

Bovine Plasma Proteins Assay

Immunoenzymetric Assay for the Measurement of Bovine Plasma Proteins Catalog # F290

Intended Use

This kit is intended for use in determining the presence of bovine plasma protein (BPP) pollutants in products purified from bovine source material. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Therapeutic agents and other biological products produced in cows must be highly purified. The manufacturing and purification process of these products leaves the potential for pollutants by other proteins normally found in bovine plasma. Such pollutants can reduce the efficacy of the therapeutic agent and result in adverse toxic or immunological reactions and thus it is desirable to reduce the plasma protein impurities to the lowest levels practical. The antibodies used in this kit were generated against normal bovine plasma, affinity stripped of bovine albumin (BSA) and bovine IgG (blgG). As such, this kit will detect the presence of BPPs other than BSA & blgG. BSA & blgG are among the major proteins in bovine plasma and can be accurately quantitated by mono-specific ELISA kits (see *Cygnus Technologies'* Cat# F030 for BSA and Cat# F070 for blgG). The microtiter plate immunoenzymetric assay method employed in this kit provides a simple to use, highly sensitive, objective, and semi-quantitative method to aid in optimal purification process development and in routine quality control of final product.

Principle of the Procedure

The BPP assay is a two-site immunoenzymetric assay. Samples which may contain BPPs are reacted in microtiter strips coated with an affinity purified capture antibody. A second horseradish peroxidase (HRP) enzyme labeled anti-bovine plasma antibody is reacted simultaneously resulting in the formation of a sandwich complex of solid phase antibody-BPP-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate, tetramethylbenzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of total BPP present.

Reagents & Materials Provided

Component	Product #
Anti-BPP:HRP Affinity purified rabbit antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F291
Anti-BPP coated microtiter strips 12x8 well strips in a bag with desiccant	F292*
Bovine plasma protein standards Bovine plasma proteins stripped of BSA & blgG in buffer, with preservative. Standards at 0, 2, 8, 25, 75, and 200 ng/mL. 1 mL/vial	F293
Stop Solution 0.5M 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 # F292.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section.
- 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 (400 - 600 rpm)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H₂SO₄. Avoid contact with eyes, skin, and clothing.
- Handle these reagents as if they were potentially infectious.
- 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. If duplicate CVs are poor or if the absorbance of the 0 standard is greater than 0.200 evaluate plate washing technique for optimal performance.

2. High Dose Hook Effect may be observed in samples with very high concentrations of BPP. Samples greater than 30 µg/mL may give absorbances less than the 200ng/mL standard. High Dose Hook samples are most likely to be encountered from samples early in the purification process. 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. Such samples should be diluted until the dilution adjusted value remains constant. The diluent used should be compatible with accurate recovery. We recommend the same diluent as is used to prepare the kit standards. This can be purchased separately from *Cygnus Technologies* as Cat # F031A. Other prospective diluents should be tested for recovery by using them to dilute the 200ng/mL standard, as described in the "Limitations" section below.

3. This kit is a very sensitive assay for BPP (< 500 pg/mL). Since bovine source materials are common reagents in many laboratories and often used at relatively high concentrations it is very important to use extreme care to avoid pollution of any of the reagents in this kit with external sources of BPP. Such pollution can manifest itself as higher background, poor precision, and/or higher values than expected.

Limitations

- The standards used in this assay are comprised of normal bovine plasma which was exhaustively immuno-affinity stripped of BSA & bIgG. Thus, this kit cannot be used to detect the presence of BSA or bIgG. *Cygnus Technologies* sells mono-specific ELISA kits for BSA (Cat# F030) and bIgG (Cat# F070). Western blot analysis of the antibodies used in this kit demonstrates that they recognize essentially all of the protein bands other than BSA & bIgG, found in bovine plasma and also detectable by one dimensional SDS electrophoresis under reducing conditions using a sensitive protein staining reagent such as silver stain. However, there can be no guarantee that this assay will detect and accurately quantitate all BPPs or protein fragments. Hence the suggested arbitrary dose units for reporting unknown samples is in "ng/mL of total immuno-reactive bovine plasma protein equivalents."
- Before exclusively relying on this assay to detect BPP it is advisable to qualify it relative to other methods of detection such as PAGE silver stain, AAE, and Western blot to ensure that this assay does not fail to react to any important impurities. The assay may not be strictly quantitative for a given BPP pollutant due to the potential for variable reactivity of different proteins. If absolute quantitation of a particular known pollutant is necessary and this pollutant does not give essentially 100% recovery relative to the standards supplied with this kit it may be necessary for the user to provide their own standards. Consult *Cygnus Technologies* Technical Services department for issues regarding quantitation.
- Certain sample matrices may interfere in this assay. High or low pH, detergents, urea, high salt concentrations, and organic solvents are some of the known interference factors. Sample matrix interference can be determined by a 'spike and recovery' experiment. For example, 1 part of the 200 ng/mL standard is diluted into 4 parts of the matrix containing no or very low BPP pollutants. This diluted standard when assayed as an unknown should give a value of ~ 30 to 50 ng/mL above the base level of the undiluted sample. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.
- Avoid the assay of samples containing sodium azide (NaN₃) which will destroy the HRP activity of the conjugate and could result in the under-estimation of BPP levels.

Assay Protocol

- The assay protocol is a simultaneous incubation of sample with HRP conjugated antibody. This yields a sensitivity (limit of detection) of ~0.3 ng/mL and requires 2.5 hours to complete.
- The protocol specifies the use of an approved orbital microtiter plate shaker for the immunological step. These can be purchased from most laboratory supply companies. 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 first immunological incubation step by one hour in order to achieve comparable results to the 2 hour shaking protocol. **Do not shake during the 30-minute substrate incubation step, as this may result in higher backgrounds and worse precision.**
- 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.
- 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. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.
- All standards, controls, and samples should be assayed at least in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to ensure 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 the absorbance of 100 μ L of substrate plus 100 μ L of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.
- Strips should be read within 10 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.
2. Pipette 100 μ L of anti-BPP:HRP (#F291) into each well.
3. Cover & incubate on orbital shaker at 400-600 rpm for 2 hours at room temperature, 24°C \pm 4°C.
4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350 μ L. Dump and tap 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 substrate.
5. Pipette 100 μ L of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. **DO NOT SHAKE.**
7. Pipette 100 μ L of Stop Solution (#F006).
8. Read absorbance at 450/650nm.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno-reactive BPP equivalents" (See Limitations section above). This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, spline, or polynomial methods. 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 15% for samples in the range of 2 to 200 ng/mL. CVs for samples less than 2 ng/mL may be greater than 15%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.006	0.006
B1	Zero Std	0.007	
C1	2ng/mL	0.034	0.033
D1	2ng/mL	0.033	
E1	8ng/mL	0.165	0.166
F1	8ng/mL	0.167	
G1	25ng/mL	0.450	0.457
H1	25ng/mL	0.464	
A2	75ng/mL	0.943	0.929
B2	75ng/mL	0.916	
C2	200ng/mL	2.231	2.111
D2	200ng/mL	1.992	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and qualification 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 process derived BPPs within or above the analytical range of this assay should be evaluated for dilutional linearity to ensure that the assay is accurate and has sufficient antibody excess for your particular BPPs. 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 qualification protocols can be obtained by contacting our Technical Services Department or on-line at our website.

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 ~0.3 ng/mL.

The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~0.500 ng/mL.

Precision

Intra (n=20 replicates) precision was determined on 3 pools with low (~8.72ng/mL), medium (~26.37ng/mL), and high concentrations (75.75ng/mL). Inter assay (n=10 assays) CV's were determined on 3 control samples at 8.16, 25.89, and 71.42ng/mL. The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	2.9%	7.7%
Medium	8.6%	14.6%
High	3.55%	6.7%

Specificity/Cross-Reactivity

In sandwich ELISA, cross reactivity can manifest itself either as a false increase in analyte levels (positive cross reactivity) or as a false decrease in analyte (negative cross reactivity) when analyte present in the sample competes with the cross reactant for the kit antibodies. Other materials that may not cross react in the true immunological sense may simulate cross reactivity due to non-specific interactions that can result in either a false increase or false decrease in the apparent analyte concentration. Because of the very defined nature of the samples proposed for testing in this assay, an extensive study into cross reactivity was not attempted. However it is recommended that each user evaluate known materials in their sample matrices for cross reactivity or non-specific interferences by testing those materials with and without BPP spiked into them. "Purified" serum proteins from humans and cows were evaluated for positive cross reactivity as indicated in the Table below. No cross reactivity is seen to the human serum proteins as evidenced by undetectable BPP levels. The very low apparent cross reactivity for BSA, and bIgG has been concluded not to be due to those proteins but rather to some contaminating BPP impurity in these preparations deemed at best 99% pure. No bands to BSA or bIgG were seen on western blot when using the antibodies employed in this kit.

Materials Tested for Cross-Reactivity to Bovine Plasma Proteins		
Analyte	Analyte concentrations tested	% Cross reactivity (wt/wt)
BSA	1mg/mL	0.004
Bovine IgG	1 mg/mL	0.020
Human serum albumin	20 mg/mL	< LOQ
Human IgG	16 mg/mL	< LOQ

Hook Capacity

Increasing concentrations of BPPs > 200 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 200 ng/mL standard, was ~31 µg/mL.

Ordering Information/ Customer Service

To place an order or to obtain additional product information contact *Cygnus Technologies*:

www.cygnustechnologies.com

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