

Kit manual of

Protein A ELISA

for samples containing IgG

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GENERAL INFORMATION

Staphylococcal Protein A (SpA) is an immunoglobulin (IgG) binding protein, which is found in the bacterial cell wall of *Staphylococcus aureus* (ref.1). SpA binds to most mammalian IgG and can be used for detection or purification of such antibodies (ref. 2). Affinity chromatography on SpA-columns is widely used for the purification of monoclonal and polyclonal antibodies. SpA may some-

times leak from the column and contaminate the preparation. Immunoglobulins react with SpA *in vivo* and may cause anaphylactic reactions (ref. 1). Contamination of SpA may also cause false results in immunological assays. Thus it is important that the antibody preparation is free from SpA before being used.

TEST PRINCIPLE

This ELISA kit is designed to detect SpA in IgG-containing solutions (e.g. monoclonal antibody preparations), in acid eluates from SpA columns, or in other liquid preparations. It is a sandwich ELISA based on microtitre strips coated with affinity-purified chicken anti-SpA IgG. SpA from the sample is bound to the microwell. Bound SpA is detected by biotinylated chicken anti-SpA IgG. A streptavidin horseradish peroxidase conjugate detects the biotin conjugate. A substrate reactive with horseradish peroxidase is added. Colour development is due to conversion of the substrate by the conjugate. A

positive result is indicated as a colour change. The colour can be read visually or by a microplate photometer at 450 nm. Samples to be tested with this assay are often of acid pH. In order for the test to work properly, such samples should be neutralised. If a sample contain mammalian IgG, SpA will interact with IgG and hence be partly blocked. A falsely lowered signal may result. To avoid this problem samples containing IgG are treated to denature immunoglobulins and expose the available SpA epitopes.

INTERACTION BETWEEN SpA AND IgG

Analysing the presence of SpA in samples containing IgG involve two major problems which has to be solved in order to obtain reliable results. **Firstly**, the Fc-reactivity of SpA with IgG of most animal species make the specific detection by immunoassay of SpA difficult. Antibodies specific to SpA will normally bind both by their specific activity and the general affinity between SpA and IgG (Fc). In this kit the problem is solved by using chicken anti-SpA IgY. Chicken antibodies are one of few immunoglobulins that do not have Fc-reactivity to SpA. **Secondly**, in samples containing mammalian IgG the immunological active epitopes of SpA are normally blocked by the non-specific binding of IgG. To overcome this problem it has been suggested to perform the analysis of SpA at low pH (ref.4), at which a certain dissociation occur between SpA and the IgG. High affinity

antibodies (e.g. human IgG and certain mouse monoclonals) however, will remain bound to SpA even below pH 3. Unfortunately, specific immunological detection of SpA is very difficult at such low pH. Consequently assay at low pH provide poor analytical performance. We have chosen to establish an assay system that is adjusted to each customer's purification situation. The standard references are made with known amounts of IgG present. The IgG solution, which is added to the standard references, should be of the same isotype as the IgG of the sample. To dissociate SpA and IgG, the samples and standard references are boiled for 4 minutes. In other words, the assay is standardised with the same IgG present as in the sample. The possible error from blocking of antigenic epitopes is therefore eliminated.

THE EFFECT OF SAMPLE PREPARATION ON RECOVERY OF SPA

SpA easily bind to glass or plastic material. In presence of 0,05% Tween 20 this binding is inhibited. It is absolutely necessary that the sample is eluted into a buffer containing Tween 20 (see test procedure) in order to recover all SpA from the sample matrix and avoid that SpA is coated

on the walls of the sample tube. The neutralisation buffer in this kit contains Tween 20 and it must be added to the sample tubes before the sample. Absence of Tween 20 may falsely lowered concentration of SpA.

MATERIALS NEEDED BUT NOT PROVIDED

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|---|----------------------------|---------------------|
| 1. Precision pipettes. 10 to 1.000 µl | 5. Disposable pipette tips | 9. Hot plate |
| 2. Centrifuge.capacity: 2.000xg | 6. Eppendorf tubes | 10. Wash bottle |
| 3. Microplate photometer | 7. Humid chamber | 11. Distilled water |
| 4. IgG solution purified by than methods then SpA | 8. 10-ml tubes | |

CONTENTS

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|---|---|
| 1) Microtitre wells 12x8 , coated with chicken anti-SpA antibodies. A plastic bag is included for storage of unused strips. | 6) Biotinylated anti-SpA IgY , 200 µl. 100 x solution. Contains preservative. |
| 2) PBS-Tween Concentrate (20x) , 2x25 ml. Sufficient for 1 L Phosphate Buffered Saline with 0,1% Tween (PBS-T). Contains preservative. | 7) HRP Conjugate , 200 µl. Streptavidin horseradish peroxidase conjugate. 100 x solution. Contains preservative. |
| 3) Tween 20 concentrate , 2 ml. | 8) Substrate solution , 25 ml. TMB/ H ₂ O ₂ solution. Contains preservative. |
| 4) Protein A reference , 200 µl. 0,5 mg/ml SpA solution. Contains preservative. | 9) Stop solution , 20 ml. 1 M HCl . (Caution. Strong Acid) |
| 5) 2 M Tris Buffer . 25 ml. | 10) Floating aid for boiling of tubes , 1 pcs. |

PRECAUTIONS

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| 1) Carefully read and follow all instructions. | 8) Do not eat, drink, or smoke where specimens or kit reagents are being handled. |
| 2) Store the kit and all reagents at +2 to +8°C (35 to 45°F). | 9) Use a separate pipette tip for each sample. |
| 3) All reagents should equilibrate to +18°C to +25°C (64 to 77°F) before use. | 10) Do not pipette by mouth. |
| 4) Unused microtitre wells should be stored sealed at +2 to +8° C. | 11) Standard references must be used for each test series. |
| 5) Do not intermix components or instruction booklets from kits with different expiration dates (= different batches). | 12) Use only distilled water for preparation of reagents. |
| 6) Be careful to prevent contamination of kit components. | 13) The Substrate solution and 2 M Tris buffer are irritating to eyes, respiratory system and skin Avoid contact with skin and eyes. |
| 7) Do not use test kit beyond expiration date. | 14) The Stop solution contains HCl. This is a strong acid and can cause burn. Handle with care. |
| | 15) This kit is <u>for research use only</u> . |

PREPARATION OF REAGENTS

1. **Protein A reference (0,5 mg/ml), Substrate solution, and Stop solution.** All of these components are ready for use.
2. **Microtitre wells.** If not all wells are used shall the remaining strips be put into the plastic bag with desiccant.
3. **Tween solution.** The Tween concentrate should be equilibrated to room temperature +18°C to +25°C (64 to 77°F). Prepare the **Tween solution** by diluting the Tween concentrate 1/5 in ultrapure water (Milli Q or double distilled) Example: Mix 1 ml of Tween concentrate in 4 ml water. Mix by Vortex.
4. **Neutralisation buffer.** Prepare the Neutralisation buffer by diluting the Tween solution 1:5 in Tris Buffer. Example: Mix 1 ml Tween solution in 4 ml Tris buffer. Mix by Vortex.
5. **Dilution buffer.** Prepare the dilution buffer by diluting the Neutralisation buffer 1/10 in **your acid elution buffer** (or equivalent, i.e. the sample matrix buffer). Example: Mix 1 ml Neutralisation buffer in 9 ml elution buffer. Mix by Vortex
6. **PBS-Tween buffer.** The PBS-Tween Concentrate (20x) should be diluted **1+19** in ultrapure water (Milli Q or double distilled). Store prepared PBS-Tween buffer at + 4°C but not for more than 3 days. PBS-Tween buffer may be stored frozen for at least one year. Example: Mix 15 ml PBS-Tween Concentrate (20x) in 285 ml water. Mix by Vortex. N.B. Check that there is no crystal precipitation in the bottle. If crystals are seen, please warm and shake the bottle well.
7. **Biotinylated anti-SpA IgY concentrate.** The Biotinylated anti-SpA IgY concentrate should be diluted 1+100 in the PBS-Tween buffer before use. If only part of the kit will be used. Dilute a sufficient amount of concentrate 1+100 in PBS-Tween buffer. The solution is not stable for more than 24 hrs. Use it on the same day. Example: Mix 120 µl Biotinylated anti-SpA IgY (concentrate) in 12 ml PBS-Tween buffer. Mix by Vortex.
8. **HRP conjugate concentrate.** The HRP conjugate concentrate should be diluted 1+100 in the PBS-Tween buffer before use. If only part of the kit will be used. Dilute a sufficient amount of concentrate 1+100 in PBS-Tween buffer. The solution is not stable for more than 24 hrs. Use it on the same day. Example: Mix 120 µl HRP conjugate (concentrate) in 12 ml PBS-Tween buffer. Mix by Vortex.

SAMPLE PREPARATION

1. **The Tween solution** *should* be added 1+400 to the tubes in which the samples are collected. Example: For 2 ml sample volume, add 5 µl Tween solution to the sample tubes before the samples are added. Mix by Vortex.
2. **Neutralisation of samples.** The samples should have neutral pH when assayed. If they are acid, neutralisation buffer should be added. Mix 1 part of Neutralisation buffer in 9 parts of the sample. Test pH and, if necessary, add additional neutralisation buffer (note the volume of buffer added in order to calculate the dilution factor).

TEST PROCEDURE

NOTE. All reagents should equilibrate to room temperature +18°C to +25°C (64 to 77°F) before use.

1. Preparation of acid eluted samples:

1.1. Establish the elution volume of your acid eluted samples. Before the SpA-column is eluted, add 1/10 of the volume of Neutralisation buffer to each sample collection tube (See 'Sample preparation'). Dilution factor: 0,9.

1.2. Determine the IgG concentration in your acid eluted samples by measure the optical density at 280 nm (OD₂₈₀). To calculate the IgG concentration in the eluted sample; divide the OD₂₈₀ value with 1,36 (mg/ml).

1.3. Dilute your neutralised samples with Dilution buffer so that all samples contain the same IgG concentration. Calculate the dilution factor (DF) for each sample. Note the DF for each sample in table 3 on page 8.

1.4. Transfer 500 µl of each sample with adjusted IgG concentration to a labelled Eppendorf tube (not included in the kit).

2. Preparation of solution A for the standard references:

2.1. Use a SpA free IgG-solution for the preparation of solution A. If the concentration of IgG is unknown, measure the optical density at 280 nm (OD₂₈₀) in same way as for the eluted samples (see 1.2.).

2.2. Add the IgG-solution to 5 ml Dilution buffer in a 10 ml tube (not included in the kit). Adjust the IgG concentration in solution A to the same as in the samples (see 1.3. above).

3. Preparation of standard references:

3.1. Label 6 Eppendorf tubes from A to F for each set of standard references. Add 990 µl of solution A to tube A and B and add 500 µl of solution A to tube C-F (see table 1).

Additional standard references can be prepared by diluting any of the standard references in Table 1 with solution A. Example: Dilute the 50 ng/ml standard reference 1:2 to obtain a 25 ng/ml standard reference.

3.2. Add 10 µl SpA reference (0,5 mg/ml) and dilute the standard reference A with solution A and mix well on vortex.

3.3. Transfer 10 µl of standard reference A to tube 'B' and mix well on vortex. Prepare standard references 'C' to 'E' according to table 1 (see below).

Table 1

Standard reference	Source solution	Solution A	SpA conc.(ng/ml)
A	10 µl of Protein A ref.	990 µl	5.000
B	10 µl of ref A	990 µl	50
C	125 µl of ref B	500 µl	10
D	125 µl of ref C	500 µl	2
E	125 µl of ref D	500 µl	0,4
F	None	500 µl	0

4. Boiling of the neutralised samples and the standard ref. B-F:

4.1. **Make a hole** with a syringe in the cap of the Eppendorf tubes with samples and standard reference B-F and place them on the floating aid for tubes.

4.2. Place the floating aid with tubes in boiling water for 4 minutes. Take the tubes from the hot water and let the tubes **cool** for 5-10 minutes at room temperature.

4.3. **Centrifuge** the standard references B-F and the samples at 2.000xg for 60 seconds. Your standard references and samples are now ready for use. Do not use the same pipette-tip for different tubes.

5. Add 100 µl of each supernatant of **the standard references B, C, D, E, and F** to appropriate wells. It is recommended to test standard references in duplicate.

6. Add 100 µl of each supernatant of **the samples** to the appropriate wells. For confirmation purposes it is recommended to test samples in duplicate.

7. **Incubate** the anti-Protein A coated microtitre wells with samples and standard references 60 minutes at room temperature +18°C to +25°C (64 to 77°F) in a humid chamber or with the wells sealed with tape.

8. Empty wells and **rinse** 4 times with PBS-Tween

buffer, filling all wells to the top for each rinse. Empty all fluid from the wells and tap the plate hard to remove the last traces of fluid.

9. Add 100 µl of (diluted) **Biotinylated anti-SpA IgY** to each well. Incubate for 1 hour at room temperature +18°C to +25°C (64 to 77°F) in a humid chamber or with the wells sealed with tape.

10. Wash the strips (see step # 8). Add 100 µl of (diluted) **HRP conjugate** to each well. Incubate for 30 minutes at room temperature +18°C to +25°C (64 to 77°F) in a humid chamber or with the wells sealed.

11. Wash the strips (see step # 8). Add 100 µl of **Substrate solution** to each well and incubate 10 minutes at room temperature +18°C to +25°C (64 to 77°F) in darkness. Begin timing after the first well is filled.

12. Stop the reaction at 10 minutes by adding 100 µl **Stop solution** to each well. Add the Stop solution in the same order as the Substrate solution was added in step # 11.

13. **Measure** the absorbance of the samples and standard references at 450 nm within 30 minutes after adding the stop reagent (use air as blank).

14. **Calculate** the mean corrected absorbance values for each unknown test sample and standard references.

VALIDITY, SENSITIVITY AND PRECISION

To ensure validity of this assay the standard reference D (2,0 ng/ml) should have a corrected absorbance value greater than 0,1. The sensitivity of the assay is in most

cases better than **1 ppm**. Coefficients of variation for duplicate measurements are typically < 10%.

INTERPRETATION OF THE RESULTS

The absorbance value of all standard references should be corrected for background before the results are calculated.

Calculate the mean value for the standard reference duplicates.

$$\text{Stand. ref. B to E OD}_{450} - \text{Standard ref. F OD}_{450} = \text{Corrected OD}_{450}$$

Table 2

Stand. ref.	SpA Conc.	Mean OD ₄₅₀ value	Corrected OD ₄₅₀ value
F	0 ng/ml	R0=	R0-R0= 0,00
E	0,4 ng/ml	R1=	*R1-R0=
D	2 ng/ml	R2=	*R2-R0=
C	10 ng/ml	R3=	*R3-R0=
B	50 ng/ml	R4=	*R4-R0=

*= Corrected OD₄₅₀ values of R1, R2, R3 and R4 should be plotted as a standard curve. Plot the absorbance vs. the concentration of the standard references in the enclosed diagram (see below). Draw a line between the standard ref-

erence points. Use the curve to determine approximate concentrations of SpA in the samples. For exact determination of sample SpA concentrations dedicated data-reduction software may be used.

Analyzed by: _____ Date/Sign: _____

Corrected
OD 450 nm

Test protocol: _____



