Molecular Devices

Threshold® Immunoassay System

ILA Application Note

Contaminant BSA assay

INTRODUCTION

Bovine serum albumin (BSA), a 63,000 Dalton protein, is a major constituent of bovine serum. Fetal calf serum containing BSA can be used in mammalian cell culture media for the production of recombinant pharmaceuticals. During the purification process of a recombinant pharmaceutical, BSA may be co-purified with the product protein, and may represent a biological hazard for clinical use. Therefore, it is necessary to assay for contaminant BSA in a final product originating from mammalian cells. An assay for BSA can also be used to monitor the purification of the product throughout the process.

This application note provides a preliminary protocol and performance of a BSA assay using a commercially available anti-BSA antibody. This note is intended as a guide and does not represent a validation of this assay, nor necessarily the optimal performance parameters.

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 BSA assay. This is because the assay buffer contains BSA, which interferes with the assay.

4 ***BSA-free Assay Buffer.*** 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 ***BSA, fraction V,*** was purchased from Boehringer Mannheim (catalog #100 350). Other sources of BSA have been tested (Boehringer Mannheim Molecular Biology grade, Pentex Miles Reagent Preparation grade, Sigma RIA grade), with comparable results.

6 ***Ovalbumin*** was purchased from Sigma Chemical Co. (catalog # A-5503).

7 ***Anti-BSA affinity purified polyclonal chicken antibody*** was purchased from O.E.M. Concepts, Toms River, New Jersey, tel: 908-341-3570.

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

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

METHODS

Labeling and storage of antibodies

The chicken anti-BSA antibody was labeled as described in ILA section of the *Threshold System Operator's Manual* and in the ILA application note *Optimizing the labeling of proteins*.

The chicken anti-BSA antibody was dialyzed overnight at 4°C against PBS to remove sodium azide prior to labeling. DNP-biotin-NHS or fluorescein-NHS haptenwere incubated with 250 μL aliquots of a 1 mg/mL antibody solution for two hours at room temperature, protected from the light. The molar coupling ratio (MCR) is defined as the number of moles of biotin or fluorescein incubated per mole of protein. The MCR used was 20:1.

The unreacted hapten was separated from the antibody by passing the reaction solution over a Pharmacia PD-10 column which had been equilibrated with 25 mL of PBS. The protein concentration, the protein recovery and the 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.

The following table shows the molar incorporation ratios obtained for four independent labeling reactions. Three different lots of antibody were used. The stock concentration of the antibodies was approximately 1 mg/mL.

Labeling#	MIR Biotin/Ab	MIR Fluorescein/Ab
1	3.1	3.2
2	4.7	4.0
3	2.5	3.3
4	3.2	4.7

Table 2: Molar incorporation ratios obtained for four 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. Aliquots of 150 µL were stored in sterile Sarstedt tubes at -20°C.

Caution: Pipet the antibodies with individually wrapped, sterile pipet tips.

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

Four concentrations of biotinylated and fluoresceinated anti-BSA antibodies (10, 20, 30 and 40 ng/test) were tested with three concentrations of BSA (0, 200 and 2000 pg/mL) to evaluate the background rates and the slope. The slope is a measurement of the change of signal as a function of change of concentration. The sandwich format and a modified simultaneous incubation protocol were used (see the ILA section of the *Threshold System Operator's Manual*).

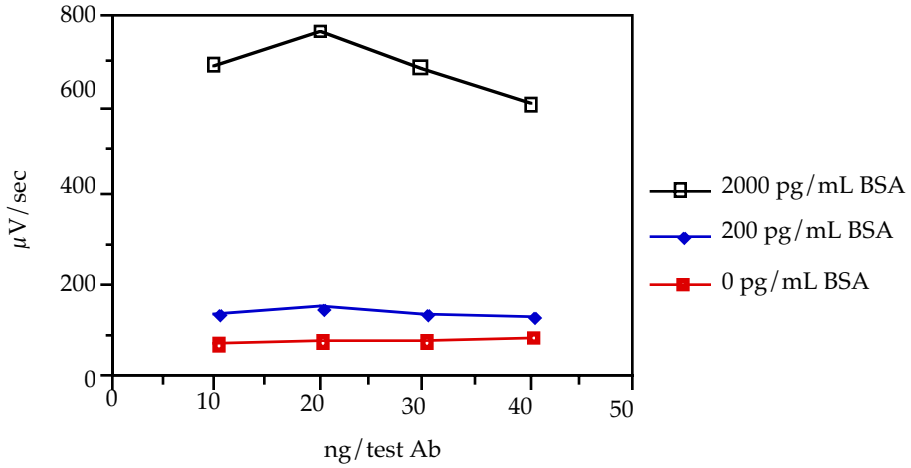

Figure 1: Loading study

A concentration of 20 ng/test (200 ng/mL) of biotinylated and fluoresceinated antibody allowed the lowest background signal (0 pg/mL BSA) and the maximum rate in the presence of BSA (200 and 2000 pg/mL BSA). The µV/sec signals observed using 30 or 40 ng/test of antibodies were lower than with 20 ng/

test because the fluoresceinated antibody exceeded the amount of Enzyme Reagent per test. An antibody concentration of 20 ng/test was used for the remainder of the experiments described in this application note.

Assay Protocol

Step 1 Prepare the BSA standard curve in polypropylene tubes. Use individually wrapped sterile pipet tips to make serial dilutions of the BSA standard (1 mg/mL stock, BSA from Boehringer Mannheim) in *BSA-free Assay Buffer*. The standards range from 10000 to 125 pg/mL of BSA. Suggested standards concentrations are 10000, 4000, 2000, 1000, 500, 250, 125 and 0 pg/mL of BSA.

Caution: This BSA assay requires the use of aseptic technique and individually wrapped sterile pipet tips. Other tips can cause high background signal. Gloves are not necessary.

Step 2 Dilute the samples with *BSA-free Assay Buffer* using individually wrapped sterile pipet tips and 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 BSA standards and samples into polypropylene tubes, using individually wrapped sterile pipet tips.

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

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

Step 7 During the 2 hour incubation, reconstitute the Enzyme Reagent with 4 mL of *BSA-free Assay Buffer*. When the incubation is complete, dispense 100 µL of Enzyme Reagent into each tube with an Eppendorf Repeater Pipetter and a sterile Combitip.

Step 8 Cover the tubes with Parafilm and incubate for 1 hour at room temperature.

Step 9 During the 1 hour incubation, reconstitute the Capture Reagent with 25 mL of *BSA-free Assay Buffer*. Prepare a 1:10 dilution of the reconstituted Capture Reagent (1 volume of Capture Reagent + 9 volumes of *BSA-free Assay Buffer*). When the incubation is complete, dispense 1 mL of the diluted Capture Reagent into each tube with an Eppendorf Repeater Pipetter and a sterile Combitip.

Step 10 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 instruction). Filter on low vacuum.

Step 11 When the wells are empty, dispense 2 mL of Wash Buffer into each well and filter on high vacuum.

Step 12 When the wells are empty, 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 BSA that would generate a 30 to 50 µV/sec increase of signal over background signal. This calculation was made using the loading study (see *Sandwich Assay Optimization* in the ILA section of the *Threshold System Operator's Manual*). The other calibrators of the standard curve were distributed over a range of approximately 2 logs (100-fold) above the lowest calibrator. Figure 2 shows a standard curve generated using BSA concentrations of 10000, 4000, 2000, 1000, 500, 250, 125, and 0 pg/mL. A quadratic equation best defines the resulting curve.

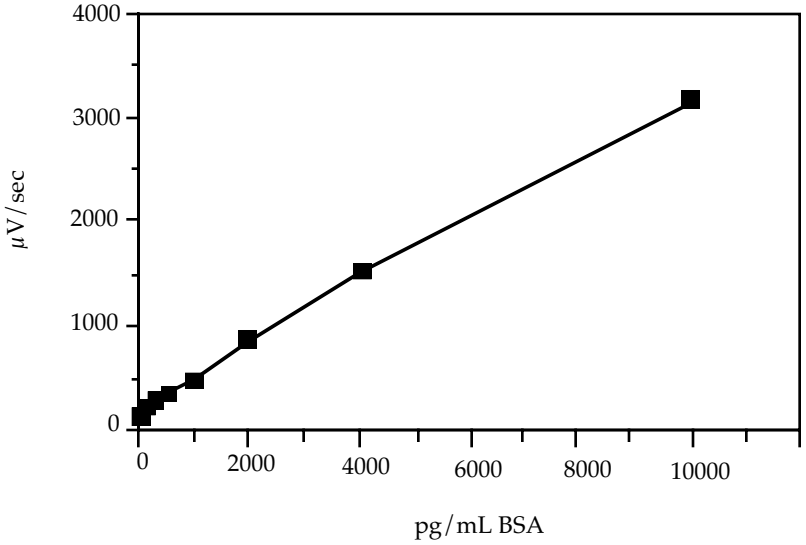

Figure 2: BSA standard curve

Incubation time

A kinetic study was performed to determine the optimum incubation time at room temperature. Three concentrations of BSA (1000, 200 and 0 pg/mL BSA) were incubated with labeled antibodies for 1 hour, 2 hours, 4 hours, and overnight. The standard curve and the on-board calibrators for each stick were incubated overnight. The Enzyme Reagent (urease conjugate) was dispensed into each tube, and all the tubes were incubated for an additional 1 hour.

The results for this kinetic study are shown in Figure 3. The overnight incubation produced an increase of signal for the standards tested, but also increased the background (0 ng/mL BSA) compared to the other incubation times. The 4 hour, 2 hour and 1 hour incubations generated the same background. However, an increase of slope was observed with an increase of the incubation time. The best sensitivity can be obtained by incubating the BSA with the antibodies for 4 hours.

However, for this application note, a 2 hour incubation with the BSA and the antibodies, then a 1 hour incubation after the addition of the urease conjugate, was used. This protocol allows satisfactory sensitivity and total assay time.

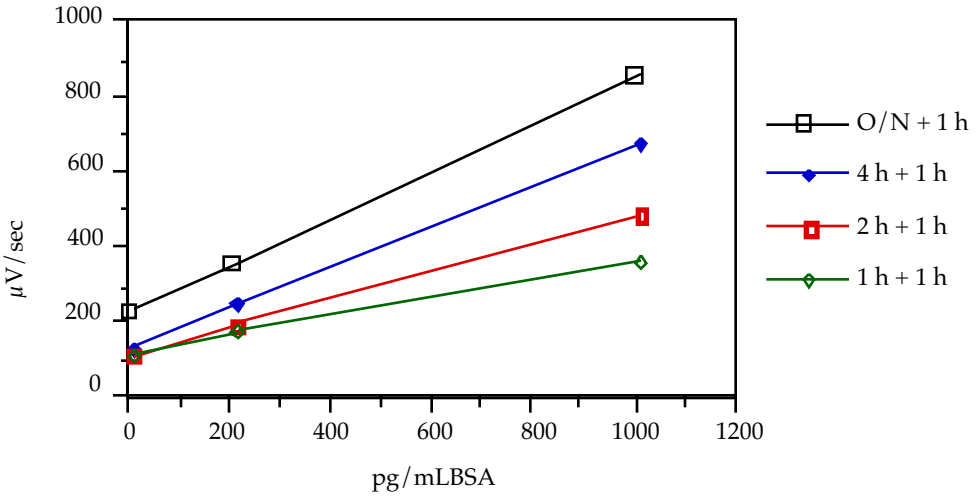

Figure 3: Kinetic study

Limit of detection:

See the *Threshold System Operator's Manual* for the evaluation of the limit of detection. This BSA assay allows detection of 125 pg/mL BSA with a 4 standard deviation separation from the background.

APPLICATIONS

The assay performance was evaluated by testing recombinant proteins that had been produced in cell culture containing fetal calf serum. Different samples were tested non-spiked and spiked with 800 pg/mL of BSA, and the spike recovery (SR) was calculated as follows:

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

Acceptable spike recovery is defined as 100% ± 20%. A good spike recovery demonstrates that the sample does not interfere in the measurement of the BSA and that the BSA quantitation is accurate.

Four recombinant pharmaceuticals were successfully tested for contaminant BSA with this assay: a murine IgG_1, a murine IgG_{2A}, a growth hormone and a therapeutic antibody.

Four lots of the therapeutic antibody were tested in the same experiment. The sample concentrations ranged from 5.3 to 6.5 mg/mL. Table 3 lists the results obtained.

Sample	Spiked (pg/mL)	Non-spiked (pg/mL)	Net Spike (pg/mL)	% Spike Recovery
BSA-free Assay Buffer	635	0	635	n/a
Lot 1 (neat)	737	83	654	103%
Lot 1 (1:2)	648	0	648	102%
Lot 2 (neat)	726	181	545	86%
Lot 2 (1:2)	688	0	688	108%
Lot 3 (neat)	610	94	516	81%
Lot 3 (1:2)	480	0	480	76%
Lot 4 (neat)	644	0	644	101%
Lot 4 (1:2)	659	29	630	99%

Table 3: Results obtained using the BSA assay on a therapeutic antibody

The Murine IgG_1 sample was tested in triplicate on 6 different days to study the day to day reproducibility. The repeatability within the same experiment was studied by testing six replicates. The final concentration of the sample was 1.4 mg/mL. Table 4 lists the results obtained.

Within assay repeatability		Day-to-day reproducibility	
Day	Quantitation (pg BSA/mL)	Day	Quantitation (pg BSA/mL)
1	1207.8	1	1189.3
	1149.9	2	948.2
	1210.2	3	1146.3
	1187.0	4	1013.1
	1204.2	5	1232.5
	1226.0	6	1040.2
Mean	1197.5	Mean	1094.9
Std. dev.	26.5	Std. dev.	111.1
C.V.	2.2%	C.V.	10.1%

Table 4: Reproducibility and repeatability of the BSA assay on a murine IgG sample

DISCUSSION

Some components of the ILA Detection Kit contain BSA: the Assay Buffer concentrate, the Enzyme Reagent, the Capture Reagent, and the biotinylated membrane on the sticks. If the protocol presented in this application note is followed, the BSA present in the Enzyme Reagent, the Capture Reagent and the biotinylated membrane does not interfere with the assay. A sequential incubation protocol, where the Enzyme Reagent is added after the streptavidin-antibody-BSA complex is captured on the membrane, produces very high and non-reproducible background signals. Preliminary work indicates that a BSA assay can also be developed using a competitive format.

SUMMARY

Data in this application note was generated using a 3 hour incubation and 20 ng/test of each labeled anti-BSA antibody. These conditions allow a dynamic range of approximately 2 logs (100-fold), and a detection limit of BSA of 125 pg/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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