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A Geno Technology, Inc. (USA) brand name

Cross-Linking Immunoprecipitation

Utilizes Protein A/G Agarose& DSS for Antibody Immobilization

(Cat. # 786-639)

INTRODUCTION

Immunoprecipitation (IP) is one of the most useful immunochemical techniques. IPs are routinely used to determine the presence and quantity of an antigen, molecular weight of a polypeptide, rate of synthesis or degradation, identify certain post translational modifications and interactions with other proteins, nucleic acids and ligands. IPs consist of four main steps:

1. Labeling of the antigen (Optional step)
2. Release of antigen by cell lysis
3. Formation of antibody-antigen complexes
4. Purification of the immune complexes.

G-Biosciences Cross-Linking Immunoprecipitation kit contains all the reagents necessary to complete all aspects of immunoprecipitation, with the exception of labeling. Based on our Classical Immunoprecipitation kit (Cat. # 786-637), the Cross-Linking Immunoprecipitation kit incorporates the homobifunctional cross-linker DSS to covalently couple the antibody to the Immobilized Protein A/G Agarose. This additional cross-linking step prevents contamination of the purified antigen with antibody. The kit is designed for effective immunoprecipitations using <10µg antibody. The kit is suitable for 50 reactions using 10µl Immobilized Protein A/G Agarose.

ITEM(S) SUPPLIED (Cat. # 786-639)

Description	**Size**
Collection Tube, 2ml	100
Control Agarose (4% agarose)	2ml
Elution Buffer	50ml
Immobilized Protein A/G Agarose	0.55ml resin
Mammalian Cell PE LB™	100ml
OneQuant™ DSS (Disuccinimidyl suberate)	8 x 2mg
IP Coupling Buffer [20X]	25ml
Spin Column	50
Caps	50

STORAGE CONDITIONS

Shipped at ambient temperature. Upon arrival, store at 4°C. Store OneQuant™ DSS desiccated at 4°C.

ADDITIONAL ITEM(S) REQUIRED

- 1X PBS
- Centrifuge tubes (1.5ml)
- DMSO

IMPORTANT INFORMATION

- Perform all steps at 4°C, unless stated otherwise.
- Perform centrifugations for 60 seconds at 1,000-2,000xg. Excessive speeds or times may result in resin clumping making it difficult to resuspend the resin.
- We recommend using an affinity purified antibody as serum, although compatible with this kit, will result in a lower antigen yield due to reduced specific antibody binding.
- For optimal results, we recommend including a protease inhibitor and phosphatase inhibitor cocktail in the lysis buffer.
  ProteaseArrest™ (Cat. # 786-108) and PhosphataseArrrest™ (Cat. # 786-450) are recommended.
- DSS is moisture sensitive. Allow to warm to room temperature before opening vials to prevent condensation formation. Store with a suitable desiccant.
- Prepare DSS immediately before use. The NHS ester reactive group readily hydrolyses becoming non-reactive. Do not prepare stock solutions and discard any unused reagents.
- Avoid buffers containing primary amines, such as Tris or glycine.

PROTOCOL

Crosslink Antibody to Resin

NOTE: *The cross-linking step is designed for 10µg antibody, but is suitable for 2-50µg.*

1. Prepare 2ml 1X IP Coupling Buffer for each immunoprecipitation (0.1ml 20X IP Coupling Buffer in 1.9ml deionized water).
2. Gently resuspend the Immobilized Protein A/G Agarose by inverting the bottle 2-3 times. Once a homogeneous slurry is formed, use a wide bore pipette tip to remove 20µl slurry and place in a 1.5ml centrifuge tube. Briefly centrifuge for 60 seconds at 1,000-2,000xg to remove the storage buffer.
3. Add 200µl 1X IP Coupling Buffer, centrifuge for 60 seconds at 1,000-2,000xg and remove the supernatant. Repeat wash step one more time.
4. Prepare 10µg affinity purified antibody by adjusting the volume to 100µl with 20X IP Coupling Buffer and deionized water. For example, for 10µl antibody, add 5µl 20X IP Coupling Buffer and 85µl deionized water. Add directly to the resin in the tube.
5. Incubate at room temperature for 0.5-1 hour with end-over-end mixing.
6. Centrifuge for 60 seconds at 1,000-2,000xg and remove and save the supernatant to determine antibody coupling.
7. Add 500µl 1X IP Coupling Buffer to the resin, centrifuge for 60 seconds at 1,000-2,000xg and discard the supernatant. Repeat wash step one more time.
8. Add 100µl dry DMSO to a vial of OneQuant™ DSS and pipette up and down to dissolve. Dilute this solution by adding 50µl to 950µl DMSO.
9. Add 2.5µl 20X IP Coupling Buffer, 9µl diluted DSS solution and 38.5µl distilled water directly to the resin.
10. Incubate at room temperature for 0.5-1 hour with end-over-end mixing.
11. Add 300µl Elution Buffer to the tube and transfer the entire contents to the spin column.
12. Snap off the bottom end cap and retain for sealing the spin column. Centrifuge for 60 seconds at 1,000-2,000xg. Save flow through to verify antibody cross-linking.
13. Add 100µl Elution Buffer to the column, centrifuge for 60 seconds at 1,000-2,000xg and discard the flow through. Repeat wash step one more time.
14. Add 200µl Mammalian Cell PE LB™ to the column, centrifuge for 60 seconds at 1,000-2,000xg and discard the flow through. Repeat wash step one more time. Proceed to the immunoprecipitation step. The resin can be stored in Mammalian Cell PE LB™ for up to 5 days. For longer storage, use 1X IP Coupling Buffer.

Lysis of Cell Suspensions

1. Pellet the cells by centrifugation at 3,000x g for 5 minutes. Remove and discard the supernatant.
2. Wash the cell pellet once with 5-10ml PBS. Pellet the cells again by centrifugation. Remove and discard the PBS wash.
3. Add ice cold Mammalian Cell PE LB™ and suspend the cell pellet. Incubate on ice for 10-30 minutes with periodic mixing. Add 10µl Mammalian Cell PE LB™ for every 1mg wet cell pellet. For large wet cell pellets add ~10% the final volume of Mammalian Cell PE LB™, vortex to suspend the pellet and then add the remaining Mammalian Cell PE LB™.
   NOTE: *Freeze/ thaw cycles are not necessary for lysis; however, one or two freeze/ thaw cycles are not detrimental to the cell extract, and often ensure complete lysis.*
4. Centrifuge the suspension at 20,000x g for 30 minutes in a refrigerated centrifuge to pellet the cell debris. Collect the clear supernatant in a fresh tube and perform a protein concentration assay.

Lysis of Adherent Mammalian Cells

1. Remove the culture medium from the adherent cells.
2. Wash the cells once with PBS. Remove the PBS wash.
3. Add an appropriate volume of ice cold Mammalian Cell PE LB™ (see table) to cover the culture surface area.

Plate Size	Volume of Mammalian Cell PE LB™
96-well plate	50-100µl/well
24-well plate	100-200µl/well
6-well plate	200-400µl/well
60mm culture plate	250-500µl
100mm culture plate	500-1,000µl

4. Incubate on ice for 5-10 minutes with periodic mixing.
5. Transfer the lysate to a micro centrifuge tube. Centrifuge the suspension at 20,000x g for 30 minutes in a refrigerated centrifuge to pellet the cell debris. Collect the clear supernatant in a fresh tube and perform a protein concentration assay.

Preclear the Lysates

1. For every 1mg lysate, transfer 80µl homogenous Control Agarose Slurry to the lysate and seal the tube.
2. Incubate at 4°C for 30-60 minutes with mixing.
3. Centrifuge the tube for 60 seconds at 1,000-2,000xg and transfer the supernatant to a clean tube.

Immunoprecipitation Procedure

NOTE: *The amount of lysate required and the length of incubation require optimization for each specific antibody-antigen system used. The following protocol uses the recommend 2-10µg affinity purified antibody.*

1. Wash the antibody coupled resin with 200µl ice cold Mammalian Cell PE LB™ and discard the flow through. Repeat the wash step once. Seal the bottom of the spin column.
2. Dilute 500-1000µg cell extract with ice cold Mammalian Cell PE LB™ to a final volume of 300-600µl.
3. Add the diluted cell extract to the spin column. Cap the top of the column and incubate at 4°C for 1 hour to overnight with end-over-end mixing.
4. Remove the bottom stopper and remove the cap from the spin column. Place the column in a 2ml collection tube and centrifuge for 60 seconds at 1,000-2,000xg. Collect the flow through and save until successful IP has been achieved.
5. Transfer the spin column to a clean tube and add 200µl Mammalian Cell PE LB™ and centrifuge for 60 seconds at 1,000-2,000xg. Repeat the wash three more times.

Immune Complex Elution

1. Place the spin column in a fresh collection tube and add 25µl Elution Buffer and centrifuge for 60 seconds at 1,000-2,000xg to collect the eluate.
2. Keep the column in the same collection tube and add 75µl Elution Buffer. Incubate at room temperature for 10 minutes.
3. Centrifuge for 60 seconds at 1,000-2,000xg to collect the eluate. Perform additional elutions as needed and analyze eluates separately to ensure complete elution of antigen.
4. To neutralize the eluates, add 5µl 1M Tris, pH9.5 to the collection tubes.

Resin Regeneration

1. Add 100µl 1X IP Coupling Buffer to the column, centrifuge for 60 seconds at 1,000-2,000xg and discard the flow through. Repeat wash step one more time. Seal the bottom of the column with the supplied plug.
2. For storage of resin, add 200µl 1X IP Coupling Buffer supplement with 0.02% sodium azide and store at 4°C.
3. Keep the column in the same collection tube and add 75µl Elution Buffer. Incubate at room temperature for 10 minutes.
4. Centrifuge for 60 seconds at 1,000-2,000xg to collect the eluate. Perform additional elutions as needed and analyze eluates separately to ensure complete elution of antigen.
5. To neutralize the eluates, add 5µl 1M Tris, pH9.5 to the collection tubes.

TROUBLESHOOTING

Issue	Possible Cause	Suggested Resolution
Antibody or antibody fragments detected with antigen	Insufficient washing resulting in unbound antibody remaining.	Increase number of elution buffer washes after cross-linking.
	Presence of reducing agents in lysis or wash buffers fragmented the antibody	Do not use buffers containing reducing agents.
No immunoprecipitation of antigen	Antigen levels to low to detect	Check protein expression and lysis efficiency by Western blotting
	Antibody not binding to the antigen	Try fresh aliquot of antibody, or a different antibody against the same antigen
	Components in Mammalian Cell PE LB™ may interfere with antigen-antibody interaction	Perform IP and washes in 1X TBS or 1X PBS
	High amounts of DSS affecting antibody structure	Ensure indicated amounts of DSS were used. Reduce amount of DSS used
Antigen failed to elute	Elution buffer not sufficient to disrupt antigen:antibody interaction	Optimize and vary the elution conditions. Vary salt concentrations and pH.
		Elute using 1X Non-Reducing SDS PAGE sample loading buffer. Add to spin column, incubate 100°C for 5-10 minutes. Spin to recover. *Note: Resins must be discarded after this elution type.*
No functional activity of antigen in downstream applications	The low pH elution may have inactivated the antigen	Elute with a high salt and neutral pH elution buffer.

RELATED PRODUCTS

1. ***Classical Immunoprecipitation*** *(Cat. # 786-637) Utilizes Protein A/G immobilized on agarose to bind antibody to immunoprecipitated proteins of interest.*
2. ***Direct Immunoprecipitation*** *(Cat. # 786-636) Utilizes our Amine Reactive HOOK™ Activated Agarose to covalently couple the antibody. The covalently coupled antibody is then retained on the agarose during elution preventing contamination of the immunoprecipitated antigen by the antibody.*
3. ***Co-Immunoprecipitation*** *(Cat. # 786-638) Utilizes our Amine Reactive HOOK™ Activated Agarose to covalently couple the antibody. The covalently coupled antibody is then retained on the agarose during elution preventing contamination of the immunoprecipitated antigen by the antibody. Used to study protein-to-protein interactions.*

For other related products, visit our website at www.GBiosciences.com or contact us.

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