Molecular Devices

Threshold® Immunoassay System

ILA Application Note

Contaminant human transferrin assay

INTRODUCTION

Human transferrin is an 80,000 Dalton glycoprotein found in human serum that facilitates transport of iron between cells. The iron-poor form, apo-transferrin, combines with the Fe^{2+} ion to become holo-transferrin, the iron-saturated form. About one-third of the transferrin normally found in serum is in the Fe^{2+} saturated form. Human transferrin can be included as a media component for mammalian cell culture used to produce biopharmaceuticals. During the purification of these biopharmaceuticals, human transferrin may co-purify with the product protein as a contaminant. Since this represents a hazard in the clinical use of the product, it may be necessary to assay for the presence of human transferrin in the final product. This assay can also be used to monitor the clearance of human transferrin throughout the purification process.

This application note provides a general protocol and performance study of an assay for human transferrin using a commercially available anti-human transferrin antibody. This note is intended as a guide, and does not represent a validation, nor the optimized performance, of this assay.

MATERIALS

1 ***Threshold® System*** from Molecular Devices Corporation (catalog #0200-0500), 1311 Orleans Drive, Sunnyvale, CA 94089, tel: 408-747-1700 or 800-635-5577.

2 ***Immuno-Ligand Assay Labeling Kit*** from Molecular Devices Corporation (catalog #R9002).

3 ***Immuno-Ligand Assay Detection Kit*** from Molecular Devices Corporation (catalog #R9003).

Note: The Assay Buffer Concentrate included in the ILA kit is not used at any time in the contaminant human transferrin assay.

4 ***BSA-free Assay Buffer.*** The Assay Buffer Concentrate provided in the ILA kit contains bovine serum albumin (BSA). Although the BSA used in the preparation of the buffer is of a high grade, it may contain contaminant transferrin that can affect the assay. An assay buffer without BSA is used in the human transferrin assay. The formulation for *BSA-free Assay Buffer* is:

	10X stock (one liter)	1X final concentration
KH_2P0_4	4.08 g	3.0 mM
K_2HP0_4	12.19 g	7.0 mM
NaCl	87.7 g	150 mM
NaN_3	0.5 g	0.005% (w/v)
Triton X-100	2.5 mL	0.025% (v/v)
pH	6.5	7.0

Table 1: Formulation for *BSA-free Assay Buffer*

Prepare 500 mL of a 1:10 dilution of the 10X solution of *BSA-free Assay Buffer* with deionized water and filter through a 0.22 μm filter. Store 10X stock at 4°C. If crystals form, warm to room temperature to dissolve them before preparing the 1X solution. To prevent contamination of the buffer, use only individually wrapped sterile pipet tips and glassware rinsed copiously with deionized water.

5 ***Human transferrin, "apo" form,*** from Boehringer Mannheim (catalog #1317-393). "Holo" and "mixed" forms of human transferrin from Boehringer Mannheim were tested, as well as the "apo" and "holo" forms from Pentex® Miles, Inc., Kankakee, IL 60901, USA, tel: 815-937-8270. The "apo" form from Boehringer Mannheim was used as the standard antigen for the experiments described in this application note.

6 ***Goat anti-transferrin (human) affinity-purified antibody,*** E•Y Laboratories (catalog # AF-010-2), San Mateo, CA 94401 US, tel: 415-342-3296, fax: 415-342-2648. Other antibodies from Accurate Chemicals and Boehringer Mannheim were also tested. European customers may consider using a human transferrin antibody isolated from chickens from Immunesystem AB (catalog # 02-020), Dag Hammarskjölds väg 26, Uppsala, P.O. Box 8012, S-750 08 Uppsala, Sweden, tel: 46-18-53 89 09, fax: 46-18-53 89 97.

7 ***Ovalbumin***, Sigma Chemical Co. (catalog #A-5503).

8 ***PD-10® Sephadex G-25® columns***, Pharmacia Biotech (catalog #17-0851-01).

9 ***Samples*** were kindly donated by customers of Molecular Devices Corporation.

METHODS

Labeling and storage of antibodies

The goat anti-transferrin antibody was labeled as described in the ILA section of the *Threshold System Operator's Manual* and the ILA application note *Optimizing the labeling of proteins*. The anti-human transferrin is supplied in buffer containing

sodium azide, a preservative, and must be removed by dialysis prior to labeling. Therefore, the antibody was dialyzed against phosphate buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.0) overnight at 4°C. DNP-biotin-NHS or fluorescein-NHS hapten was incubated with 250 µg of the antibody for 2 hours at room temperature protected from light. The molar coupling ratio (MCR) is defined as the number of moles of biotin or fluorescein hapten per mole of protein (antibody) used in the labeling reaction. An MCR of 20:1 was used for the fluorescein and biotin labelings.

The unreacted hapten was separated from the labeled antibody by passing the reaction mixture over a Pharmacia PD-10 column which had been equilibrated with 25 mL of PBS. The protein concentration, protein recovery, and molar incorporation ratio (MIR) were calculated as described in the ILA section of the *Threshold System Operator's Manual*. The MIR is defined as the average number of moles of hapten covalently bound per mole of protein (antibody). Table 2 shows the molar incorporation ratios obtained for three independent labeling reactions. The stock concentration of the antibody was approximately 0.5 mg/mL.

Labeling #	MIR Biotin/Ab	MIR Fluorescein/Ab
1	4.7	5.4
2	4.3	4.1
3	4.4	4.2

Table 2: Molar incorporation ratios obtained for three labelings

After labeling, the antibodies were diluted to 10 µg/mL in *BSA-free Assay Buffer* with 1 mg/mL ovalbumin as a carrier protein. The aliquots were stored at -20°C in Sarstedt sterile tubes.

Determining the optimal concentration of antibodies per test (loading study)

Three concentrations of biotinylated and fluoresceinated anti-human transferrin antibodies (10, 20, and 40 ng/test) were tested with four concentrations of human transferrin (0, 0.2, 2.0, and 20 ng/mL) to evaluate the background rate and the "slope" of the reaction. The slope is a measurement of the change in signal as a function of change in antigen concentration. A sandwich format with a sequential incubation protocol was used with a 2 hour incubation at room temperature (see the ILA section of the *Threshold System Operator's Manual*).

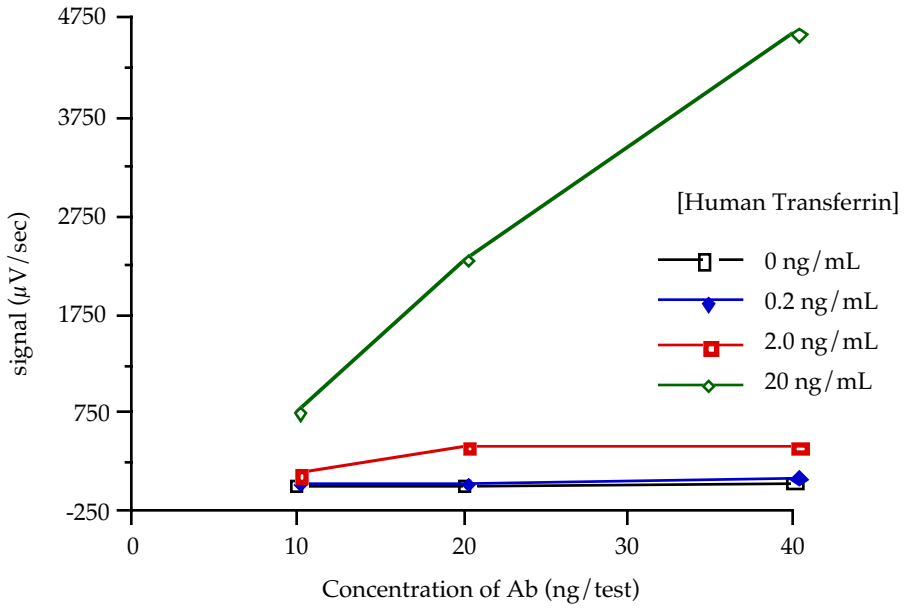

Figure 1: Loading study

The data in Figure 1 show that the antibody combination containing 20 ng/test (200 ng/mL) of each of the labeled antibodies generated acceptable background signal (at 0 ng/mL human transferrin) and also generated satisfactory signal increases at the 0.2, 2, and 20 ng/mL antigen levels. An antibody concentration of 20 ng/test of each antibody was used for the remainder of the experiments cited in this application note.

Assay protocol

Step 1 Prepare the human transferrin standard curve in polypropylene tubes. Make dilutions of human transferrin standard (10 mg/mL stock concentration) in *BSA-free Assay Buffer*. The standards range from 0.2 ng/mL to 20 ng/mL, with intermediate standards at 0.5, 1.0, 2.0, 5.0, and 10 ng/mL.

Step 2 Dilute samples with *BSA-free Assay Buffer* in polypropylene tubes.

Step 3 Prepare a mixture of the biotinylated and fluoresceinated antibodies, each at a concentration of 20 ng/test (200 ng/mL) in *BSA-free Assay Buffer* in a single polypropylene tube.

Step 4 Dispense 100 μL of each human transferrin standard and each sample into the appropriate tubes.

Step 5 Dispense 100 μL of the antibody combination prepared in Step 3 into the tubes using an Eppendorf Repeater Pipetter and a Combitip®.

Step 6 Cover the tubes with Parafilm®, shake the rack to mix, and incubate for 2 hours at room temperature.

Step 7 Reconstitute the Capture Reagent with 25 mL of *BSA-free Assay Buffer*. Prepare a 1:10 dilution of the reconstituted Capture Reagent with *BSA-free Assay Buffer* (1 volume Capture Reagent + 9 volumes *BSA-free Assay Buffer)*. When the incubation is complete, dispense 1 mL of the diluted

Capture Reagent into each tube using an Eppendorf Repeater Pipetter and a Combitip.

Step 8 Transfer the reaction mixtures to the filtration units. The filter bases and filter blocks may be either new or re-used (see ILA Detection Kit package insert for cleaning instructions). Filter on low vacuum.

Step 9 During the filtering step, reconstitute the Enzyme Reagent with 4 mL of *BSA-free Assay Buffer* per vial. Prepare a 1:10 dilution of the reconstituted Enzyme Reagent (1 volume of Enzyme Reagent + 9 volumes of *BSA-free Assay Buffer*).

Step 10 When the wells of the filtration units are empty, dispense 2 mL of Wash Buffer into each well and filter on high vacuum. Turn off the vacuum.

Step 11 Dispense 1 mL of the diluted Enzyme Reagent into each well with an Eppendorf Repeater Pipetter and a Combitip and filter on low vacuum.

Step 12 When the wells of the filtration units are empty, dispense 2 mL of Wash Buffer into each well and filter on high vacuum. Turn off the vacuum and read the sticks.

ASSAY CHARACTERIZATION

Standard curve

The lowest calibrator of the standard curve was selected by calculating the concentration of human transferrin that would generate a 30-50 µV/sec increase in signal over the background signal. This calculation was made based on the results of the loading study (see "*Sandwich Assay Optimization*" in the ILA section of the *Threshold System Operator's Manual*). The other standard curve calibrators were distributed over a 2 log (100 fold) range above the lowest calibrator (see Figure 2). A quadratic equation best defined the standard curve.

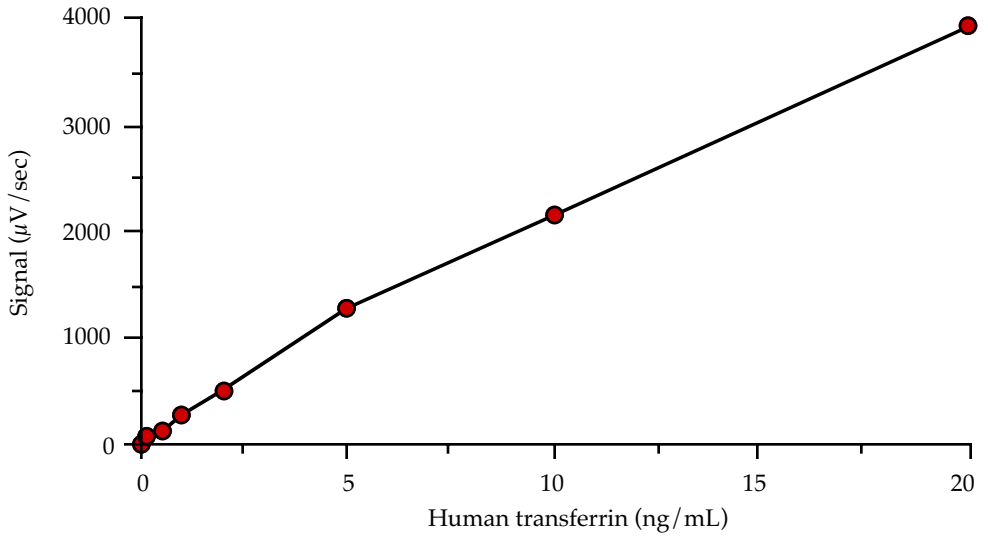

Figure 2: Standard curve

Incubation time

A kinetic study was performed to determine the optimum incubation time. Six concentrations of human transferrin (20, 10, 5, 1, 0.5, and 0.2 ng/mL) were incubated with labeled antibodies for 2 hours, 1 hour, 0.5 hour, and 0.25 hour. All incubation time periods generated similar background signals. Figure 3 shows that an increase in slope was observed when the incubation time increased.

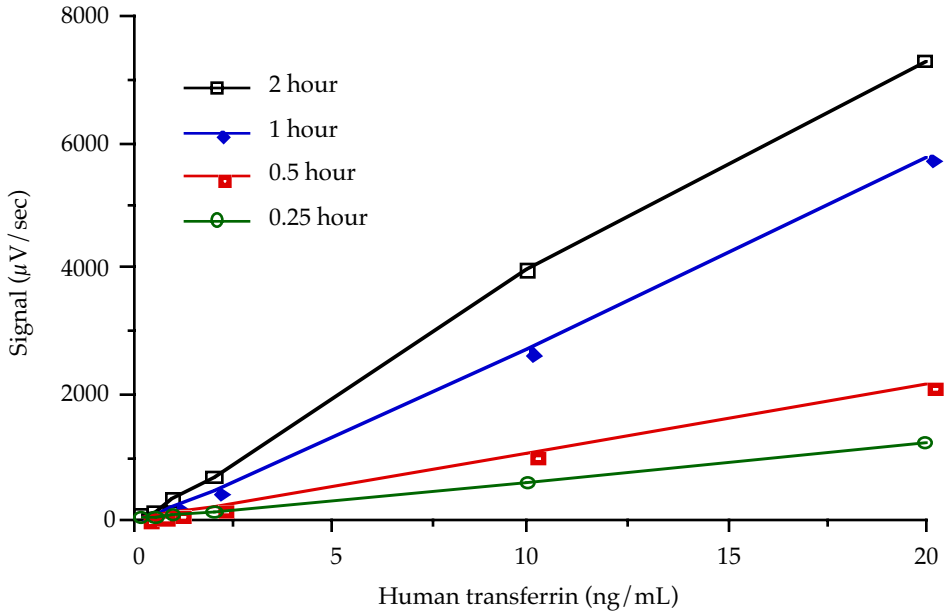

Figure 3: Kinetic study

Limit of detection

See the *Threshold System Operator's Manual* for information on evaluating the limit of detection. This specific assay allows detection of 0.2 ng/mL human transferrin with a four standard deviation separation from the background.

APPLICATIONS

The assay performance was evaluated by testing different protein samples for human transferrin contamination. The samples were tested non-spiked and spiked with a known concentration of human transferrin, and the percentage spike recovery (% SR) was calculated as follows:

$$\frac{mean\ (ng/mL)\ spiked\ sample - mean\ (ng/mL)\ non\text{-}spiked\ sample}{mean\ (ng/mL)\ spiked\ buffer - mean\ (ng/mL)\ non\text{-}spiked\ buffer} \times 100 = \%SR$$

Acceptable spike recovery is defined as 100 ± 20%, and is used to determine whether a sample interferes in the accurate quantitation of contaminant human transferrin.

Murine IgG samples

Two different murine IgG antibody samples were tested for contaminant human transferrin. Both samples were evaluated with and without a 1000 pg/mL spike of human transferrin. Sample #1 was tested at a 1:50,000 dilution and Sample #2 was tested at a 1:500 dilution. The results are listed in Table 3.

Sample	Spiked (pg/mL)	Non-spiked (pg/mL)	Net Spike (pg/mL)	Spike Recovery
BSA-free Assay Buffer	1014.7	0.0	1014.7	---
Sample #1	2454.2	1491.9	962.3	95%
Sample #2	2470.9	1512.1	958.8	95%

Table 3: Analysis of murine IgG for contaminant human transferrin

The levels of human transferrin in Sample #1 and Sample #2 were determined to be 74.6 μg/mL and 756 ng/mL, respectively.

Assay precision

The two murine IgG samples specified above were tested in triplicate on six different days to study the day-to-day reproducibility. The repeatability within the same experiment was studied by testing six replicates. The following tables demonstrate good within assay repeatability and day-to-day reproducibility.

Sample #1 Within Assay Repeatability		Sample #2 Within Assay Repeatability	
Day	Quantitation (pg/mL)	Day	Quantitation (pg/mL)
1	1436.9	1	1505.7
	1530.8		1440.0
	1480.4		1425.3
	1549.1		1427.6
	1612.4		1517.9
	1430.1		1434.2
Mean	1506.6	Mean	1458.4
Std. Dev.	70.7	Std. Dev.	41.8
C.V.	4.7%	C.V.	2.9%

Table 4: Repeatability of the human transferrin assay

Sample #1 Day-to-Day Reproducibility		Sample #2 Day-to-Day Reproducibility	
Day	Quantitation (pg/mL)	Day	Quantitation (pg/mL)
1	1393.0	1	1353.1
2	1407.8	2	1430.7
3	1470.8	3	1568.0
4	1469.0	4	1599.2
5	1506.6	5	1458.5
6	1491.9	6	1512.1
Mean	1456.5	Mean	1486.9
Std. Dev.	45.9	Std. Dev.	91.3
C.V.	3.2%	C.V.	6.1%

Table 5: Reproducibility of the human transferrin assay

SUMMARY

The data presented in this application note were generated using a 2 hour incubation and 20 ng/test of each labeled anti-human transferrin antibody. These conditions allow a dynamic range of two logs (100 fold), and a detection limit for human transferrin of 0.2 ng/mL. The assay may be adjusted to meet different requirements: the incubation time can be reduced for a faster assay, or increased for a more sensitive assay. Validation of assay performance should be determined for each product tested.

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