

# Seize<sup>®</sup> X Protein G Immunoprecipitation Kit

45210

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Number	Description
45210	<p><b>Seize<sup>®</sup> X Protein G Immunoprecipitation Kit</b>, contains sufficient reagents to prepare four antibody immobilization reactions and 40 immunoprecipitations</p> <p><b>Kit Contents:</b></p> <p><b>ImmunoPure<sup>®</sup> Immobilized Protein G Plus</b>, 1 ml of settled gel supplied as a 50% slurry (e.g., 400 µl of 50% slurry is equivalent to 200 µl of settled gel)</p> <p><b>Binding/Wash Buffer: BupH<sup>™</sup> Modified Dulbecco's PBS Pack</b>, 1 pack, results in 0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M potassium phosphate and 0.01 M KCl, pH 7.4 when reconstituted</p> <p><b>ImmunoPure<sup>®</sup> IgG Elution Buffer</b>, 50 ml, pH 2.8, contains primary amine</p> <p><b>Handee<sup>™</sup> Spin Cup Columns</b>, 12 each</p> <p><b>Handee<sup>™</sup> Microcentrifuge Tubes</b>, 72 each</p> <p><b>ImmunoPure<sup>®</sup> Lane Marker Sample Buffer, Non-reducing, (5X)</b>, 5 ml, 0.3 M Tris•HCl, 5% SDS, 50% glycerol, lane marker tracking dye; pH 6.8</p> <p><b>DSS (disuccinimidyl suberate)</b>, 8 x 2 mg microtubes</p> <p><b>Storage:</b> Upon receipt store kit at 4°C. Store DSS desiccated at 4°C. Kit is shipped at ambient temperature.</p>

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## Introduction

The Seize<sup>®</sup> X Immunoprecipitation Kit offers an improvement over the classical method of immunoprecipitation (IP) by immobilizing the antibody to the Protein G support using the cross-linker DSS. This procedure results in a permanent affinity support with a properly oriented antibody. The crude sample is then incubated with the immobilized antibody to form the immune complex. The affinity support is washed by centrifugation using a Handee<sup>™</sup> Spin Cup Column and the remaining antigen is dissociated from the antibody using an elution buffer. The primary antibody does not contaminate the final antigen preparation and the immobilized antibody support is preserved for future IPs. Depending on the stability of the immobilized antibody, the prepared affinity support may be used 2-10 times, thus conserving precious antibody. The kit also contains a 5X sample buffer, which may be used to prepare eluted samples for SDS-PAGE without significant dilution of the sample.

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Once the antibody is immobilized, small-scale antigen purifications can be performed for a variety of applications such as protein assays, ELISAs, binding studies, Western blotting, etc. Different binding and elution buffer conditions may be substituted to preserve the activity of the antigen if the kit buffer conditions are not suitable for a particular application. The researcher also has the option to perform the classical method of IP by omitting the antibody cross-linking step. The Seize<sup>®</sup> X Method prevents antibody contamination of the antigen, conserves precious primary antibody and offers faster and easier IPs.

## Important Product Information

- Perform all steps at room temperature unless otherwise indicated. The steps may be performed at 4°C but will take longer for completion.
- Perform all gel centrifugation steps for 1 minute at medium speed (i.e., 3,000-5,000 x g). Centrifuging at greater speeds may cause the gel to clump and make resuspending the gel difficult.

## Procedure for the Seize<sup>®</sup> X Protein G Immunoprecipitation Kit

### A. Binding of Antibody to Immobilized Protein G

1. Equilibrate the Immobilized Protein G and reagents to room temperature.
2. Add 500 ml of ultrapure water to the dry-blend buffer. To store excess buffer, add a preservative such as 0.02% sodium azide and store at 4°C.
3. Gently swirl the bottle of ImmunoPure<sup>®</sup> Immobilized Protein G to obtain an even suspension. Add 0.4 ml of the Immobilized Protein G (50% slurry) into one of the Handee<sup>™</sup> Spin Cup Columns and place inside a Handee<sup>™</sup> Microcentrifuge Tube and centrifuge.

**Note:** Less Immobilized Protein G may be used (i.e., 0.1-0.4 ml of the 50% slurry); however, the amount of DSS should then be scaled proportionately.

4. Discard flow-through and replace spin cup into the tube.
  5. Wash gel by adding 0.4 ml of Binding/Wash Buffer to the gel. Cap tube and resuspend gel by inverting with gentle shaking. Centrifuge tube. Discard flow-through and replace spin cup into tube. Repeat this step once.
  6. Place spin cup into a new microcentrifuge tube. Apply 50-500 µg of purified antibody prepared in 0.3-0.4 ml of Binding/Wash Buffer to the prepared gel.
- Note:** For this step, alternative buffers may be substituted in place of Binding/Wash Buffer, provided no primary 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.
7. Cap the microcentrifuge tube and place it on a rocker for at least 15 minutes to allow the antibody to bind to the gel.
  8. Centrifuge the tube. If desired, save the flow-through to estimate the amount of antibody bound to the Protein G.
  9. Place spin cup into another microcentrifuge tube and add 0.5 ml of Binding/Wash Buffer. Invert tube 5-10 times. Centrifuge tube and discard flow-through. Repeat this step two additional times using the same collection tube.
  10. Transfer the spin cup into a new microcentrifuge tube and add 0.4 ml of Binding/Wash Buffer.

### B. Cross-linking the Bound Antibody

**Note:** Conventional IP may be performed by omitting cross-linking the bound antibody. If cross-linking is omitted, the antibody will co-elute with the antigen during the elution step.

**Note:** The DSS cross-linker is moisture-sensitive. Keep DSS in foil pouch after use. Dissolve DSS in DMSO or DMF immediately before use. DSS is not compatible with amine-containing (e.g., Tris, glycine) buffers.

1. Puncture the foil covering of a single tube of DSS with a pipette tip and add 80 µl of DMSO or DMF. Use the pipette to thoroughly mix the solution (i.e., draw up and expel the solution) until the DSS is dissolved.
2. Add 25 µl of the DSS solution to the spin cup containing the bound antibody support. The tube containing the reconstituted DSS can be discarded from the strip by pushing tube from the bottom, away from the strip.

**Note:** Because DSS is a hydrophobic molecule, a microprecipitate may form when it is added to the aqueous medium, which results in a cloudy appearance. Nevertheless, the reaction will proceed efficiently and the microprecipitate may disappear during conjugation.

**Note:** Reconstituted DSS cannot be reserved for subsequent reactions. Once reconstituted, the DSS must be used immediately. Discard any unused reconstituted DSS.

- Place the top cap on the tube and gently mix for 30-60 minutes. Centrifuge tube and discard flow-through.
- Add 500  $\mu$ l of ImmunoPure<sup>®</sup> Elution Buffer (see note) to the spin cup. Cap tube and invert it 10 times. Centrifuge tube and discard flow-through. Place the spin cup back into the microcentrifuge tube.

**Note:** The pH of the elution buffer is 2.8 and will elute IgG that is not covalently coupled to the Immobilized Protein G. The majority of polyclonal antibodies and most monoclonal antibodies can tolerate low pH conditions for short durations. However, if an antibody is known to be intolerant of pH conditions between 2.5-3.0, use the Gentle Elution Buffer System, which is a high-salt, neutral pH elution system. The ImmunoPure<sup>®</sup> Gentle Elution Buffer is NOT compatible with phosphate-based buffers. (Product No. 21030 – ImmunoPure<sup>®</sup> Gentle Ag/Ab Binding and Elution Buffer System; kit contains 100 ml Gentle Binding Buffer and 100 ml Gentle Elution Buffer.)

- Repeat Step 4 four additional times to quench the reaction and to remove excess DSS and uncoupled antibody.
- Place the spin cup in a new microcentrifuge tube and wash gel two times using 500  $\mu$ l Binding/Wash Buffer.
- Proceed to Section C. Antigen Immunoprecipitation. If sample is not to be immunoprecipitated at this time, proceed to Section E. for storage conditions.

### C. Antigen Immunoprecipitation

**Note:** The antigen amount and the incubation time needed are dependent upon the antibody-antigen system used and must be optimized for each system.

- Dilute sample 1:1 with Binding/Wash Buffer and add sample to be purified to the spin cup. Recommended sample loading in the spin cup is 0.2-0.5 ml. For larger sample volumes, transfer antibody-coupled gel to a separate tube containing the sample.
- Incubate sample with gentle mixing for at least 1 hour. Typical incubation times range from 1 hour to overnight. For overnight incubations, incubate sample at 4°C.
- Centrifuge the tube. If the incubation was performed in a separate tube, transfer the sample back to the spin cup. Individual 0.5 ml aliquots may be applied and centrifuged until the entire sample has been processed.
- Place the spin cup into a new microcentrifuge tube and add 0.5 ml of Binding/Wash Buffer to the spin cup. Cap the tube and invert it 10 times. Centrifuge tube and discard flow-through, or save it for future analysis. Repeat this wash two additional times using the same collection tube. After the final wash, place the spin cup into a new microcentrifuge tube and wash one additional time.

**Note:** To avoid contamination from residual proteins, before eluting the purified material verify that the gel has been thoroughly washed by performing a protein assay (e.g., A<sub>280</sub>, Coomassie Plus<sup>™</sup> Protein Assay or BCA<sup>™</sup> Protein Assay) on the flow-through from the final wash. There should be minimal protein in the final wash fraction. If the material has not been adequately washed, repeat Step 4 before proceeding. Additional washes may be necessary for samples containing high protein concentrations.

### D. Elution of Immunoprecipitated Antigen

**Note:** Before using the purified material in functional applications, neutralize the pH after the antigen has been eluted. The Elution Buffer has a pH of 2.5-3.0 and can be neutralized by adding 10  $\mu$ l of 1 M Tris, pH 9.5 per 200  $\mu$ l of Elution Buffer. Alternatively, if the protein or antibody is sensitive to low pH, use a neutral pH system, such as ImmunoPure<sup>®</sup> Gentle Elution Buffer (Product No. 21027). When using the Gentle Elution Buffer, wash gel in Step C.4 with 25 mM Tris, 0.15 M NaCl; pH 7.2.

If performing SDS-PAGE, it is not necessary to neutralize the eluted samples. However, the dye in the sample buffer may change color caused by the low pH, but this color change will disappear after the sample enters into the gel.

- Add 190  $\mu$ l of ImmunoPure<sup>®</sup> Elution Buffer to the spin cup. Cap tube and invert it 10 times. Centrifuge the tube.

**Note:** Smaller elution volumes may be used if the contents of the spin cup are first transferred to a microcentrifuge tube. Elution volumes must be sufficient to resuspend the gel.

- Repeat Step 1 until desired sample is eluted. Sample should elute within the first three fractions. Do not pool fractions. Assess the first three fractions by SDS-PAGE. To preserve activity of the immobilized antibody, proceed to Section E immediately following the last elution step.

**E. Gel Regeneration and Storage Conditions**

1. Add 0.5 ml of Binding/Wash Buffer to the spin cup. Cap tube and invert it 10 times. Centrifuge tube and discard flow-through. Repeat this step once.
2. Add 0.5 ml of Binding/Wash Buffer to the spin cup. Place spin cup into a microcentrifuge tube, cap tube and wrap with laboratory film to prevent gel from drying. For long-term storage, add sodium azide at a final concentration of 0.02%.
3. For convenience, place the wrapped microcentrifuge tube containing the immobilized antibody into the foam insert of the Seize<sup>®</sup> X Kit Box and store at 4°C.

**Note:** A sufficient number of microcentrifuge tubes is supplied for 40 IPs if the storage tube is also used during the wash steps of the IP procedure. If more tubes are required, any 1.5 ml microcentrifuge tube may be substituted.

**F. Sample Preparation for SDS-PAGE**

1. Pipette 20 µl of the sample into a microcentrifuge tube.
2. Equilibrate sample buffer (dark pink-colored solution) to room temperature. Gently mix the sample buffer by inverting 5-10 times. Pipette approximately 5 µl of the sample buffer into the microcentrifuge tube.

**Note:** The sample buffer is 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 µl of a 1 M DTT solution (MW=154.25) to the 25 µl sample containing sample buffer and mix well.

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

**Troubleshooting**

<b>Problem</b>	<b>Possible Cause</b>	<b>Solution</b>
Antibody leaching from gel (i.e., antibody detected together with eluted antigen)	Too much antibody was added to the gel	Wash the antibody-coupled gel with Elution Buffer until no antibody elutes (as determined by protein assay or measuring the absorbance at 280 nm), re-equilibrate column with Binding/Wash Buffer and re-apply antigen sample
	Gel was not washed sufficiently with elution buffer before antigen was applied	
Antigen does not immunoprecipitate	Sample does not contain enough antigen to detect (cell lysis incomplete, protein not expressed, etc.)	Verify protein expression and/or lysis efficiency by SDS-PAGE or Western blot of the crude lysate
	Antibody not cross-linked to the Immobilized Protein G	Analyze wash fractions from Section A and Section B by using a protein assay or by measuring the absorbance at 280 nm to verify that antibody initially bound to the gel and that the antibody was not eluted during the cross-linking elution step
	Antibody is sensitive to low pH and has lost activity (extremely rare)	Prepare more antibody-coupled support and then use a high-salt, neutral pH elution buffer such as Product No. 21027
	The antibody-antigen interaction does not elute using acidic conditions	Use a neutral pH elution buffer, guanidine•HCl, urea, lithium bromide, potassium thiocyanate or nonionic detergents to elute antigen  <b>Note:</b> Using denaturants may cause the antibody to lose activity and, therefore, the antibody-coupled gel cannot be reused

## Additional Information

Please visit the Pierce web site for additional information relating to this product including the following items:

- Tech Tip: Binding characteristics of Proteins L, A, G and A/G to immunoglobulins
- Tech Tip: Optimize Elution Conditions for Immunoaffinity Chromatography
- Frequently Asked Questions for DSS
- Tech Tip: Protein Stability and Storage

## Related Pierce Products

25200-25244	Precise™ Protein Gels (see catalog or web site for a complete listing)
45335	Seize® Primary Immunoprecipitation Kit
45332	Seize® Primary Mammalian Immunoprecipitation Kit
45215	Seize® X Protein A Immunoprecipitation Kit
45220	Seize® X Bacterial Immunoprecipitation Kit
45225	Seize® X Mammalian Immunoprecipitation Kit
45230	Seize® X Yeast Immunoprecipitation Kit
69700	Handee™ Spin Cup Column Columns
69715	Handee™ Microcentrifuge Tubes
23225	BCA™ Protein Assay Kit*
23236	Coomassie Plus™ -The Better Bradford Assay Kit

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\*BCA Technology is protected by U.S. Patent # 4,839,295.

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