

Seize™ X Protein A Immunoprecipitation Kit

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Product Description

Number

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Description

Seize™ X Protein A Immunoprecipitation Kit

Contains sufficient reagents to immobilize four primary antibodies and to perform a total of 40 immunoprecipitations.

Kit Contents:

1. **ImmunoPure® Plus Immobilized Protein A**, 2 ml of 50% slurry or 1 ml of settled gel. Example; 400 µl of a 50% slurry is equivalent to 200 µl of settled gel.
2. **Microcentrifuge Tubes**, 72
3. **BupH™ Modified Dulbecco's PBS Pack (Binding/Wash Buffer 1)**
Reconstitute the contents of the BupH™ Pack with 500 ml of distilled water. The resultant buffer will be composed of: 0.14 M NaCl, 0.008 M Na₂PO₄, 0.002 M Potassium Phosphate, 0.01 M KCl, pH 7.4.
4. **BupH™ Tris Buffered Saline Pack (Quenching/Wash Buffer 2)**
Reconstitute contents of the BupH™ Pack with 500 ml of distilled water. The resultant buffer will be composed of 25 mM Tris, 0.15 M NaCl, pH 7.2.
5. **ImmunoPure® Elution Buffer**, pH 2.8 buffer (contains primary amine), 50 ml
6. **Spin X Cups and Tubes**, 12
7. **Sample Buffer (5X)**, (0.3 M Tris•HCl, 5% SDS, 50% Glycerol, Lane Marker Tracking Dye, pH 6.8)
8. **Disuccinimidyl Suberate (DSS)**, 4 x 13 mg

Note: DSS is moisture-sensitive. It should be stored refrigerated and desiccated. Be sure to warm vials to room temperature before opening. DSS should be dissolved in DMSO or DMF immediately before use. DSS is not compatible with any amine-containing buffers (e.g., Tris, glycine, etc.).

Storage: This kit should be stored at 4°C. Disuccinimidyl Suberate (DSS) should be stored desiccated at 4°C.

Summary of Seize™ X Protein A Immunoprecipitation Kit Procedures

- A. Binding of Antibody to Immobilized Protein A
- B. Cross-linking of Bound Antibody to Protein A Support
- C. Immunoprecipitation of the Antigen
- D. Elution of Immunoprecipitated Antigen
- E. Regeneration of Antibody Gel and Storage Conditions
- F. Preparation of Samples for Loading onto an SDS-PAGE Gel

Note: All of these steps are performed at room temperature.

Introduction

The Seize™ X Immunoprecipitation Kit offers an improvement on the classical method of immunoprecipitation. Classic immunoprecipitation requires that a primary antibody binds to the antigen of interest in a crude preparation to form an antigen-antibody complex. This complex is then incubated with Protein A immobilized to a solid support. The Protein A binds the antibody via the antibody Fc region, allowing the entire complex to be separated from the crude mixture by simple centrifugation. The solid support forms a pellet and is physically separated from the remaining crude preparation (or lysate) in the supernatant. The pellet is washed and finally boiled at 100°C with electrophoresis loading buffer (with or without reducing agents). The proteins are then analyzed via SDS-PAGE to observe what has been precipitated out of the crude sample. Problems associated with this classical method include: 1.) contamination of the purified antigen with the precipitating antibody, 2.) destruction of the Protein A support, 3.) destruction of precious primary antibody, and 4.) poor antigen recovery.

The Seize™ X Immunoprecipitation Kit overcomes these problems by immobilizing the primary antibody to the Protein A support using a cross-linking agent, DSS. This creates a permanent affinity support upon which the antibody is properly oriented with the antigen-binding sites facing away from the solid support. The crude sample can then be incubated with the immobilized antibody to form the immune complex. The affinity support is then washed by centrifugation using a Spin X device (porous cup inside of a microcentrifuge tube). Crude material is quickly washed away. The remaining antigen is then dissociated from the antibody using an elution buffer and centrifugation. This step serves two purposes: The antibody does not contaminate the final antigen preparation and the immobilized antibody support is preserved for future immunoprecipitations (IPs).

Depending on the stability of the antibody immobilized to the matrix, the prepared affinity support may be utilized 2-10 times, thus conserving precious antibody samples. In addition, the kit contains a 5X sample buffer, which can be used to prepare eluted samples for SDS-PAGE gels without significant dilution of the sample.

Compared to the classic immunoprecipitation method, the Seize™ X Immunoprecipitation Kit: 1.) prevents contamination of the antigen with the antibody, 2.) results in better recovery of the immunoprecipitated antigen, 3.) conserves precious primary antibody and 4.) offers faster and easier immunoprecipitations. The Seize™ X Kit provides enough reagents to immobilize four different antibodies and to perform 40 immunoprecipitations (or 10 IPs per antibody). Once the immobilized antibody matrix has been prepared, subsequent immunoprecipitations can be performed in a matter of minutes.

A final benefit of the Seize™ X Immunoprecipitation Kit is flexibility. The researcher has the option to use the classical method of immunoprecipitation by not performing the antibody cross-linking step. Once the antibody is immobilized, these kits can also be used for small-scale purifications of the antigen for a variety of applications such as protein assays, ELISAs, binding studies, Western blotting, etc. Different binding and elution buffer conditions can be used to preserve the activity of the antigen if the kit buffer conditions are not suitable. Please contact the Pierce Technical Assistance department should you have questions on the use of this kit for your laboratory applications.

Procedure

A. Binding of Antibody to Immobilized Protein A

1. Gently invert bottle of ImmunoPure[®] Plus Immobilized Protein A. Add 0.4 ml of the immobilized protein A (50% slurry) into one of the Spin X cups and place inside a microcentrifuge tube. Spin the tube for 1 minute at maximum speed in a microcentrifuge.
2. Remove the Spin X cup from the microcentrifuge tube and pour out the buffer collected in the tube. Place the Spin X cup back into the tube.
3. Wash the gel by adding 0.4 ml of Binding/Wash Buffer 1 to the Spin X cup containing the immobilized protein A. Cap the microcentrifuge tube and mix gently end over end or place on a rocker for ~1-2 minutes. Spin the tube for 1 minute at maximum speed in a microcentrifuge.
4. Remove the Spin X cup from the tube and discard remaining buffer from the tube. Place the cup back into the microcentrifuge tube.
5. Repeat Steps 3 and 4.
6. Remove the Spin X cup from the microcentrifuge tube and place into a new microcentrifuge tube.
7. Add purified antibody to the Spin X cup. Recommended loading is 0.1 mg-1.2 mg of purified antibody prepared in 0.3-0.4 ml of Binding/Wash Buffer I.
Note: For this step, alternative buffers may be substituted in place of Binding/Wash Buffer 1, provided no amines (e.g., Tris, glycine, etc.) are present, the pH is between 7-8.5 and the salt concentration is not greater than 0.25 M.
8. Cap the microcentrifuge tube and place the tube on a rocker to allow the antibody to bind to the matrix. Incubate for at least 15 minutes.
9. Spin the microcentrifuge tube for 1 minute at maximum speed in a microcentrifuge.
Note: Flow-through can be quantitated to determine the amount of antibody bound to the resin.
10. Place the Spin X cup into another microcentrifuge tube and add 0.5 ml of Binding/Wash Buffer 1 into the cup. Gently invert the tube end over end 5-10 times.
11. Spin the microcentrifuge tube for 1 minute at maximum speed in a microcentrifuge. Discard the flow-through in the tube.
12. Repeat Steps 10 and 11 two additional times using the same collection tube.
13. Transfer the Spin X cup into a new microcentrifuge tube and add 0.5 ml of Binding/Wash Buffer 1. Proceed to Section B- Cross-linking of Bound Antibody.

Note: Conventional immunoprecipitation can be performed by omitting Section B- Cross-linking of Bound Antibody. If Section B is omitted, the antibody will co-elute with the antigen during the elution step.

B. Cross-linking of Bound Antibody

Note: The DSS cross-linker is moisture-sensitive. It should be stored refrigerated and desiccated. Be sure to warm vials to room temperature before opening. DSS should be dissolved in DMSO or DMF immediately before use. DSS is not compatible with amine-containing (e.g., Tris, glycine, etc.) buffers.

1. Dissolve the contents of one vial of DSS in 1.0 ml of DMSO or DMF for a final concentration of 13 mg/ml.
2. Immediately add 0.1 ml of the DSS solution prepared in Step 1 to the Spin X cup containing the bound antibody support. Discard the unused portion of DSS.

Note: Upon addition of the DSS dissolved in DMF or DMSO to an aqueous reaction medium, the solution may appear cloudy due to the formation of a microprecipitate. This is normal for a hydrophobic cross-linker. The reaction still will proceed efficiently and may result in the disappearance of the microprecipitate during the conjugation process.

Note: Reconstituted DSS may not be used again for subsequent reactions. Once reconstituted, it must be used immediately.

3. Place the top cap on the microcentrifuge tube and gently mix by inversion for 30-60 minutes. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Empty the microcentrifuge tube.
4. Add 500 µl of the Quenching/Wash Buffer 2 to the Spin X cup. Gently mix for 5-10 minutes. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Empty the microcentrifuge tube.
5. Add 500 µl of the Quenching/Wash Buffer 2 to the Spin X cup, cap the tube and gently mix end over end 10 times. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Place the Spin X cup into a new microcentrifuge tube.
6. Add 500 µl of ImmunoPure[®] Elution Buffer** to the Spin X cup. Gently mix end over end 10 times. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Empty the tube. Place the Spin X cup back into the microcentrifuge tube.

** **Note:** The pH of this buffer is 2.8 and will elute IgG from immobilized protein G that is not covalently attached to the resin. The majority of polyclonal antibodies and most monoclonal antibodies can tolerate low pH conditions for short durations. However, if your antibody is known to be intolerant of pH conditions between 2.5-3.0, you will want to utilize our Gentle Elution Buffer System, which is a high-salt, neutral pH elution system. The Gentle Elution Buffer is NOT compatible with phosphate-based buffers. (Pierce Product No. 21030 – Gentle Ag/Ab Binding and Elution Buffer System; Kit contains 100 ml Gentle Binding Buffer and 100 ml Gentle Elution Buffer.)

7. Repeat Step 6 four additional times. This removes excess DSS and uncoupled antibody. After the final elution, place the Spin X cup in a new microcentrifuge tube for next step.
8. Add 500 µl of Quenching/Wash Buffer 2 to the Spin X cup, cap the tube and gently mix the tube end over end 10 times.
9. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Remove the Spin X cup and empty the microcentrifuge tube.
10. Repeat Steps 8 and 9 two additional times. After the final wash, place the Spin X cup in a new microcentrifuge tube.

Proceed to Section C – Immunoprecipitation **or** if sample is not to be immunoprecipitated at this time, proceed to Section E – Regeneration of Gel/Storage Conditions.

C. Immunoprecipitation of the Antigen

1. Dilute the sample 1:1 with Binding/Wash Buffer 1 and add the sample to be purified to the Spin X cup. Recommended loading of sample is 0.2-0.5 ml. Incubate the sample with the gel using gentle rocking motion for 1-10 minutes.
2. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Repeat Steps 1 and 2 until the entire sample has been applied.

Note: Larger sample volumes (>0.5 ml) may not be needed depending on the concentration of your target protein.

3. Place the Spin X cup into a new microcentrifuge tube and add 0.5 ml of Quenching/Wash Buffer 2 to the Spin X cup. Gently invert the tube end over end 10 times.
4. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Discard flow-through buffer collected in the microcentrifuge tube.
5. Repeat Steps 3 and 4 two additional times using the same collection tube. After the final wash, place the Spin X cup into a new microcentrifuge tube and repeat Steps 3 and 4 one additional time.

Note: Before proceeding to elute the purified material, verify that the gel has been thoroughly washed to avoid contamination from residual proteins by checking the solution that flowed through in the last wash. (i.e., A_{280} , Coomassie[®] Protein Assay or BCA Protein Assay). There should be no protein in the final wash fraction. If the material has not been washed adequately, repeat Step 5 before proceeding. Extra washes are necessary only for samples containing high protein concentrations.

D. Elution of Immunoprecipitated Antigen

1. Add 190 μ l of ImmunoPure[®] Elution Buffer** to the Spin X cup, cap tube and gently invert the tube end over end 10 times. Spin the tube for 1 minute at maximum speed in a microcentrifuge

****Purification Options:**

- A. Before using the purified material in functional applications, neutralization of the elution buffer is necessary. Elution buffer pH conditions are between pH 2.5-3.0, but this can be neutralized with the addition of 10 μ l of a 1M Tris solution, pH 9.5, after elution. Alternatively, if the protein or antibody is intolerant to the low pH the Gentle Elution Buffer (neutral pH system) Prod. No. 21027, can be used instead.
 - B. If performing SDS-PAGE analysis, it is not necessary to neutralize the eluted samples. However, many low pH buffers will change color due to the low pH, but this color change will disappear after the sample runs into the gel.
2. Repeat Step 1 until desired sample is eluted. Sample should be eluted within the first three fractions. Do not pool fractions. Analyze each fraction by SDS-PAGE (see Section F) or Western blotting.
 3. Immediately following the last elution step, proceed to Section E- Regeneration of Gel/Storage Conditions of this instruction book. Immediately proceeding to the Regeneration of Gel/Storage Condition step will extend the life of the antibody-coupled resin.

E. Regeneration of Gel/Storage Conditions

1. Add 0.5 ml of Quenching/Wash Buffer 2 to the Spin X cup. Cap tube and gently invert 10 times.
2. Spin the tube for 1 minute at maximum speed in a microcentrifuge. Empty the microcentrifuge tube.
3. Repeat Steps 1 and 2.
4. Add 0.5 ml of Wash Buffer 1 or 2 to the Spin X cup. Cap and store. For long-term storage, add sodium azide to a final concentration of 0.02%.
5. For convenience, place the capped microcentrifuge tube with the antibody resin into the foam insert of the Seize[™] X Kit box for storage.

Note: A sufficient number of microcentrifuge tubes is supplied in the kit for 40 IPs if the storage tube is also used during the wash steps of the IP procedure. If the number of tubes is not sufficient, any 1.5 ml microcentrifuge tube can be substituted.

F. Preparation of Samples for Loading onto an SDS-PAGE Gel

1. Pipette a 20 μ l aliquot of the sample into a microcentrifuge tube.

2. Allow sample buffer (dark pink-colored solution) to come to room temperature. Gently mix the sample buffer by inverting 5-10 times. Pipette approximately a 5 μ l aliquot of the sample buffer into the microcentrifuge tube.

Note: The sample buffer is fairly viscous and may require that the pipette tip be “snipped” to allow the solution to be drawn up into the tip. Cap the microcentrifuge tube and gently mix by inverting the tube 5-10 times. This sample buffer does not contain reducing agents. To prepare the sample for a reducing gel, add 2-3 μ l of a 1 M DTT solution (M.W. 154.25) to the 25 μ l sample containing sample buffer.

3. Place the microcentrifuge tube in a microcentrifuge tube holder and place the holder in boiling water. Incubate the sample in the boiling water for ~5 minutes.
4. Allow the sample to cool to room temperature. The sample is now ready to be loaded onto the electrophoresis gel.

Binding Capabilities of Immobilized Protein A

Antibody	Binding Capabilities
Human IgG1, IgG2 and IgG4	Strong
Human IgG3	Weak
Rabbit IgG	Strong
Mouse IgG2a, IgG2b and IgG3	Strong
Mouse IgG1	Weak
Goat IgG	Weak
Bovine IgG2	Strong
Bovine IgG1	Weak
Sheep IgG	Weak
Monkey IgG (Rhesus)	Strong
Donkey IgG	Moderate
Guinea Pig IgG	Strong
Cat IgG	Strong
Chicken IgG	No Binding
Rat IgG1	Weak
Rat IgG2a and Rat IgG2b	No Binding
Rat IgG2c	Strong
Dog IgG	Strong

Troubleshooting Guide:

Problem: Leaching antibody from the resin or co-elution of the antibody

Probable causes:

1. Too much antibody was loaded onto the resin (exceeded 1.2 mg recommendation)
2. The resin was not washed sufficiently with elution buffer prior to applying sample (See Steps 6 and 7 under the section Cross-linking of Bound Antibody)

Solution to 1 and 2: Wash resin with additional elution buffer until no additional antibody elutes from the resin (either by protein assay or by absorbance at 280 nm), re-equilibrate the column with Binding/Wash Buffer 1 and re-apply sample.

Problem: Antigen does not immunoprecipitate

Probable causes:

1. Sample does not contain large enough quantities of the antigen to detect.
(The lysis of the cells may not have been complete or the protein may have not been expressed.) Verify expression of protein of interest and efficiency of lysis by running an SDS-PAGE gel of the crude lysate.
2. Antibody is not cross-linked to the matrix.
(Verify that antibody initially bound to the resin by checking wash fractions from Section A either by A_{280} or with a protein assay to ensure the antibody initially bound and to also verify that the antibody was not eluted during the elution wash step in the cross-linking Section B.)
3. Antibody has lost its titer.
4. Antibody is sensitive to low pH and was destroyed during the wash steps (extremely rare).
5. Antibody has high affinity for the antigen and does not elute under acidic conditions. Solution: Use guanidine HCl, urea, lithium bromide, potassium thiocyanate or nonionic detergents to elute the antigen.⁸

Note: Using denaturants may cause the immobilized antibody to lose activity.

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