INSTRUCTIONS

Seize® X Mammalian Immunoprecipitation Kit

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45225

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Number	Description
45225	**Seize X Mammalian Immunoprecipitation Kit,** contains sufficient reagents to prepare four antibody immobilization reactions and 40 immunoprecipitations

Kit Contents:

Immobilized Protein G Plus, 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)

BupH™ Modified Dulbecco's PBS Pack, 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

IgG Elution Buffer, 50 ml, pH 2.8, contains primary amine

Handee™ Spin Cup Columns, 12 each, columns contain 0.45 µm cellulose acetate filters

Handee Microcentrifuge Tubes, 72 each

Lane Marker Sample Buffer, Non-reducing, (5X), 5 ml, 0.3 M Tris•HCl, 5% SDS, 50% glycerol, lane marker tracking dye; pH 6.8

No-Weigh™ DSS (disuccinimidyl suberate), 8 × 2 mg microtubes

M-PER® Mammalian Protein Extraction Reagent, 25 ml

Storage: Upon receipt store kit at 4°C. Store DSS desiccated at 4°C. Kit is shipped at ambient temperature.

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Introduction

The Seize X Immunoprecipitation Kit offers an improvement over the classical method of immunoprecipitation (IP) by immobilizing the antibody to the Protein G support using the crosslinker 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 Spin Cup Column and the interacting 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 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.

Once the antibody is immobilized, small-scale antigen purifications can be performed for a variety of applications such as protein assays, ELISAs, binding studies and Western blotting. Different binding and elution buffer conditions may be substituted 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 antibody crosslinking. The Seize X Method prevents antibody contamination of the antigen, conserves antibody and offers faster and easier IPs.

This kit also includes the M-PER Mammalian Protein Extraction Reagent for lysing eukaryotic cells quickly and gently. M-PER Reagent is more efficient at extracting proteins than the freeze-thaw method and is gentler than most other lysis methods, yielding greater amounts of native protein.

General Guidelines for Mammalian Cell Lysis using M-PER Reagent

- **Adherent Cells vs. Cell Pellets:** M-PER Reagent effectively lyses plated cells, cells pelleted from suspension cultures or scraped cells. For direct, in-plate lysis of adherent cells, protein extraction efficiency is similar to freeze/thaw methods. For lysis of pelleted cells (from cell suspension or scraped adherent cells), protein extraction efficiency is typically 25% greater than that achieved with freeze-thaw (three cycles) and 20% greater than sonication (2 minutes with 50% pulse) methods.
- **Cell Lines:** M-PER Reagent has been tested on representatives of several different cell types. Complete lysis of adherent cells was observed with, but is not limited to, the following cell lines: COS-7, NIH3T3, Hepa 1-6, 293, CHO, MDA, MB 231 and FM2 cells.
- **Additives:** For immunoassays such as ELISA or RIA, extracts prepared in M-PER Reagent alone will generate satisfactory results. However, the addition of 150 mM NaCl to the cell lysate has been shown to improve results.
- **Volume for Cell Lysis:** Volumes listed in Table 2 are optimal for cell lysis without scraping cells. If more concentrated extracts are preferred, a smaller volume may be used; however, scraping the cells is necessary for maximal recovery.
- **Compatibility with Protein Assays:** A feature that distinguishes M-PER Reagent from other detergent-based lysis methods is its compatibility with both Coomassie Plus™ (Product No. 23236) and BCA™ Protein Assay (Product No. 23225) to determine protein extraction efficiency.

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 × *g*). Centrifuging at greater speeds may cause the gel to clump and make resuspending the gel difficult.

Procedure for the Seize X Mammalian Immunoprecipitation Kit

A. Binding of Antibody to Immobilized Protein G

1. Equilibrate the Immobilized Protein G Plus and reagents to room temperature.
2. Combine 500 ml of ultrapure water with the BupH Modified Dulbecco's PBS. To store excess buffer, add a preservative such as 0.02% sodium azide and store at 4°C.
3. Gently swirl the bottle of immobilized Protein G to obtain an even suspension. Add 0.4 ml of the 50% slurry 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.

4. Centrifuge the tube and discard flow-through. Place spin cup back into the tube.
5. Wash gel by adding 0.4 ml of PBS 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.
6. Place the spin cup into a new microcentrifuge tube. Apply 50-500 µg of purified antibody prepared in 0.3-0.4 ml of PBS.

   Note: For this step, alternative buffers may be used provided no primary amines (e.g., Tris, glycine, etc.) are present, the pH is 7.0-8.5 and the salt concentration is ≤ 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 wash to estimate the amount of antibody bound to the Protein G.
9. Place the spin cup into another microcentrifuge tube and add 0.5 ml of PBS. 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.
10. Transfer spin cup into a new microcentrifuge tube and add 0.4 ml of PBS.

B. Crosslinking the Bound Antibody

Note: To perform conventional immunoprecipitation, omit crosslinking. If crosslinking is omitted, the antibody will co-elute with the antigen during the elution step.

Note: DSS is moisture-sensitive. Store the unused microtubes in the foil pouch. 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 No-Weigh 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 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.

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

   Note: Once reconstituted, the DSS must be used immediately. Discard any unused reconstituted DSS.
3. Place cap on the tube and gently mix for 30-60 minutes. Centrifuge the tube and discard the flow-through.
4. Add 500 µl of Elution Buffer (see note) to the spin cup. Cap tube and invert 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 elutes IgG that is not covalently coupled to the Protein G. Most antibodies can tolerate low pH conditions for a short duration; however, if an antibody has known intolerance of pH 2.5-3.0, use the Gentle Elution Buffer System, which is a high-salt, neutral pH system. The Gentle Elution Buffer is NOT compatible with phosphate-based buffers. (Product No. 21030 – Gentle Ag/Ab Binding and Elution Buffer System; kit contains 100 ml of binding buffer and 100 ml elution buffer.)
5. Place the spin cup in a new microcentrifuge tube and wash gel two times with 500 µl of PBS.
6. Proceed to Section C. If sample is not to be immunoprecipitated at this time, proceed to Section F for storage conditions.

C. Mammalian Cell Lysis

Protocol I: Lysis of Adherent Mammalian Cells

1. Carefully remove (decant) culture medium from adherent cells.
2. Optional wash: If the culture medium contained phenol red or other reagents that could interfere with subsequent protein analysis, wash the cells once with PBS (Product No. 28372; 0.1 M phosphate, 0.15 M NaCl, pH 7.2) or PBS.
3. Add the volume of M-PER Reagent as indicated in Table 1 to the cells. Gently shake the reaction for 5 minutes.

Table 1. Suggested volume of M-PER Reagent to use for different sizes of standard culture plates.

Plate Size/Surface Area	Volume of M-PER Reagent
100 mm 60 mm	250-500 µl
6-well plate	200-400 µl per well
24-well plate	100-200 µl per well
96-well plate	50-100 µl per well

4. Collect lysate and transfer to a microcentrifuge tube. Centrifuge samples at ~13,000 × *g* for 5-10 minutes to pellet debris.
5. Transfer supernatant to a new tube for further analysis.

Protocol II: Lysis of Mammalian Cells in Suspension

1. Centrifuge the cell suspension at 2,500 × *g* for 10 minutes to pellet cells. Discard the supernatant.
2. Optional wash: If the culture medium contained phenol red or other reagents that could interfere with subsequent protein analysis, wash cells once by suspending the cell pellet in a desired wash buffer (e.g., PBS). Centrifuge at 2,500 × *g* for 10 minutes to pellet cells.
3. Add M-PER Reagent to the cell pellet (500 µl of reagent is sufficient for lysing 50 mg of wet cell pellet). For optimal results use a 10:1 v/w ratio.

   Note: If a large amount of cells is used, first add 10% the final recommended volume of M-PER Reagent to the cell pellet. Pipette the mixture up and down to suspend. Add the remaining volume of M-PER Reagent to the cell suspension.
4. Shake the reaction gently for 10 minutes. Remove cell debris by centrifugation at 27,000 × *g* for 15 minutes.
5. Transfer supernatant to a new tube for further analysis.

D. Immunoprecipitation of the Antigen

Note: The amount of antigen needed and the incubation time are dependent upon the antibody-antigen system used and require optimization for each specific system.

1. Dilute the mammalian extract sample (prepared in section C) at least 1:1 with PBS. Recommended total sample volume in the Handee Spin Cup is 0.2-0.5 ml.
2. Centrifuge to remove the PBS 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.

   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 PBS. Gently invert the tube end-over-end 10 times and centrifuge the tube. Repeat this step two additional times (three times total).

   Note: Evaluate the washes (e.g., A_{280}, 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 necessary only for samples containing high protein concentrations.

E. Elution of Immunoprecipitated Antigen

Note: After elution and before using the purified material in functional applications, neutralize the pH of the eluted sample. 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 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 disappears after the sample enters the gel.

1. Add the appropriate volume (see Table 2) of 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 elute 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 F immediately following the last elution step.

F. Regeneration of Gel and Storage Conditions

1. Add 0.5 ml of PBS 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 PBS 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.

G. Preparation of Samples for SDS-PAGE

1. Pipette 20 µl of the sample into a microcentrifuge tube.
2. Equilibrate Lane Marker Sample Buffer 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 tube and gently mix by inverting the tube 5-10 times. 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 the 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 and load it into the electrophoresis gel.

Additional Information

Please visit the Pierce website for additional information 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

Troubleshooting

Problem	Possible Cause	Solution
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), equilibrate column with PBS and re-apply antigen sample
	Gel was not washed sufficiently with elution buffer before antigen was added	
Antigen does not immunoprecipitate	Sample does not contain enough antigen to detect (cell lysis incomplete, protein not expressed,)	Verify protein expression and/or lysis efficiency by SDS-PAGE or Western blot of the crude lysate
	Antibody not crosslinked 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 crosslinking elution step
	Antibody is sensitive to low pH and has become inactive (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 **Note:** Using denaturants may inactivate the antibody and, therefore, the antibody-coupled gel will no longer immunoprecipitate the target

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**

45210 **Seize X Protein G Immunoprecipitation Kit**

45215 **Seize X Protein A Immunoprecipitation Kit**

69702 **Handee Spin Cup Columns,** 50 units, contains 0.45 µm cellulose acetate filter

69720 **Handee Microcentrifuge Tubes, 2 ml,** 72 tubes

23235 **Micro BCA Protein Assay Reagent Kit**

23236 **Coomassie Plus - The Better Bradford™ Assay Kit**

General References

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Current versions of product instructions are available at *www.piercenet.com.* For a faxed copy, call 800-874-3723 or contact your local distributor.