Molecular Devices

Threshold® Immunoassay System

ILA Application Note

Contaminant bovine IgG assay

INTRODUCTION

Bovine IgG, a 160,000 Dalton protein, is one of the constituents of bovine serum. Fetal calf serum containing bovine IgG is commonly used in mammalian cell culture media for the production of recombinant pharmaceuticals. During the purification process of a recombinant pharmaceutical, bovine IgG may be co-purified with the product, and may represent a biological hazard for clinical use. Therefore, it may be necessary to assay for contaminant bovine IgG in a final product originating from mammalian cells. An assay for bovine IgG can also be used to monitor the purification of the product throughout the process.

MATERIALS

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

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. 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 ***Bovine IgG, whole molecule*** was purchased from Chemicon International Inc. (catalog #PP03). tel: 909-676-8080. Other sources of bovine IgG have been tested (Jackson ImmunoResearch, Calbiochem Corp.). The bovine IgG from Chemicon provided the best response. The ratio of IgG subclasses in the IgG standard may vary from supplier to supplier, and may explain the different responses observed between the different antigens tested.

6 ***Goat anti-bovine IgG affinity purified polyclonal antibody*** (heavy and light chains) was purchased from Jackson ImmunoResearch Labs Inc., tel: 610-869-4024. Other sources of antibody have been tested (rabbit anti-bovine IgG from Jackson ImmunoResearch, chicken anti-bovine IgG from Chemicon International). The goat antibody from Jackson ImmunoResearch provided the best response.

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

8 ***Streptavidin*** was purchased from Scripps Laboratories (catalog #S1214), tel: 619-546-5800. A stock solution of 25 mg/mL of streptavidin in deionized water was prepared. The storage temperature was 4°C.

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

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

METHODS

Labeling of antibody

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

The goat anti-bovine IgG antibody is supplied in phosphate buffer and does not contain preservatives. Therefore the antibody was not dialyzed prior to labeling. DNP-biotin-NHS or fluorescein-NHS hapten was incubated with 250 µg of antibody, 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 hapten per mole of protein (antibody) used in the labeling reaction. The MCR used was 10:1 for the fluorescein and 20:1 for the biotin.

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 (10 mM phosphate, 150 mM NaCl, pH 7.0). 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. Table 2 shows the molar incorporation ratios obtained for four independent labeling reactions. The stock concentration of the antibody was 2 to 3 mg/mL.

Labeling#	MIR Biotin/Ab	MIR Fluorescein/Ab
1	3.7	3.2
2	4.1	3.1
3	4.6	5.8
4	4.6	5.2

Table 2: Molar incorporation ratios obtained for four labelings

All labelings were performed using 250 µg of antibody, molar coupling ratios of 10:1 for the fluorescein and 20:1 for the biotin, and a 2 hour incubation time.

After labeling, the antibodies were diluted to 20 µg/mL in *BSA-free Assay Buffer* with 1 mg/mL ovalbumin as a carrier protein. Aliquots of 150 µL were stored at -20°C in Sarstedt sterile tubes.

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

Four concentrations of biotinylated and fluoresceinated anti-bovine IgG antibodies (20, 40, 60 and 80 ng/test) were tested with three concentrations of bovine IgG (0, 200 and 2000 pg/mL) to evaluate the background rate and the slope. The slope is a measurement of the change of signal as a function of change of antigen concentration. The sandwich format and a sequential incubation protocol were used (See the ILA section of the *Threshold System Operator's Manual*).

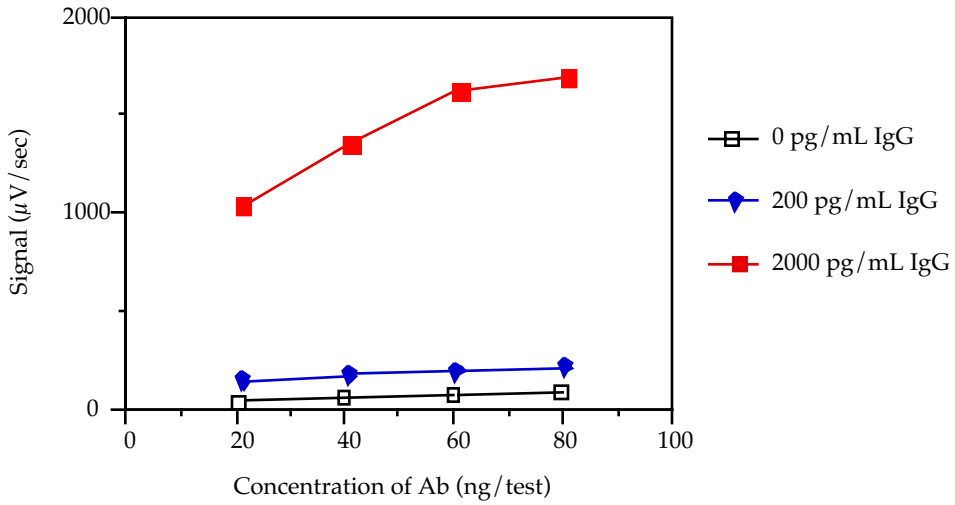

Figure 1: Loading study

Figure 1 shows that a concentration of 40 ng/test (400 ng/mL) of biotinylated antibody and 40 ng/test (400 ng/mL) of fluoresceinated antibody allowed low background signal (with 0 pg/mL bovine IgG) and satisfactory rates in the presence of bovine IgG (with 200 and 2000 pg/mL bovine IgG). An antibody concentration of 40 ng/test was used for the remainder of the experiments in this application note.

Assay Protocol

Step 1 Prepare the bovine IgG standard curve in polypropylene tubes. Make serial dilutions of the bovine IgG standard (approximately 20 mg/mL stock bovine IgG from Chemicon International) in *BSA-free Assay Buffer*. The standards range from 5000 to 50 pg/mL of bovine IgG. Suggested standard concentrations are 5000, 2000, 1000, 500, 200, 100, 50 and 0 pg/mL of bovine IgG.

Caution: A few instances of high background have been observed when using regular pipet tips (and not individually wrapped sterile tips). Nevertheless, the contamination occurrences could never be reproduced or conclusively attributed to the regular pipet tips.

Step 2 If necessary, dilute the 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 40 ng/test (400 ng/mL) in *BSA-free Assay Buffer* in a single polypropylene tube.

Step 4 Dispense 100 μL of bovine IgG standards and samples into polypropylene 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 Dilute the streptavidin with *BSA-free Assay Buffer* (stock solution is 25 mg/mL) to 2 µg/mL. When the incubation is complete, dispense 1 mL into each tube with 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 in 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 in each well, and filter on high vacuum.

Step 13 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 bovine IgG that would generate a 30 to 50 µV/sec increase of 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 calibrators of the standard curve were distributed over a range of 2 log (100^{-} fold) above the lowest calibrator. A quadratic equation best describes the data (see Figure 2).

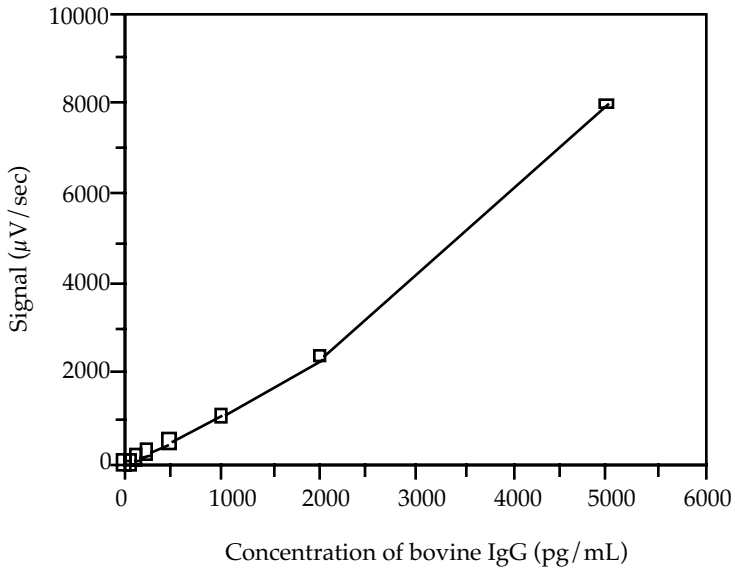

Figure 2: Bovine IgG standard curve

Incubation time

A kinetic study was performed to determine the optimum incubation time. Three standard curves of bovine IgG (5000, 2000, 1000, 500, 200, 100, 50 and 0 pg/mL bovine IgG) were incubated with labeled antibodies for 4 hours, 1 hour or 30 minutes.

The 4 hour, 1 hour and 30 minute incubations generated similar background signals. Figure 3 shows that an increase of slope was observed with an increase of the incubation time. A 30 minute incubation may be sufficient for the assay: it provides adequate sensitivity and slope.

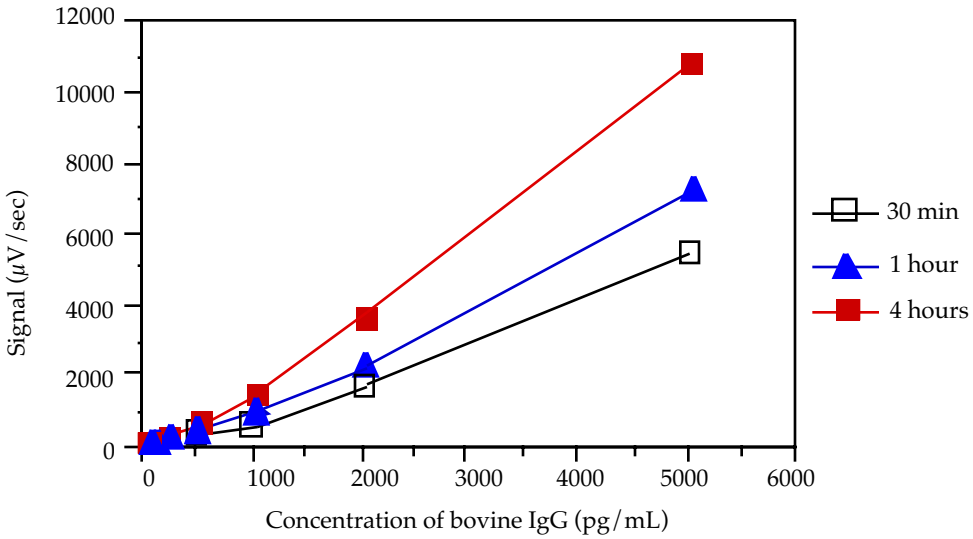

Figure 3: Effect of the incubation time

Limit of detection

See the *Threshold System Operator's Manual* for information on evaluating the limit of detection. This bovine IgG assay allows detection of 50 pg/mL bovine IgG with a 4 standard deviation separation from the background (data not shown).

Faster and less sensitive assay

Some applications may require a bovine IgG assay with less sensitivity and shorter assay time. The standard curve can be shifted to the ng range by decreasing the incubation time and adding unlabeled goat anti-bovine IgG antibody to the reaction mixture. Figure 4 displays the standard curve obtained using a 10 minute incubation. The labeled antibodies (40 ng/test) were mixed with 400 ng/test of unlabeled antibody. An aliquot of 100 µL of this antibody

mixture was then dispensed in the tubes containing the different concentrations of antigen. The standard curve ranged from 100 to 1 ng/mL. The total assay time was 1 hour.

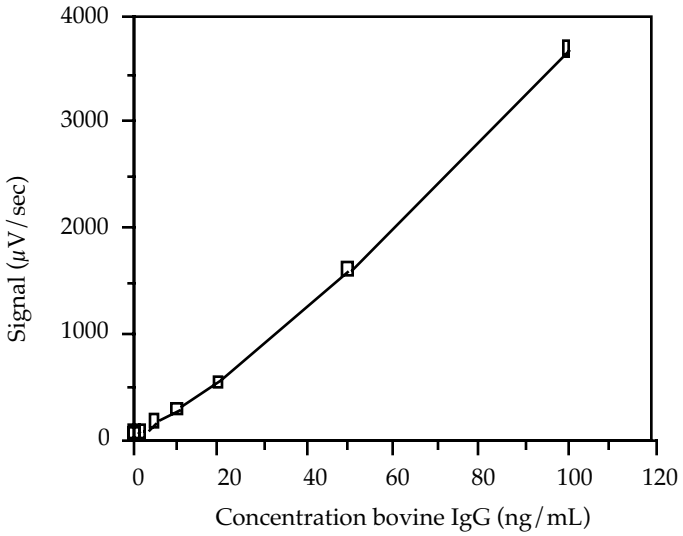

Figure 4: Bovine IgG standard curve with the fast assay protocol

Cross-reactivity

Bovine IgG is potentially homologous with IgG from other species, such as human and murine. The most common therapeutic antibodies are monoclonal human or murine IgG. The anti-bovine IgG antibodies used in this assay may react with the product antibody during the assay, and generate positive signal that may not represent true bovine contaminant, but rather inter-species cross-reactivity.

The potential cross-reactivity with murine IgG was evaluated by reacting various amounts of a murine IgG (referred to as cross-reactivity control) in the presence or absence of a fixed concentration of bovine IgG. If the sample antibody is a monoclonal generated from a cell line, the best cross-reactivity control would be the product antibody purified from mouse ascites to ensure that it contains no bovine IgG. If this is unavailable, the cross-reactivity control should be as similar as possible to the sample antibody to be analyzed, but without bovine contamination.

In one experiment, the sample antibody to be analyzed was a monoclonal murine IgG_1 purified from a recombinant cell line. The cross-reactivity control was a polyclonal murine IgG_1 isolated from mice. The polyclonal cross-reactivity control antibody was tested at 4 concentrations (11.3 mg/mL, 2.3 mg/mL, 1.1 mg/mL and 0.23 mg/mL) in the presence or absence of 1000 pg/mL of bovine IgG. The percentage of cross-reactivity was relatively high (approximately 5 to 10 ppm: for a total amount of 1 mg of polyclonal murine IgG, 5 to 10 ng were cross-reacting with the anti-bovine IgG antibodies). Such cross-reactivity between a polyclonal murine IgG and a polyclonal anti-bovine IgG is not surprising. Fortunately a therapeutic antibody sample is typically a monoclonal, and the expected probability of cross-reactivity is very low.

To test this in another experiment, the cross-reactivity control was a monoclonal murine IgG_1 purified from mouse ascites. Four concentrations of the cross-reactivity control monoclonal murine IgG_1 were tested (10 mg/mL, 2 mg/mL, 1 mg/mL and 0.2 mg/mL) in the presence or absence of approximately 1 ng/mL of bovine IgG. The assay results are displayed in Figure 5.

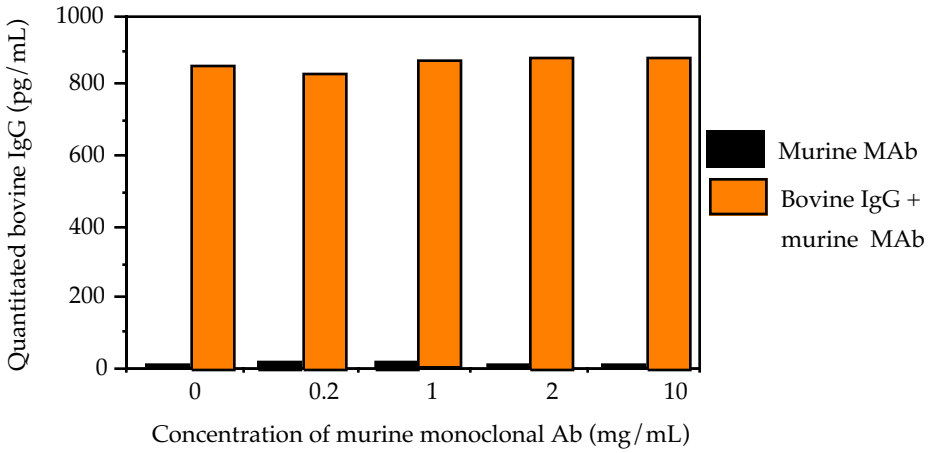

Figure 5: Cross-reactivity between bovine IgG and monoclonal murine IgG_1

The quantitated value for the monoclonal murine IgG_1 cross-reactivity control is below the limit of detection for all four concentrations tested in the absence of the bovine IgG spike. In the presence of 1 ng/mL bovine IgG, the quantitated value for the cross-reactivity control was comparable to the quantitated value for the buffer control (0 mg/mL cross-reactivity control) for all four concentrations. The polyclonal anti-bovine IgG antibodies do not bind to the monoclonal murine IgG. There is no cross-reactivity between the monoclonal murine IgG_1 sample tested and bovine IgG.

The potential cross-reactivity with human antibodies was evaluated using polyclonal human IgG isolated from human blood. We were unable to obtain a monoclonal human antibody that had not been in contact with bovine contaminants. Four concentrations of the polyclonal human cross-reactivity control (17.6 mg/mL, 3.5 mg/mL, 1.8 mg/mL and 0.4 mg/mL) were tested in the presence or absence of 1 ng/mL of bovine IgG. The cross-reactivity was 0.8 ppm: from a total amount of 1 mg of human polyclonal antibody control, 0.8 ng cross-reacted with the anti-bovine IgG antibodies. As with the murine antibody, the probability of cross-reactivity is expected to be significantly lower in the presence of a monoclonal human antibody.

APPLICATIONS

The assay performance was evaluated by testing different protein samples. The samples were tested non-spiked and spiked with approximately 1 ng/mL of bovine IgG, and the spike recovery (SR) was calculated as follows:

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

Acceptable spike recovery is defined as 100% ± 20%. Acceptable spike recovery means that the sample does not interfere in the measurement of the bovine IgG and that the bovine IgG quantitated in the sample is accurate.

BSA Samples

Two sources of BSA were tested for contaminant bovine IgG with the regular protocol assay. If BSA is used in the preparation of buffers or media, the IgG concentration may be of interest. The original sample concentrations were 840 μg/mL (supplier #1) and 930 μg/mL (supplier #2). Both BSA samples were tested non-spiked and spiked with approximately 1 ng/mL of bovine IgG. The supplier #1 BSA was tested at a 1:10 dilution, and the supplier #2 BSA at a 1:100 dilution.

Sample	Spiked (pg/mL)	Non-spiked (pg/mL)	Net Spike (pg/mL)	Actual (ng/mg BSA)	% Spike Recovery
BSA-free Assay Buffer	927	13	914		
BSA supplier #1	1357	444	913	5.2	100%
BSA supplier #2	1328	544	784	58.5	86%

Table 3: Results obtained for the BSA solutions using the regular bovine IgG assay protocol

The BSA solutions contained 5.2 ng of contaminant bovine IgG per mg of BSA for supplier #1 and 58.5 ng of contaminant bovine IgG per mg of BSA for supplier #2. The bovine IgG assay allows the comparison of BSA purity from two different vendors.

The positive response of the BSA samples in the assay may be due to two phenomena: bovine IgG contaminating the BSA solution, and/or the presence of contaminating anti-BSA antibodies in the anti-bovine IgG antibodies used in this assay. The second possibility may be minimal compared to the actual bovine IgG contamination in the BSA solution, but should be considered. If the bovine IgG immunogen used to immunize the goats is contaminated with BSA, the anti-bovine IgG antibodies purified from the goats will contain some anti-BSA antibodies. These antibodies will react with the sample BSA during the assay. These potential problems can be eliminated by saturating the anti-BSA antibodies or using a monoclonal anti-bovine IgG antibody.

Fetal calf serum

Fetal calf serum (FCS) was tested for bovine IgG with the regular protocol assay. Quantitating bovine IgG in fetal calf serum may be of interest if serum is used in the preparation of cell culture media. Each dilution tested (10^{-3} to 10^{-7}) was tested both non-spiked and spiked with approximately 1 ng/mL of bovine IgG.

Sample	Spiked (pg/mL)	Non-spiked (pg/mL)	Net Spike (pg/mL)	Actual (μg/mL FCS)	% Spike Recovery
BSA-free Assay Buffer	773	19	754		
10^{-3}	15497	15002	**	–	–
10^{-4}	2711	1935	776	19	103%
10^{-5}	975	165	810	17	107%
10^{-6}	786	10	776	*	103%
10^{-7}	777	12	765	*	101%

Table 4: Results obtained for fetal calf serum using the regular bovine IgG assay protocol. ** quantitation beyond the standard curve. * no detectable bovine IgG at this dilution.

The fetal calf serum tested contained an average of 18 μg/mL of bovine IgG.

Murine IgG sample

A murine IgG_1 sample was tested for contaminant bovine IgG with the regular protocol (tested at 1:50 and 1:100 dilutions), and compared using the fast and less sensitive assay protocol (tested neat). The original sample concentration was 14 mg/mL of murine IgG_1.

Sample	Spiked (pg/mL)	Non-spiked (pg/mL)	Net Spike (pg/mL)	Actual (ng/mL)	% Spike Recovery
BSA-free Assay Buffer	835	0	835		
Murine IgG_1 1:50	1727	763	964	38	115%
Murine IgG_1 1:100	1243	336	907	34	109%

Table 5: Results obtained for a murine IgG_1 sample using the regular bovine IgG assay protocol

When using the regular assay protocol, the mean concentration of bovine IgG detected in the sample is 36 ng/mL, which represents 2.6 ppm (2.6 ng of bovine IgG in 1 mg of murine IgG sample). The same sample was tested neat in the fast and less sensitive assay: the concentration of contaminant bovine IgG was 31 ng/mL, which is comparable to the result obtained using the regular protocol assay. This amount represents the actual contamination of bovine IgG, since the control monoclonal murine antibody produced from ascites showed no cross-reactivity between bovine and murine for this antibody subclass.

Assay precision

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 0.28 mg/mL. The following table lists the results obtained.

Within assay repeatability		Day-to-day reproducibility	
Day	Quantitation (pg bov. IgG/mL)	Day	Quantitation (pg bov. IgG/mL)
1	679	1	717
	664	2	732
	694	3	685
	712	4	743
	702	5	684
	692	6	679
Mean	691	Mean	707
Std. dev.	17	Std. dev.	28
C.V.	2.5%	C.V.	4.0%

Table 6: Reproducibility and repeatability of the bovine IgG assay

SUMMARY

Data in this application note were generated using a one-hour incubation time and 40 ng/test of each labeled anti-bovine IgG antibody. These conditions allow a dynamic range of 2 logs (100-fold), and a 50 pg/mL detection limit for bovine IgG. 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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