Seize X Mammalian Immunoprecipitation Kit
(Pierce, cat #: 45225)

This kit has a cross linker **DSS** that can bind the antibody of interest to protein G coated beads (by a covalent bond), thus, during elution of the immunoppt, only the corresponding antigen should be eluted wheras the antibody still on the beads and can be used in further experiments (2-10 times).

First things to do:
dissolve one envelope of dry-blend bind/wash buffer (Bup H Modified Dulbecc's PBS: 0.14M NaCl, 0.008M sodium phosphate, 0.002M potassium phosphate and 0.01M KCl, pH 7.4) in 500ml of ultrapure water. Add a preservative such as 0.02% sodium and store at 4C for long storage.

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.

Binding of the antibody to immobilized protein G:

1. Equilibrate the Immobilized Protein G (50% slurry) and reagents to room temperature.

2. Gently swirl the bottle Protein G to obtain an even suspension. Add 0.4ml (0.2ml) of Protein G gel beads into one of the Handee™ Spin Cup Columns and place inside a Handee™ Microcentrifuge Tube.
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.

3. Centrifuge the tube and discard flow-through.

4. Wash gel by adding same volume of washing/binding buffer (i.e. 0.4 ml; in my case 0.2ml) to the spin cup, cap the tube and resuspend gel by inverting tube with gentle shaking. Centrifuge tube and discard flow-through. Place spin cup back into the tube. Repeat this step once.

5. Place the 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. [my Ab conc is 5µg/µl, I used 100µl + 200µl of bind buffer].
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 7.0-8.5 and the salt concentration is not greater than 0.25 M.

6. Cap the microcentrifuge tube and place it on a rocker for at least 15 minutes to allow the antibody to bind to the gel.

7. Centrifuge the tube. If desired, save the wash to estimate the amount of antibody bound to the Protein G.

8. Place the spin cup into another microcentrifuge tube and add 0.5 ml of Binding/Wash Buffer. Invert the tube 5-10 times.
Centrifuge the tube and discard the flow-through. Repeat this step two additional times using the same collection tube.

9. Transfer spin cup into a new microcentrifuge tube and add 0.4 ml of Binding/Wash Buffer.

B. Cross-linking the Bound Antibody

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 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 0.4ml beads, scale it if less amount is used; even I started with 0.2ml beads and thus I only need 12.5µl of DSS, I still use 25µl in 0.4ml) prepared in step 1 to the spin cup containing the bound antibody support. The tube containing reconstituted DSS can be discarded from the strip by pushing tube from the bottom, away from the strip.

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

3. Incubate for 30-60 minutes. Centrifuge the tube and discard the flow-through.

4. Add 500 µl of Elution Buffer (see note) the spin cup. Cap the tube and invert it 10 times. Centrifuge the tube and discard the flow-through. Place the spin cup back into the microcentrifuge tube. Repeat this step four additional times to quench the reaction and to remove excess DSS and uncoupled antibody.

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® Gentle Elution Buffer is NOT compatible with phosphate-based buffers. (Product No. 21030 – ImmunoPure® Gentle Ag/Ab Binding and Elution Buffer System; kit contains 100 ml Gentle Binding Buffer and 100 ml Gentle Elution Buffer.)

5. Place the spin cup in a new microcentrifuge tube and wash gel two times with 500 µl Binding/Wash Buffer.

6. Proceed to Section C. If sample is not to be immunoprecipitated at this time, proceed to Section F for storage conditions.

D. Immunoprecipitation of the Antigen

1. Dilute the mammalian extract sample at least 1:1 with Binding/Wash Buffer. In total volume between 0.2-0.5 ml. I used 100µl of 1%CHAPS S. mansoni extract+ 100µl of wash/bind buffer.

2. Centrifuge to remove the Binding/Wash Buffer used to store the antibody-coupled support.
3. Place spin cup into a new microcentrifuge tube. Add the sample to the antibody-coupled gel in the spin cup column. Incubate with gentle end-over-end mixing or rocking for at least 1 hour. Typical incubation times range from 2 hours to overnight. For overnight incubations, incubate sample at 4ºC. I incubate for 2-2.5hrs at RT using end-over-end rotor.

Note: For large sample volumes, transfer the antibody-coupled gel to a separate tube containing the antigen solution.
After incubation, centrifuge 0.5 ml aliquots through the spin cup until the entire sample has been processed.

4. Centrifuge the tube and discard the flow-through or save it for future analysis.

5. Place the spin cup into a new microcentrifuge tube and add 0.4 ml of Binding/Wash Buffer. Gently invert the tube end-over-end 10 times and centrifuge the tube. Repeat this step two additional times (three times total). Instead, I do 5 times washes with 0.4ml each wash.

Note: Evaluate the washes (e.g., A280, SDS-PAGE or Micro BCA™ Protein Assay) to determine the optimal number of washes for the specific system. There should be no protein in the final wash fraction. Extra washes are usually only necessary for samples containing high protein concentrations.

E. Elution of Immunoprecipitated Antigen

Note: Before using the purified material in functional applications, neutralize the pH after the antigen is eluted. The Elution Buffer has a pH of 2.5-3.0 and can be neutralized by adding 10 µl of 1 M Tris, pH 9.5 per 200 µl of Elution Buffer.

Alternatively, if the protein or antibody is sensitive to the low pH, use a neutral pH system, such as ImmunoPure® Gentle Elution Buffer (Product No. 21027). When using the Gentle Elution Buffer, wash the gel in steps D.5 with 25 mM Tris, 0.15 M NaCl; pH 7.2.

If performing SDS-PAGE analysis, 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 the gel.
1. Add the appropriate volume (see Table 2) of ImmunoPure® Elution Buffer to the gel in the spin cup, cap the tube and

gently tap tube to mix. Centrifuge the tube.

Table 2. Amount of Elution Buffer to add to the antibody-coupled gel.

1. Add the appropriate volume (see Table 2) of ImmunoPure® Elution Buffer to the gel in the spin cup, cap the tube and gently tap tube to mix. Centrifuge the tube.

Table 2. Amount of Elution Buffer to add to the antibody-coupled gel.

Recommended Volume of Elution Buffer	Amount of Antibody-Coupled Gel (50% Slurry)	Amount of Antibody-Coupled Settled Gel
200 µl	400 µl	200 µl
100 µl	200 µl	100 µl
50 µl	100 µl	50 µl

2. Repeat Step 1 until the antigen is eluted. Antigen should be eluted within the first three fractions. Do not pool fractions.
Assess the amount of protein in the first three fractions by SDS-PAGE. Use GelCode® Blue Stain Reagent (Product No.
24590) for fast results without destaining.

3. To preserve activity of the immobilized antibody, proceed to Section E immediately following the last elution step.

F. Regeneration of Gel 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 flowthrough. 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® 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.