ELISA

Protein A Immunoassay

Catalog Number BPPA00

For the detection of natural and recombinant Protein A constructs.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Protein A, also known as Staphylococcus aureus protein A (SpA) is a 40-60 kDa surface protein originally found in the cell wall of the bacterium Staphylococcus aureus (1). The carboxyl terminal end of Protein A is anchored to the Staphylococcus cell wall by the transpeptidase enzyme sortase. It is encoded by the *spa* gene and consists of five homologous 6.7 kDa immunoglobulin domains (E, D, A, B, and C) joined by conserved linkers of six to nine residues (2). Protein A has been shown to stimulate inflammatory responses by interacting with several proteins including tumor necrosis factor alpha receptors, Willebrand factor, and C1qR.

Protein A is most known for interacting with the Fc region of the antibody heavy chain. Although it binds all mouse immunoglobulin (IgG) subtypes (IgG_1, IgG_{2a}, IgG_{2b} and IgG_3), in humans, it binds to IgG_1, IgG_2 and IgG_4 but not IgG_3 (2). Protein A fails to bind IgG_3 because of a single histidine to arginine change at amino acid 435 (2). Protein A reacts with Ig from a variety of other species including rhesus macaque, guinea pig, porcine, bovine, and goat.

Protein A is used in a wide variety of applications (1). Because of its ability to bind immunoglobulins, it is commonly immobilized on a chromatography support for antibody purification. Protein A affinity chromatography is used regularly in the pharmaceutical industry as it allows for the high binding affinity and purity of monoclonal antibodies (mAbs) (3). Antibodies are eluted from Protein A under acidic conditions. The low pH mechanism of elution involves acquisition of a positive charge between a highly conserved histidyl residue at the center of the Protein A binding region of IgG and the complementary histidyl residue in Protein A. The positive charge causes the histidyl residues to repel each other to weaken the hydrophobic association (4). There are also pharmaceutical applications for Protein A, including autoimmune diseases, rheumatoid arthritis, and idiopathic thrombocytopenic purpura (1).

INTENDED USE

This Protein A ELISA is intended for detection of natural and recombinant Protein A in Bioprocess manufacturing applications. There are occasions where Protein A or its fragments leach from a purification column and remain bound to the Fc region of a therapeutic antibody. This must be avoided in order to preclude any adverse effects of Protein A on the patient, particularly in instances where antibodies are manufactured for pharmaceutical uses. This ELISA can be utilized as a Protein A ligand leaching quality control step in the mAb development workflow.

PRINCIPLE OF THE ASSAY

The Protein A Immunoassay is a one-step, 2.5 hour, quantitative solid-phase ELISA designed to measure natural and recombinant Protein A. A monoclonal antibody specific for Protein A has been pre-coated onto a microplate. An HRP-linked monoclonal antibody specific for Protein A is added to the wells followed by the pretreated standards and samples. Standard, samples and conjugate are incubated for 2 hours on a microplate shaker. Following a wash step to remove any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of Protein A bound. The color development is stopped, and the intensity of the color is measured.

A pretreatment buffer is necessary to dissociate the Protein A from antibody product sample without the need for boiling. This treatment eliminates interference from the antibody product sample and allows Protein A to be detected and quantified by the assay.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH AND MANUFACTURING USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by other factors present in samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- **In order to achieve optimal performance, do not allow the pipette tip to touch the inside of the well while loading standards, controls, samples, or blanks.**
- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Protein A Microplate	899161	96 well polystyrene microplate (12 strips of 8 wells) coated with a human monoclonal antibody specific for Protein A.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Pretreatment Plate	899020	96 well polystyrene microplate.	
Protein A Standard	899163	2 vials of recombinant Protein A in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Protein A Conjugate	899162	21 mL of a monoclonal antibody specific for Protein A conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Pretreatment I	899055	11 mL of pretreatment solution.	
Calibrator Diluent RD5-18	895335	21 mL of buffer with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 25 mL and 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- **Polypropylene** tubes for dilution of standards

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

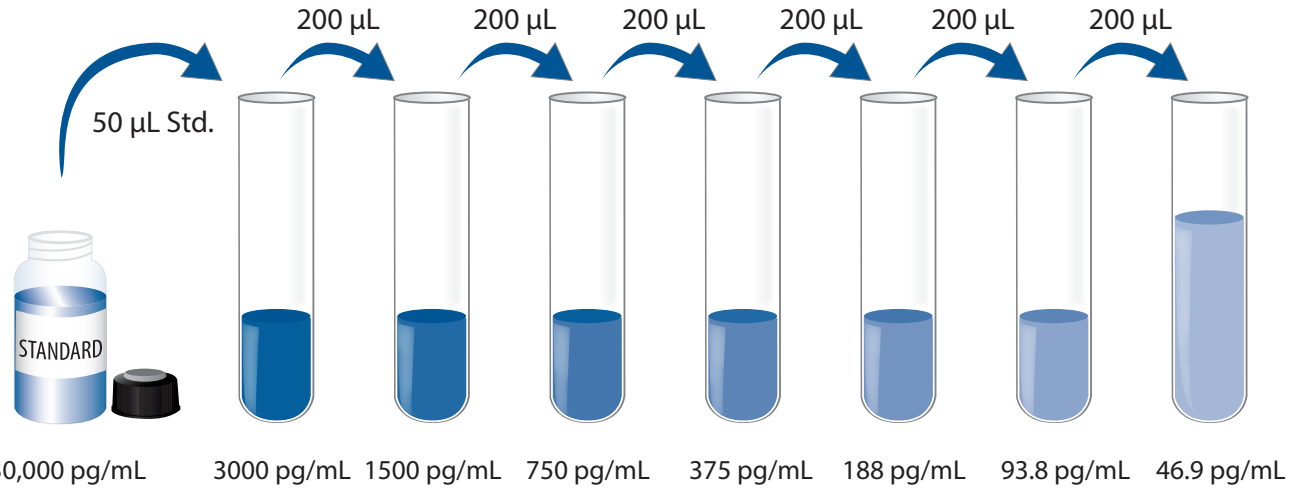
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-18 (diluted 1:2) - Add 10 mL of Calibrator Diluent RD5-18 to 10 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD5-18 (diluted 1:2).

Protein A Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Protein A Standard with deionized or distilled water. This reconstitution produces a stock solution of 30,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes. Mix well prior to making dilutions.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5-18 (diluted 1:2) into the 3000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 3000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-18 (diluted 1:2) serves as the zero standard (0 pg/mL).



SAMPLE PREPARATION

Dilute Protein A purified antibody samples to a concentration of ≤ 3.0 mg/mL in Calibrator Diluent RD5-18 (diluted 1:2). Prepare sample dilutions prior to pretreatment step.

PRETREATMENT PROCEDURE

All standards, controls, samples, and blanks should be pretreated.

Note: *If crystals have formed in the Pretreatment I buffer, warm briefly at 37 °C and mix gently until crystals have completely dissolved.*

1. Place 100 µL of every standard, sample, control, and blank into the Pretreatment Plate.
2. Add 50 µL of Pretreatment I. Mix by pipetting up and down for a minimum of 5 times.
3. Incubate for 5-30 minutes at room temperature on benchtop.
4. Proceed to Assay Procedure section.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, samples, and blanks be assayed in duplicate.

1. Prepare all standards, controls, samples or blanks as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 150 µL of Protein A Conjugate to each well.
4. Transfer 50 µL of pretreated standards, controls, samples, and blanks from the Pretreatment Plate to the Protein A Microplate. **Ensure reagent addition to plate wells is uninterrupted.** Cover with the adhesive strip provided. Incubate for **2 hours** at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

Note: *For optimal assay performance, dispense sample into microplate well without touching the pipette tip to the side of the well or the conjugate.*

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
7. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

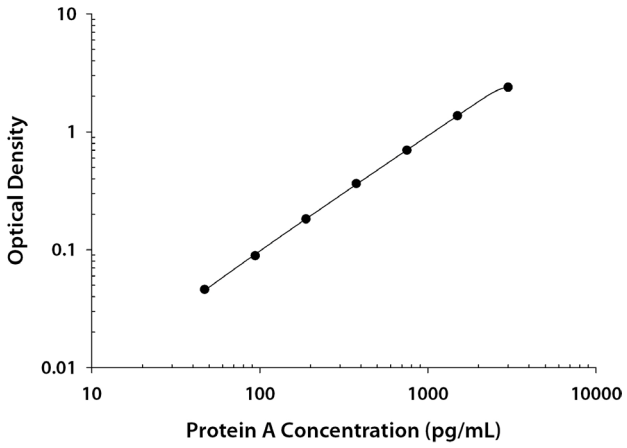
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the Protein A concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

For diluted samples the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.005 0.007	0.006	—
46.9	0.044 0.047	0.046	0.040
93.8	0.087 0.090	0.089	0.083
188	0.181 0.183	0.182	0.176
375	0.353 0.375	0.364	0.358
750	0.696 0.699	0.698	0.692
1500	1.364 1.380	1.372	1.366
3000	2.282 2.494	2.388	2.382

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	157	507	1519	148	458	1442
Standard deviation	4.90	13.6	42.3	12.2	28.9	86.8
CV (%)	3.1	2.7	2.8	8.3	6.3	6.0

RECOVERY

The recovery of Protein A spiked into biosimilar therapeutic antibody samples to levels throughout the range of the assay was evaluated. Each user should qualify their sample matrices for accurate recovery. For sample matrices other than PBS, further qualify for accurate recovery.

Sample Type	Average % Recovery	Range
Biosimilar Therapeutic Antibody (n=4)	101	88-113%

LINEARITY

To assess the linearity of the assay, biosimilar therapeutic antibody samples spiked with high concentrations of Protein A were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Biosimilar Therapeutic Antibody (n=4)
1:2	Average % of Expected	107
	Range (%)	103-116
1:4	Average % of Expected	112
	Range (%)	107-123
1:8	Average % of Expected	108
	Range (%)	102-114
1:16	Average % of Expected	104
	Range (%)	100-111

HIGH DOSE HOOK

No high dose hook effect was observed for Protein A concentrations up to 40 ug/mL.

LIMIT OF DETECTION

The limit of detection (LoD) is the lowest concentration where the analyte can be detected 95% of the time (a 5% likelihood of a false negative). Alternatively stated, LoD is the true value where the likelihood of a false negative measurement is 5%.

The LoD for Protein A is 16.1 pg/mL, determined consistent with the guidelines in CLSI document EP17 based on the proportions of false positivies (α) less than 5% and false negatives (β) less than 5%; using 128 determinations, with 64 blank and 64 low level samples; and a limit of blank (LoB) of 4.03 pg/mL.

LIMIT OF QUANTITATION

The limit of quantitation (LoQ) is the lowest concentration at which Protein A can be reliably detected where recovery is between 80-120% and precision is less than or equal to 20% CV. The LoQ was calculated based on the Westgard Model according to CLSI document EP17-A2. The lower limit of quantitation (LLoQ) for the assay is 23.4 pg/mL.

CALIBRATION

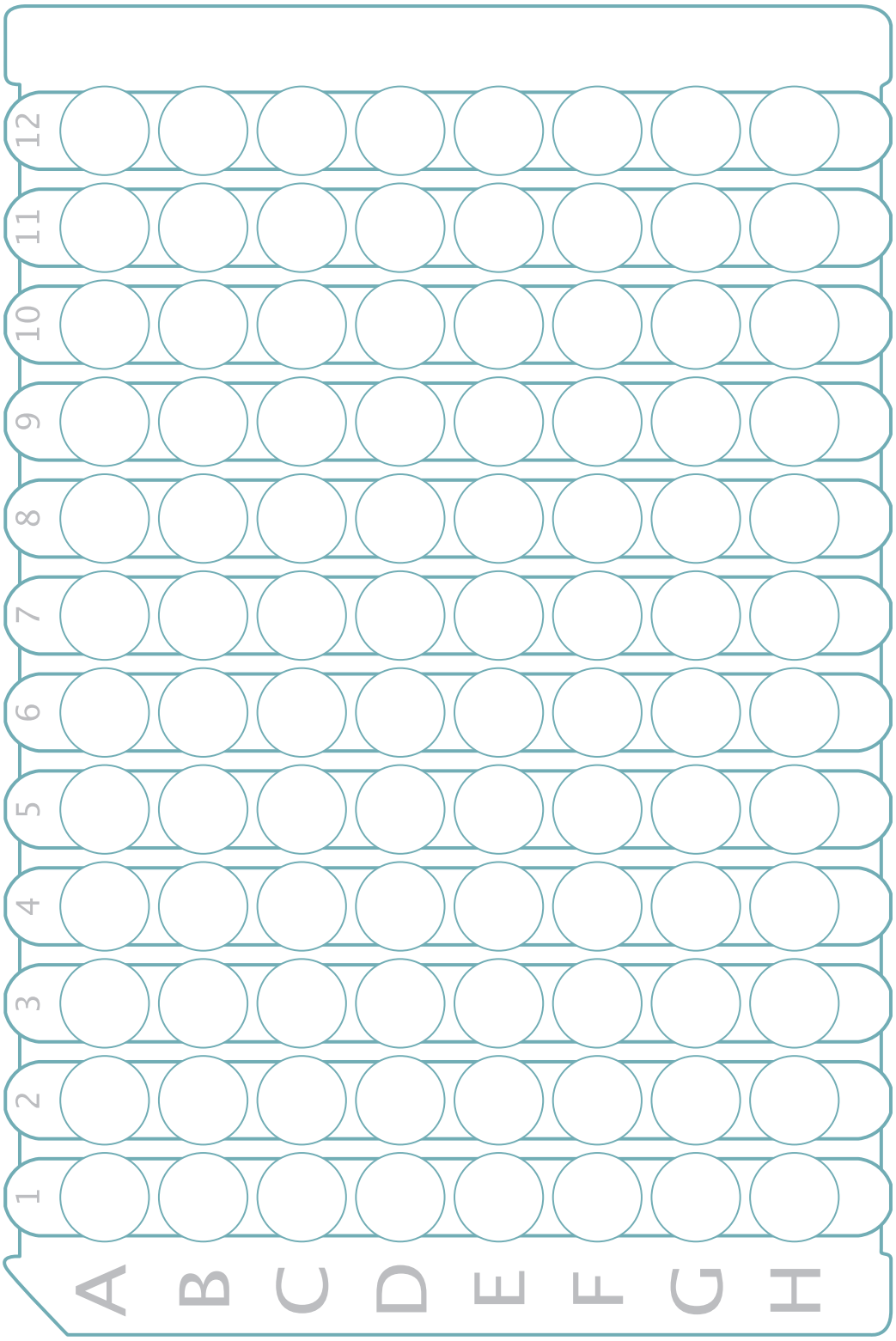
This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant Protein A produced at Bio-Techne®.

REFERENCES

1. Rigi, G. *et al.* (2019) Biotechnology and Applied Biochemistry. **66**:454.
2. Mazigi, O. *et al.* (2019) Protein Engineering Design and Selection. **32**:359.
3. Ramos-de-la-Pena, A.M. *et al.* (2019) Journal of Separation Science. **42**:1816.
4. Chabar, D.S. *et al.* (2020) Biologicals **63**:1.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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