Product Manual

Protein A ELISA Kit

Catalog #: ADI-900-057

96 well enzyme-linked immunosorbent assay
For use with natural and recombinant Protein A

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Product Manual

TABLE OF CONTENTS

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

Product Manual

INTRODUCTION

The Protein A ELISA kit is a complete kit for the quantitative determination of natural and recombinant Protein A in neutralized buffers.

Staphylococcus aureus Protein A is a cell wall constituent that is characterized by its binding affinity to the Fc portion of some immunoglobulins, especially the IgG class. Protein A is a 42 kDa protein that has four repetitive domains rich in aspartic and glutamic acids but devoid of cysteine[1]. The IgG binding domain (domain B) consists of three antiparallel alpha-helices, the third of which is disrupted when the protein is complexed with Fc[2].

Protein A participates in a number of different protective biological functions including anti-tumor, toxic and carcinogenic activities. There are antifungal and antiparasitic properties in addition to its ability to act as an immunomodulator[3]. Staphylococcal Protein A (with other surface proteins) is able to induce a Th1 type of response by eliciting the production of cytokines such as IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, and IL-4[4]. Protein A is used during the microscopic *in situ* visualization of biologically important molecules and to purify antisera. Extracorporeal therapeutic immunoadsorption techniques utilize Protein A in the treatment of proteinuria in nephrotic syndrome and severe autoimmune diseases such as rheumatoid arthritis, coeliac disease, and systemic lupus erythematosis[5-9]. Protein A from the Cowan I strain of *Staphylococcus aureus* has therapeutic and prophylactic applications in the control of Leishmania infections in animals. The anti-leishmanial effects may be mediated through the activation of macrophages resulting in enhanced phagocytosis of the parasites[10]. Protein A induced TNF-α and IL-2 is associated with the control of splenic cell apoptosis in mice[11].

PRINCIPLE

1. Samples and standards are added to wells coated with a chicken antibody specific for Protein A. The plate is then incubated.
2. The plate is washed, leaving only bound Protein A on the plate. A yellow solution of biotinylated chicken antibody to Protein A is then added. This binds the Protein A captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution streptavidin-HRP conjugate is added to each well, binding to the biotinylated antibody, which is attached to the Protein A. The plate is again incubated.
4. The plate is washed to remove excess streptavidin-HRP conjugate. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450nm. The amount of signal is directly proportional to the level of Protein A in the sample.

MATERIALS SUPPLIED

Component	Size	Product No.	Description
Protein A Clear Microtiter Plate	One Plate of 96 Wells	80-0348	A plate of break-apart strips coated with purified chicken antibody specific to Protein A
Assay Buffer 13	55 mL	80-1500	Tris buffered saline containing proteins and detergents
Protein A Antibody	10 mL	80-0346	A yellow solution of biotinylated chicken antibody to Protein A
Protein A Conjugate	10 mL	80-0347	A blue solution of streptavidin conjugated to horseradish peroxidase
Protein A Standard	One vial	80-0621	A solution of 10,000 pg/mL of recombinant Protein A
Wash Buffer Concentrate	100 mL	80-1287	Tris buffered saline containing detergents
TMB Substrate	10 mL	80-0350	A solution of 3,3’,5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide
Stop Solution 2	10 mL	80-0377	A 1N solution of hydrochloric acid in water
Protein A Assay Layout Sheet	1 each	30-0100	One assay layout sheet for assay planning
Plate Sealer	2 each	30-0012	A plate sealer to be used for incubation/shaking steps

STORAGE

All components of this kit are stable at 4°C until the kit's expiration date.

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 100 µL and 1,000 µL
3. Disposable test tubes for dilution of samples and standards
4. Precision pipets for volumes between 5 µL and 1,000 µL.
5. Repeater pipets for dispensing 100 µL
6. Disposable beakers for diluting buffer concentrates.
7. Graduated cylinders.
8. A microplate shaker.
9. Lint-free paper for blotting.
10. Microplate reader capable of reading at 450 nm.
11. Graph paper for plotting the standard curve.
12. Beaker for boiling samples
13. Microcentrifuge tubes for boiling samples

PRECAUTIONS

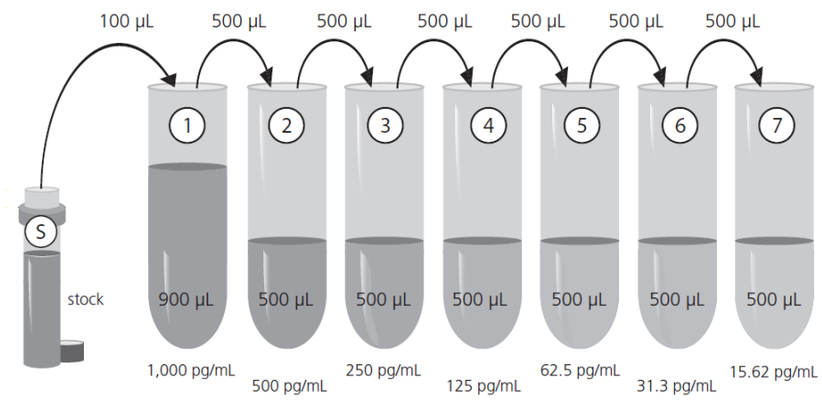
1. **For Research Use Only. Not for use in diagnostic purposes.**
2. Use a safety pipetting device for all pipetting. Never pipet by mouth.
3. Interpretation of the results is the sole responsibility of the user.
4. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
5. **Do not mix components** from different kit lots of use reagents beyond the expiration date of the kit.
6. Activity of the conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.
7. The standard should be handled with care due to the known and unknown effects of the antigen.
8. Protect the substrate from prolonged exposure to light.
9. Stop solution is caustic. Keep tightly capped.

REAGENT PREPARATION

Wash Buffer:

Prepare the Wash Buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

Protein A Standards:



Allow the 10,000 pg/mL Protein A Standard to warm to room temperature. Label seven 12 x 75 mm polypropylene tubes #1 through #7. Pipet 900 µL of Assay Buffer 13 into tube #1. Pipet 500 µL of Assay Buffer 13 into tubes #2 through #7. Add 100 µL of the 10,000 pg/mL standard into tube #1 and vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

Note: If samples contain IgG, standards must be boiled. See Sample Handling section for details.

Diluted standards should be used within 30 minutes of preparation.

The concentrations of Protein A in the tubes are labeled above.

SAMPLE HANDLING

The Protein A ELISA kit is compatible with natural and recombinant Protein A samples in neutralized buffers. Samples containing antibodies must be prepared in the following manner prior to running the assay.

1. Determine the concentration of antibody present in the eluted samples. Dilute all samples to 1 mg/mL with Assay Buffer 13.
2. Prepare standard curve as described on Page 4.
3. Aliquot a minimum of 0.5 mL of each sample and standard into a microcentrifuge tube with a hole in the lid. This volume will allow for duplicates of each sample and standard to be measured in the assay. Include an additional tube with Assay Buffer 13 only (0 pg/mL).
4. Incubate samples, standard curve, and buffer for 5 minutes in a boiling water bath.
5. Allow samples to cool for 5-7 minutes at room temperature. Centrifuge samples for four minutes at 13,800 x g at room temperature.
6. Use supernatants from the cooled sample and standard tubes directly in the assay.

Depending on the species and type of antibody present in the sample it may be necessary to modify the above protocol. For example, samples containing human IgG, an antibody with high affinity for Protein A, require dilution to 50 µg/mL hIgG and an additional 15 minutes of boiling to achieve accurate Protein A concentrations.

ASSAY PROCEDURE

NOTE:

(1) Bring all reagents to room temperature for at least 30 minutes prior to opening.

(2) All standards and samples should be run in duplicate.

(3) Pipet the reagents to the sides of the wells to avoid possible contamination.

(4) Prior to the addition of the antibody, conjugate, and substrate, ensure that there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

(5) Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100 µL of the Assay Buffer into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
3. Pipet 100 µL of the samples into the appropriate wells.
4. Seal the plate. Incubate at room temperature on a plate shaker (~500 rpm) for 1 hour.
5. Empty the contents of the wells and wash by adding 400 µL of Wash Solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of yellow antibody into each well, except for the Blank.
7. Seal the plate. Incubate at room temperature on a plate shaker (~500 rpm) for 1 hour.
8. Wash as above (Step 5).
9. Add 100 µL of blue conjugate to each well, except the Blank.
10. Seal the plate. Incubate at room temperature on a plate shaker (~500 rpm) for 30 minutes.
11. Wash as above (Step 5).
12. Pipet 100 µL of substrate solution into each well.
13. Incubate for 15 minutes at room temperature on a plate shaker (~500 rpm).
14. Pipet 100 µL of stop solution to each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of Protein A in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

$$\textbf{Average Net OD} = \textbf{Average OD} - \textbf{Average Blank OD}$$

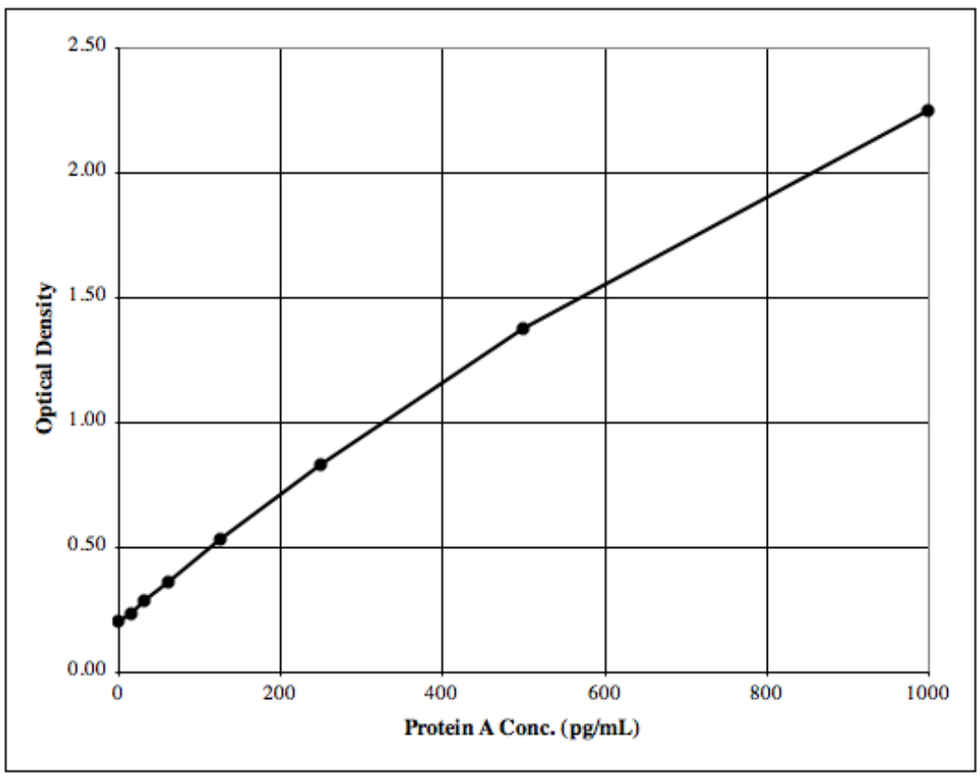
2. Plot the Average Net OD for each standard versus Protein A concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	Protein A (pg/mL)
S0	0.202	0
S1	2.243	1000
S2	1.374	500
S3	0.825	250
S4	0.533	125
S5	0.356	62.5
S6	0.28	31.3
S7	0.233	15.6
Unknown 1	0.324	48.2
Unknown 2	1.123	379.9

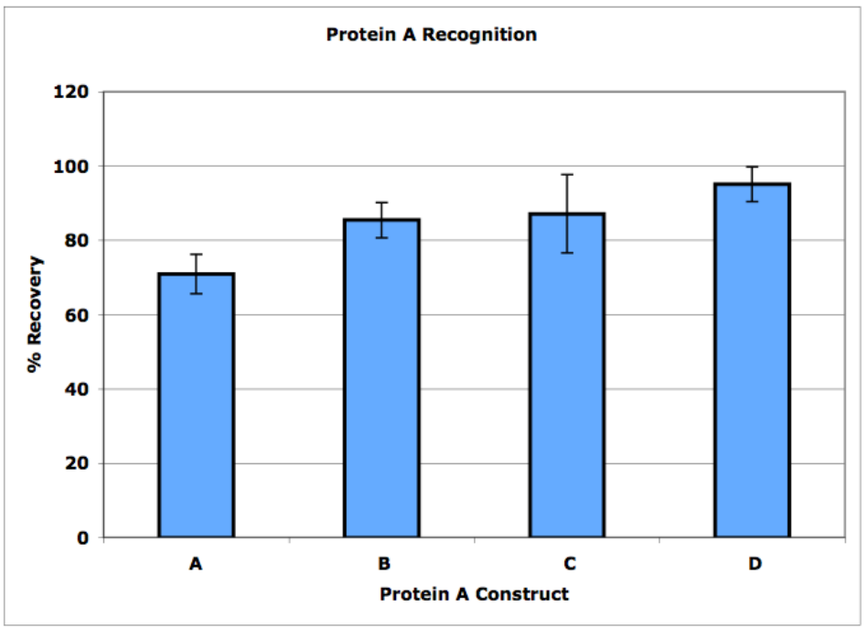


PERFORMANCE CHARACTERISTICS

Specificity

The Protein A ELISA Kit recognizes natural and recombinant forms of Protein A. Four Protein A constructs, described in the table below, were evaluated (post boiling) in the assay.

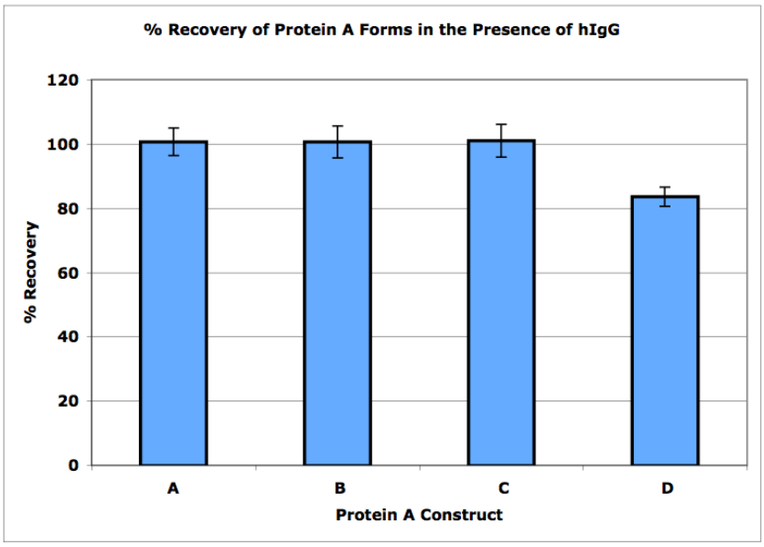
Resulting optical densities were interpolated off of the kit standard curve. Percent expected was calculated by dividing observed concentration by expected concentration (A-B: n=9, C-D: n=12, graphical data represents statistical mean ± 1 SD).



Human Immunoglobulin G Experiment

Four Protein A constructs, described in the table below, were evaluated in the presence of human IgG. Due to the high affinity of hIgG to Protein A the sample handling protocol on Page 6 was modified. Prior to boiling, samples were diluted to 50 µg/mL hIgG in the kit assay buffer. Samples were then boiled for 20 minutes. Percent recovery was calculated by dividing the observed recovery in the presence of hIgG by the observed recovery from the assay buffer (A-B: n=9, C-D: n=12, graphical data represents statistical mean ± 1 SD).

SPIKE AND RECOVERY



Assay recognition of different Protein A constructs, post boiling. Resulting concentrations were interpolated from kit standard curve. Percent recovery calculated by dividing observed concentration by expected concentration. Graphical data represents statistical mean ± 1 standard deviation.

A. Natural Protein A from *S. Aureus*, n=9
B. Recombinant Protein A from *E. coli*, n=9
C. Recombinant Cys-Protein A from *E. coli*, n=12
D. Recombinant alkaline-resistant Protein A variant from *E. coli*, n=12

Sensitivity

Sensitivity was calculated as the ratio of the mean OD plus 2 standard deviations of 16 replicates of the 0 pg/mL standard to the mean of 16 replicates of the lowest standard, multiplied by the concentration of that standard (15.62 pg/mL). This value was determined to be 9.01 pg/mL.

Linearity

Protein A was spiked into acidic 0.1 M Citric Acid and 0.1 M Glycine to model samples eluted off of Protein A columns. These samples were neutralized with a 2 mM Tris buffer and then serially diluted 1:2 in the kit assay buffer. The results are shown in the tables below.

Citric Acid

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	N/A	636.6	N/A
1:2	318.3	313.8	99
1:4	159.2	157.1	99

1:8	79.6	79.2	99
1:16	39.8	39.9	100
1:32	19.9	21.3	107

Glycine

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	N/A	697.7	N/A
1:2	348.8	332.6	95
1:4	174.4	161.2	92
1:8	87.2	88.7	102
1:16	43.6	52.5	120

PRECISION

Intra-assay precision was determined by assaying 16 replicates of three buffer controls containing Protein A in a single assay.

Concentration (pg/mL)	%CV
127.9	5.2
364.3	6.4
727.8	5.5

Inter-assay precision was determined by measuring buffer controls of varying Protein A concentrations in multiple assays over several days.

Concentration (pg/mL)	%CV
103.6	13.4
209.7	8.1
378.3	8.0

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