop solution as in step 5.3.

7.10 Read absorbance at 450/630 nm, blanking on the zero standard. Refer to ***SOP 22100, Operation of the Labsystems iEMS Microtiter Plate Reader/ Dispenser MF Using Ascent Software version 2.4.2,*** for analysis of the data.

NOTE: Read the plates within 30 minutes after adding the stop solution since the color will fade over time.

8.0 Data Analysis and Acceptable Criteria

After the Ascent Bovine Transferrin Results (Attachment II) have been printed from the ELISA run, the results will need to be manually entered into an Excel Summary Worksheet (Attachment III). The Summary Worksheet is also in a Microsoft Excel format, and the requested information is transcribed from the report generated by the plate reader and calculated by the Excel application. The acceptable spike recoveries and %CV's are calculated in this Summary Worksheet.

8.1 Go to windows “Start.” Select programs and click on “Microsoft Excel.”

8.2 Click on “File” and “Open”. Select the QC/PA server

8.3 Click on the “QC Public” folder. Click on the “ELISA Results” folder. Select the specific ELISA test being analyzed; for example, BSA Template, Murine IgG Template, E. coli Host Cell Protein Template, etc.

8.4 Fill in the top portion of the summary sheet with all appropriate information, including QC number, Analyst, Date, Sample ID, Lot Number, Kit Lot Number and Expiration Date.

8.5 Fill in the “Bovine Transferrin Concentration” section with appropriate measured concentration for replicate 1 and 2 from the calculation column of the Ascent ELISA Results (Attachment II). The “Corrected Concentration,” “Average,” and “% CV” will automatically be calculated on the spreadsheet. These numbers can be compared to those on the Ascent ELISA Results. They should be approximately the same depending upon rounding numbers. The “%CV” should be less than 25% for samples > 4ng/mL.

8.6 Fill in the "Expected" and "Found" Positive Control and the "% CV" from the Ascent ELISA Results. The positive control will be one of the standards that the analyst manually made up and included in the standard curve (see step 6.2.1). The "%CV" should be less than 25%. The "Found" positive control should be 100% ± 30 of what is "Expected."

8.7 Fill in the "Found" Negative Control from the Ascent ELISA Result. The negative control is the diluent used to make up the sample dilutions. This number should be close to 0 or less than minimum (< min).

8.8 Fill in the "Found" Spike Control and the "% CV." The spike control is the diluent used to make up the dilutions spiked with a known amount of standard (see step 6.3.3). The "%CV" should be less than 25%. The "Found" spike control should be 100% ± 30 of what is "Expected."

8.9 Fill in replicate 1 and 2 for each dilution with the "Spiked Test Article Concentrations." These numbers are found on the Ascent ELISA Results under the "Calc." column for the spiked samples. These numbers are not corrected for dilutions. The "Average" and "% CV" will automatically be calculated and should be approximately the same as on the Ascent ELISA Results. The % CV should be less than 25%.

8.10 The "Percent Spike Recovery of Test Article" will be automatically calculated. The %CV should be less than 25% and the average recovery should be 100% ± 30.

8.11 If %CV and recoveries for the assay controls do not meet criteria, then the assay is invalid and will need to be rerun**.**

9.0 Documentation

9.1 Print the Excel Summary Worksheet (Attachment III) and attach it to the QC Test Request Form with a copy of the Ascent Bovine Transferrin ELISA Results (Attachment II).

9.2 The lowest dilution with the acceptable criteria listed above can be recorded on the QC Test Request Form.

NOTE: If the results are less than the lowest standard, it is recorded as < 2 ng/mL.

9.3 Record all solution preparation on the Form 23105-01, Bovine Transferrin ELISA Preparation Form (Attachment IV), and include with the QC Test Request form.

10.0 References

10.1 Cygnus Technologies Bovine Transferrin Kit Instructions.

10.2 Cygnus Suggested Manual Microtiter Plate Washing Procedure.

10.3 **SOP 22100,** *Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser MF Using Ascent Software version 2.4.2.*

10.4 **SOP 22702,** *Solutions Used in Process Analytics.*

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 1

Sample ELISA Worklist

H	G	F	E	D	C	B	A	↑
32 ng/mL Standard	16 ng/mL Standard	8 ng/mL Standard	4 ng/mL Standard	2 ng/mL Standard	0.5 ng\mL Standard	0 ng/mL Standard	BLANK 0ng/mL Std	1
32 ng/mL Standard	16 ng/mL Standard	8 ng/mL Standard	4 ng/mL Standard	2 ng/mL Standard	0.5 ng/mL Standard	0 ng/mL Standard	BLANK 0ng/mL Std	2
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED NEGATIVE CONTROL BSA diluent	NEGATIVE CONTROL BSA diluent	3
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED NEGATIVE CONTROL BSA diluent	NEGATIVE CONTROL BSA diluent	4
								5
								6
								7
								8
								9
								10
								11
								12

Assay:

Sample ID:

Date:

Analyst:

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 2

Sample Ascent Bovine Transferrin ELISA Results

Ascent Software Curve Fit1 11/25/03 11:24 AM

Session: C:\ASCENT\103003.SEE
Instrument: EMS Reader MF V2.9-0D
User name: K.Leib
Started at: 10/30/03 0:32:8PM
Actual temperature: Amb.temp.

Layout map for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	Blank	Blank	nc	nc	_014	_014				
B	Cal1	Cal1	snc	snc	s014	s014				
C	Cal2	Cal2	buf	buf						
D	Cal3	Cal3	sbuf	sbuf						
E	Cal4	Cal4	_014	_014						
F	Cal5	Cal5	s014	s014						
G	Cal6	Cal6	_014	_014						
H			s014	s014						

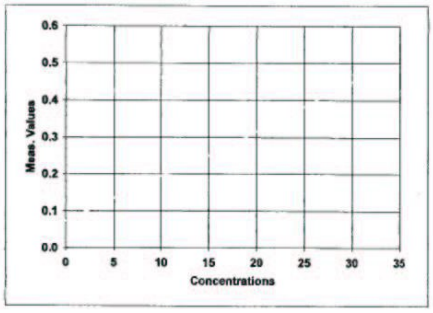
Source data for calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet: Measure1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10
A	0.071	0.071	0.069	0.068	1.891	0.515				
B	0.074	0.072	0.138	0.134	0.502	0.475				
C	0.095	0.099	0.081	0.077						
D	0.130	0.114	0.132	0.121						
E	0.174	0.169	0.185	0.176						
F	0.261	0.262	0.239	0.229						
G	0.534	0.420	0.207	0.198						
H			0.288	0.289						

Sheet: Measure1, Assay: Assay1

Name	Meas.	Conc.
Cal1	0.074	
	0.072	
	0.073	0.00
Cal2	0.095	
	0.099	
	0.097	2.00
Cal3	0.130	
	0.114	
	0.122	4.00
Cal4	0.174	
	0.169	
	0.172	8.00
Cal5	0.261	

	Conc.	Meas.	CalcConc.	Residual
Cal1	0	0.073	0	0
Cal2	2	0.097	2	0
Cal3	4	0.122	4	0
Cal4	8	0.172	8	0
Cal5	16	0.262	16	0
Cal6	32	0.477	32	0



This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 3

Sample EXCEL Summary Worksheet

Summary of Bovine Transferrin ELISA Results

QC Number:	QC-00000	**Analyst:**	K.Leib	**Date:**	9/19/2002
Sample ID:		Project Name		**Lot #:**	LXX0000
Kit Lot#:	28082	**Expiration Date:**	7/31/2003		

I. Bovine Transferrin Concentration (ng/ml)
Acceptable Criteria: %CV <25% for samples >4ng/ml

Dilution Factor	Measured Concentration		Corrected Concentration		Average	%CV
	Replicate 1	Replicate 2	Replicate 1	Replicate 2		
1.00	14.62	14.48	14.62	14.48	**14.55**	0.68
5.00	4.63	5.43	23.15	27.15	**25.15**	11.25
50.00	123.19	1.42	6159.50	71.00	**3115.25**	138.20

Final Result: **25.15ng/ml**

Corrected Concentration = Measured Concentration x dilution factor

*n/c = not calculated

Calculated Concentration of "Bovine Transferrin Spiking Solution": 32.0

Correlation Coefficient of Standard Curve: point to point

Acceptable Criteria: **%Recovery (100% ± 30) and %CV <25%**

	Expected	Found	%Recovery	%CV
Avg. Positive Control (ng/ml):	4.00	3.95	98.75%	6.23
Avg. Negative Control (ng/ml): (dilution buffer only)	0.00	<min		
Avg. Spike Control (ng/ml): (spiked dilution buffer)	8.00	6.54	81.75%	6.53

[0.0125ml x (conc. of "Bovine Transferrin Spiking Sol'n." (in ng/ml))] / 0.05 ml

Spiked Test Article Concentrations (ng/ml; not corrected for dilution):
Acceptable Criteria: %CV <25%

Dilution	Replicate 1	Replicate 2	Average	%CV
Neat	134.08	21.30	77.69	102.65
1:5	8.27	10.14	9.21	14.36
1:50	6.14	7.04	6.59	9.66

Percent Spike Recovery of Test Article:
Acceptable Criteria: Average Recovery (100% ± 30) and %CV <25%

Dilution	Replicate 1	Replicate 2	Average	%CV
Neat	706.99	112.94	409.96	102.46
1:5	72.09	83.99	78.04	10.79
1:50	6.12	77.66	41.89	120.77

Calculated by: Spiked test article concentration, ng/ml/[[(0.0375 ml) * (measured concentration, ng/ml) + (0.0125ml)* (Concentration of "Bovine Transferrin Spiking Solution", ng/ml)] / 0.05 ml]

Analyst/Date: _____

Reviewed By/Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 4

Bovine Transferrin ELISA Preparation Form

FNLCR, BDP
Form No.: 23105-01
SOP No.: 23105
Revision 02: JUN 14 2017

BOVINE TRANSFERRIN ELISA PREPARATION FORM

QC Number: _____ **Operator**:_____ **Date**:_____ **Kit Catalog #:** _____

Plate Reader MEF Number:_____ **Calibration Due Date:** _____

Diluent:

Name: _____ Lot#_____ Exp. Date: _____

Test sample Dilutions:

Test Sample #1
Name:_____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution:_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution:_____
Volume of diluent:_____µL
Volume of initial diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted sample _____µL

Test sample #2
Name:_____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution:_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution:_____
Volume of diluent:_____µL
Volume of initial Diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluent sample _____µL

Test sample #3
Name:_____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution:_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 4 (Continued)

Bovine Transferrin ELISA Preparation Form

FNLCR, BDP
Form No.: 23105-01
SOP No.: 23105
Revision 02: JUN 14 2017

Second dilution:_____
Volume of diluent:_____µL
Volume of initial diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted sample _____µL

Positive Control
Preparation of Positive Control
Expiration Date: _____
Concentration: _____ ng/mL
Std: _____µL
Sample Dilution Buffer:_____µL

Negative Control Spike
Preparation of Negative Control Spike
Expiration Date: _____
Concentration: _____ ng/mL
Std: _____µL
Sample Dilution Buffer:_____µL

Template Procedure

anti-Bovine Transferrin:HRP Antibody
Lot# _____
Expiration Date:_____
Volume of antibody added to wells:_____
Incubation Time:_____
Washing times:_____

Substrate
Lot #_____
Expiration Date:_____
Volume of substrate added to wells:_____
Incubation Time:_____

Stop solution
Lot #_____
Expiration Date:_____
Volume of stop solution added to wells:_____
Pipette#: _____

Data Storage
File Name:_____

Performed by/Date: _____
Reviewed by/Date: _____

Attachment 5

CYGNUS TECHNOLOGIES

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 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

Component	Product #
Anti–bovine transferrin:HRP Sheep polyclonal antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F123
Anti-bovine transferrin coated microtiter strips 12x8 well strips in a bag with desiccant	F122*
Bovine Transferrin Standards Bovine transferrin in a protein matrix with preservative. Standards at 0, 0.5, 2, 8, and 32ng/mL. 1mL/vial	F121
Stop Solution 0.5N 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 # F122.

Storage & Stability

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

* The substrate reagent should not be used if its stopped 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 (recommended Cat # F031A)
Distilled water
1 liter wash bottle for diluted wash solution

Attachment 5 (Continued)

Precautions

* For Research or Manufacturing use only.

* Stop reagent is 0.5N H_2SO_4. Avoid contact with eyes, skin, and clothing. At the concentrations used in this kit, none of the other reagents are believed to be harmful.

* 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 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 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.

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. These can be purchased from most laboratory supply companies. Alternatively you can purchase an approved, pre-calibrated shaker directly from *Cygnus Technologies*. 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_3) 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_3 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 insure that all incubation times are the same for each well.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 5 (Continued)

* 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. 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.

Assay Protocol

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

2. Pipette 100µL of anti-bovine transferrin:HRP (#F123) into each well.

3. Cover & incubate on rotator 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 well from a squirt bottle or by pipetting in ~350 µL. Dump and bang 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 TMB 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 blanking on the Zero standard.

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.*

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL Bovine Transferrin
1A	Zero Std	0.002		
1B	Zero Std	-0.002	0.000	
1C	0.5ng/mL	0.035		
1D	0.5ng/mL	0.038	0.037	
1E	2ng/mL	0.162		
1F	2ng/mL	0.170	0.166	
1G	8ng/mL	0.650		
1H	8ng/mL	0.672	0.661	
2A	32ng/mL	1.952		
2B	32ng/mL	1.928	1.940	
2C	sample 1	0.005		
2D	sample 1	0.010	0.008	<0.2ng/mL
2E	sample 2	0.164		
2F	sample 2	0.169	0.167	2ng/mL

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 qualification and 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

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Attachment 5 (Continued)

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 bovine transferrin > 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*:

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This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]