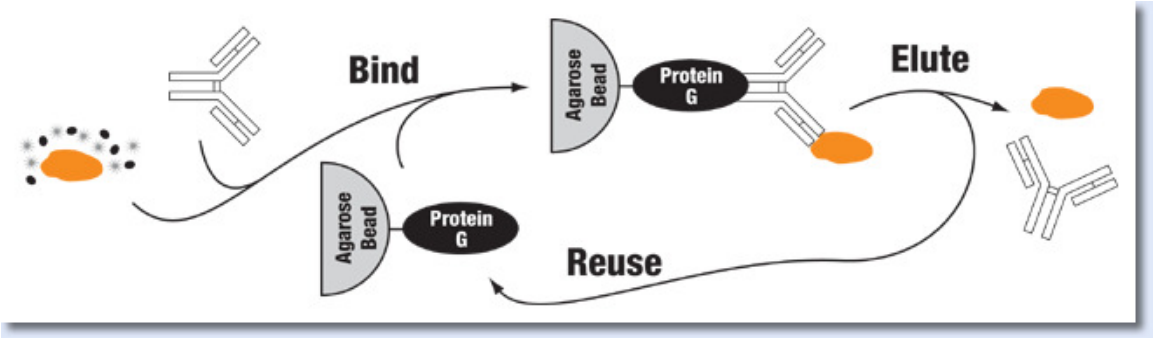
TECH TIP 1

Co-immunoprecipitation (co-IP) Troubleshooting Guide

TR0001.1

Introduction



I. IP Workflow

II. General Considerations for immunoprecipitation (IP)/Co-IP

III. Troubleshooting

Immunoprecipitation: From Start to Finish

Immunoprecipitation methods (IP, co-IP, ChIP and others) typically require a great deal of optimization and troubleshooting. While in most cases the critical factor is the antibody, several other factors can significantly influence the outcome of the IP. These guidelines will help you to obtain the best possible results from your IP.

The first section provides general recommendations, which should be considered when you are setting up your IP. The troubleshooting section describes alternatives in greater detail.

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I. IP Workflow

When starting an IP, the following workflow will help to minimize the time for optimization:

Cell Lysis: Cell lysis from tissue or cell culture is straightforward for Western blotting applications in which harsh lysis conditions (e.g., an SDS-sample loading buffer) will not interfere with the results, as the sample will be denatured during processing. For an IP/co-IP, the lysis should be relatively mild so as not to interfere with the antibody-antigen binding, but harsh enough to efficiently extract proteins from the cells. It can be especially challenging to find the perfect compromise for membrane proteins. The non-denaturing Thermo Scientific Pierce® Cell Lysis Reagents, which are all tested for IP, are a good choice for obtaining high protein yields while retaining the proteins' activity.

Pre-Clearing: No resin is totally inert, and the more complex the sample, the more background binding can occur. (Lists from tissue typically give significantly more background problems than lysates from cell culture.) The pre-clearing step will reduce the background that is due to adhesion of sample components to the resin. This step should not be omitted without very good reason.

Binding: The idea of the binding step is to generate the ternary complex of resin, antibody and antigen (protein). The buffers used in this step (as well as in the washing step) are crucial elements for success. The order of addition of these three components can also be critical. Antibodies can be pre-bound to resins (covalently or non-covalently) and the lysate can then be added to the immobilized antibody.

Alternatively, the (non-immobilized) antibody can be incubated with the lysate to form the antibody:antigen complex in solution. Next, the Immobilized Protein A or G Resin is added to purify the antibody:antigen complex from the mixture. You may want to compare both protocols to see which provides the best results in your particular biological system.

Washing Buffer: Ideally, washing will break all nonspecific interactions while preserving the specific interaction between antibody and antigen (and antigen and binding partners for co-IP). Washing with additional Lysis Buffer is common, as it typically contains mild denaturants that can help break nonspecific interactions. If background is a problem, increasing the stringency of the wash buffer may help obtain a more purified antigen and antigen-binding partners from the sample.

Elution: Usually accomplished with very harsh conditions such as boiling of the beads in a Reducing SDS-Sample Loading Buffer. When using immobilized antibodies, milder conditions (pH-shift) are applied to avoid disruption of the antibody. In this case, only antigen (antigen and binding partners for co-IP) are eluted free of antibody contamination.

Detection: Typically performed by Western blotting. In co-IP experiments often very small amounts of protein are available for detection, so high-sensitivity Western blotting substrates are required. Consult the thermo Scientific Pierce Western Blotting Handbook and Troubleshooting Guide for detailed suggestions. To allow optimization, always check the following control samples together with the desired eluate:

- Lysate in IP-Buffer — positive control confirms sample contains protein of interest. If not detectable, your sample may not have the protein of interest. However, consider dimerization/multimerization of protein if bands are not visible at the size of the monomer.
- IP Flow-Through — confirms whether antigen or binding partners bound to immobilized antibody beads.
- 1st Wash Step — provides information on whether Wash Buffer is excessively stringent.
- Post-Elution Bead-Boil — boiling beads following elution in Reducing SDS-Sample Loading Buffer to confirm efficiency of the elution.

II. General Considerations for IP/co-IP

This section provides protocol details on some key steps of the IP process.

Pre-clearing: Incubate samples with an inert resin (in column or batch format) such as underivatized agarose or Protein A-agarose for 30 minutes at 4°C.

IP Buffers: The stringency of the buffer used during the binding step can be critical, enhancing binding without interfering due to excess stringency.

- High stringency: RIPA buffer (not recommended for co-IP as it is partly denaturing due to the anionic detergent SDS).
- Medium stringency: NP-40 or Triton® X-100 based buffers in TBS pH 7.4 (up to 1% detergent). CHAPS-based buffers for membrane proteins (up to 1-2%).
- Low stringency: PBSCM (PBS buffer (100 mM sodium phosphate, 150 mM NaCl, pH 7.2) with 1 mM $CaCl_2$ + 1 mM $MgCl_2$) or Modified Dulbecco's PBS (8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM NaCl and 10 mM KCl, pH 7.4). Often used as IP-binding buffers.

In addition to the stringency of the binding buffer, you may need to include additives, if required for the protein: protein interaction, especially for co-IP. For example, if ATP is required for two proteins two interact and bind, this should be included in the buffer used for the binding step.

***In vivo* Crosslinking**:

- Several articles have been published, that demonstrate how a mild *in vivo* crosslinking treatment can provide superior results in ChIP experiments.[1,2]
- Alternatively, photo-amino acids, which are zero-length linkers, can provide an excellent way to capture the binding before starting a co-IP.[3]
- A label-transfer method is a valuable alternative or can act as a confirmation method for co-IP, when it is possible to use a (recombinant) bait protein, instead of an antibody, to bind the prey proteins.[10]

Choice of Protein A or Protein G Resin:

- Protein G is often considered a more universal IgG Binding Protein than is Protein A, but different species, and subtypes of species, do vary in their binding to these proteins. Following are some examples:
  - Rabbit IgG binds well to both Protein A and G.
  - Mouse IgG_1 binds only weakly to Protein A and moderately well to Protein G.
  - Other mouse IgG subclasses bind well to both proteins.
- Immobilized Protein A/G, a fusion protein that has binding sites of both Protein A and G in its sequence, can be used to ensure binding of a wider array of IgG.

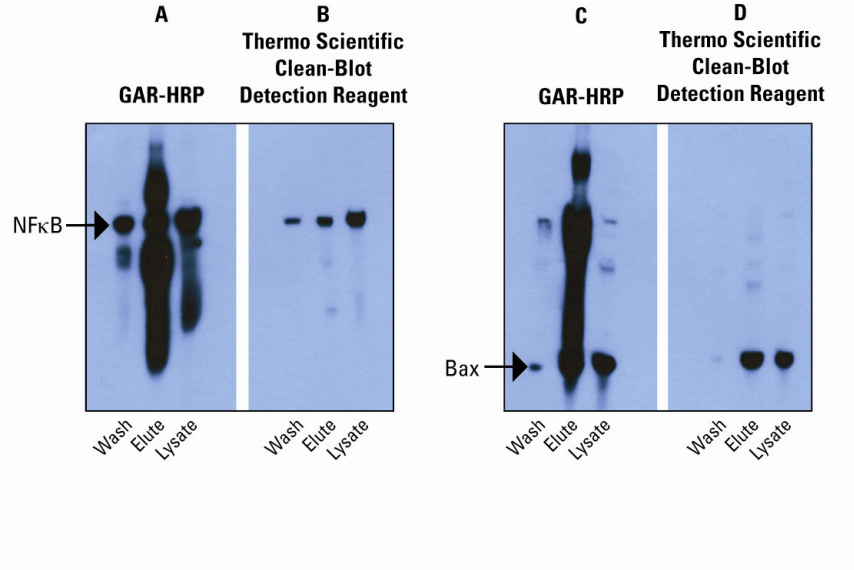
Protein A/G Blocking:

- Blocking of Protein A and Protein G Resin is often performed with 1-4% BSA for 1 hour (or 1-5% fish gelatin) at RT or 4°C.
- Methods to effectively block the Protein A or G Resin without introduction of foreign proteins (which might interfere with later analysis) are outlined below[4]:
  - Protein A-Agarose: 50 mM Tris, 200 mM glycine, 1% Tween®-20, pH 10,6 treated overnight at RT
  - Protein G-Agarose: 50 mM Tris, 200 mM ethanolamine, 1% Tween-20, pH 10,6 treated overnight at 4°C

The IP Starter Combo:
The Thermo Scientific Pierce Classic IP Kit (#45213) + the Thermo Scientific Clean-Blot IP Detection Reagents (#21230-HRP conjugated, #21233-AP conjugated and #21232-HRP conjugated Kit)

The Pierce Classic IP Kit provides everything you need to perform a standard immunoprecipitation, but avoids the single, most troublesome part of an IP: the careful withdrawal of sample and wash supernatants. This step is replaced by easy-to-use Thermo Scientific Pierce Spin Cup Columns, which allow for more efficient and easier separation.[18]

The Clean-Blot IP Detection Reagents overcome the detection of unwanted antibody fragments (28 kD and 55 kD) on the Western blot and simplify interpretation of the IP because they do not bind to denatured IgG that has undergone electrophoresis under reducing SDS-PAGE (see Figure):



Reveal your target protein with Thermo Scientific Clean-Blot Detection Reagent (HRP). To demonstrate unmasking of the target protein, we performed IP and Western blot experiments. NFκB and Bax were immunoprecipitated from A549 lysate using Protein A/G Agarose Resin and rabbit anti-NFκB (Panels A and B) and rabbit anti-Bax (Panels C and D). Panels A and C were detected with goat anti-rabbit HRP, which masked the target. Panels B and D were detected with the Clean-Blot Detection Reagent (HRP), revealing the target protein.

III. Troubleshooting

IP troubleshooting involves adaptations of the general protocol, including optimization of buffer composition, appropriate volume of sample and buffers to use, as well as the length of time for incubation and the number of washing steps, etc. Many of the components of the Pierce Classic IP Kit and Clean-Blot IP Detection Kit are also available separately to enable such adaptations:

- Immobilized Protein A (#20333)
- Immobilized Protein G (#20398)
- Pierce Spin Cup Columns (#69725)
- Clean-Blot IP Detection Reagent (#21230-HRP conjugated. Also available as #21233-AP conjugated)

The main challenge is to determine the appropriate conditions for the specific binding between the antigen that the antibody pulls down and its interacting partner(s). Polyclonal antibodies often perform better in IPs than monoclonal antibodies. Don't hesitate to test different antibodies to find the best one for your specific IP.

1. No Proteins Detected following IP

Confirm that the Western blot works well. Appropriate positive and negative controls should be used, including a whole-cell lysate as positive control. Then check the following:

The lysis step:

- Perform all steps at 4°C to reduce proteolysis, dephosphorylation and denaturation. This is especially important for the binding step which is typically incubated overnight (or at least 2 hours) at 4°C.
- Use protease inhibitors (e.g., #78430). When working with phosphoproteins also add phosphatase inhibitors (e.g., #78420).
- Try different lysis buffers (harsher or less harsh) and, as mentioned above, use a whole-cell lysate as positive control to confirm that protein extraction works (especially important when working with membrane proteins). The least stringent lysis buffer that gives an acceptable yield of the desired protein can be considered "optimal".
- For co-IPs, use only gentle vortex steps and wide pipette tips. Be very careful not to disrupt any protein complexes. In addition, if any additives are required for the proteins to interact, these should be included in the binding buffer to enhance capture of the protein complex.

The binding step:

- Avoid reducing agents because they can cleave the antibody.
- Use the "classic" IP method without covalent antibody immobilization on beads. The immobilization can reduce the antibody's affinity to the antigen and prevent IP. This is more likely to happen with monoclonal than with polyclonal antibodies. Direct immobilization of the antibody to a bead, as in the Thermo Scientific Pierce Co-IP Kit (#23600) typically results in less functional loss to the antibody than does crosslinking the antibody to Protein A or G, but may still reduce the ability of the antibody to bind its antigen. A useful method to prevent the antibody losing its affinity to the antigen is to mask the antibodies binding site before immobilization.
- Avoid the pre-clearing step referred to in *Additional Considerations, Pre-clearing*, above.
- Increase the amount of antibody and/or sample (e.g., incubate lysate aliquots sequentially until a sufficient amount of protein has come in contact to the beads). To keep this practical, incubation of the binding step is typically done for 2 hours at 4°C. Do not use samples that are very highly concentrated because they are more likely to encounter background problems.
- Over-express your bait protein, being careful that expression levels are not so high that the protein cannot be properly folded in the cells, resulting in artifacts or loss of binding.
- Try different binding conditions. Refer to *Additional Considerations, IP Buffers* above (e.g., buffers with different concentrations of detergents or different detergents, especially when working with membrane proteins).
- Try *in vivo* crosslinking (to stabilize protein complexes in case of co-IPs) as mentioned in *Additional Considerations, In-vivo Crosslinking*.

The washing step:

- Use less stringent washes (using e.g. PBSCM) If necessary, cut down on the number of washing steps. Refer to *Additional Considerations, IP Buffers*

The elution step:

- If elution was done with a pH-shift, also check if boiling the beads in SDS-sample loading buffer (Laemmli-buffer) results in a higher protein yield. However, background will rise as antibody fragments will appear in the eluate.

The detection step:

- Increase the sensitivity using one of the following options:
  - Western blotting substrates that are more sensitive (such as the Thermo Scientific SuperSignal® West Femto Substrate, (#34095), which is 100X more sensitive than an ECL Substrate and might even help to avoid an IP at all.).
  - Metabolic labeling of proteins (e.g., by radioactive or heavy amino acids – the latter only for MS-based detection methods).

2. High Background (Specific or Nonspecific)

Several components in an IP or co-IP can cause binding of the support, which can be specific or nonspecific binding. Specific binding occurs when an immobilized molecule has a similar sequence or docking site as a sample component's target. Nonspecific binding can be due to charges, contact of hydrophobic surfaces, etc. Following is a summary of the most common causes of nonspecific binding and ways to overcome them.

The lysis step:

- Try to avoid frozen cells, using fresh material whenever possible. If frozen material has to be used, use frozen lysates instead of cells. Frozen cells may be less of a problem for co-IPs: however,
- Reduce sample complexity by extracting proteins from selected cellular compartments using Thermo Scientific Pierce Reagents optimized for this purpose:
  - NE-PER® Kit (#78833) for nuclear proteins[5]
  - Mem-PER® Reagent Kit (#89826) or Pierce Cell Surface Labeling Kit (# 89881) for membrane proteins[6,9]
  - T-PER® Reagent (#78510) for tissue extracts[11]
  - Lysosome Enrichment Kit for Tissues and Cultured Cells (#89839)
  - Mitochondrial Extraction Kits (#89874:for cultured cells and #89801: for Tissue)

The binding step:

- If biological fluids are used as samples (biological fluids may contain antibodies which will bind to Protein A or G, the Pierce Co-IP Kit doesn't use Protein A or G, but relies on direct immobilization of the antibody to the resin)[13], use the Pierce Co-IP Kit or Seize Primary Kit.
- Use a pre-clearing step, as described above in *Additional Considerations, Pre-clearing*.
- Remove protein aggregates (spin at 100.000 g for 30 minutes.).
- When using primary antibody, shorten the incubation step to 45 minutes.
- When using Protein A Resin, shorten the incubation step to 30 minutes.
- Block Protein A- (or Protein G-) agarose beads see *Additional Considerations, Protein A/G Blocking* above.
- Try a different matrix. We offer the following Thermo Scientific Matrices:
  - UltraLink® Protein A (or G) instead of agarose-based supports[9]
  - MagnaBind™ Protein A (or G) or MagnaBind Goat Anti-Mouse or Goat Anti-Rabbit IgG Beads instead of agarose-based supports.

Magnetic beads are especially recommended for IPs with cytoskeletal (or other filamentous) proteins because these proteins tend to easily precipitate in centrifugation steps[7]. The magnetic beads coated with secondary antibodies have made co-IP possible when it had been previously impossible to perform these experiments successfully with conventional matrices[12].

The washing step:

- Problems related to actin contamination can be avoided by adding 10 mM ATP to lysis and wash buffers[8].
- Adjust washing stringency and steps. Move toward a higher stringency buffer and increase the number of washing steps (refer to general tips above). Use up to 1% Tween-20 (a nonionic detergent), up to 0.2% SDS (an anionic and therefore, charged detergent), or up to 1 M NaCl. Alternating these high stringency washes with distilled water may help to reduce background or nonspecific binding, as described in *Additional Considerations, IP-Buffers* above.
- Transfer the matrix to a fresh tube after the last washing step in order to avoid carry-over of contamination.

The elution step:

- To recover the proteins during the elution step, typically a pH 2.5 – 3.0 buffer is used. This alternative to boiling the resin in a Laemmli-buffer will prevent contamination of the sample by Protein A or G and non-immobilized antibody fragments. Other "mild" elution conditions using detergents or reducing agents can be tested as well.

The detection step:

- High background in Western blots can be a result of the massive presence of antibody fragments in the sample. Use Clean-Blot IP Reagents (see "the Starter Combo" and also Part III.) to overcome this problem.
- Depending on the sample source, contamination by lipids, carbohydrates or nucleic acids can generate significant background. This problem occurs much more often when working with tissues than when working with cultured cells. Thermo Scientific Pierce SDS-PAGE Sample Prep Kit (#89888) can eliminate these contaminations and others such as detergents, salts, urea or glycerol.

3. Antibody Fragments Blocking the Signal of Interest

In some cases, detection of the antibody used to pull out sample components during the IP or co-IP will interfere with interpretation of the results from a Western blot or during mass spectrometry analysis. There are two major methods to avoid this problem: antibody immobilization or a detection reagent that does not recognize the denatured antibody fragment.

Antibody immobilized on resin

- Protein A or G binding of antibody followed by covalent attachment with a crosslinker; orients the antigen-binding sites outward, but can result in significant loss of antibody function. This choice is compatible with unpurified antibodies (use Thermo Scientific Seize X Kits).
- Direct immobilization of antibody onto an activated resin - non-oriented, but greater retention of antibody function. This choice requires a purified antibody because the resin reacts with amines found on all proteins. (Use Seize Primary or Co-IP Kits.)

Detection reagent specific to native antibody

- Clean-Blot IP Reagents and Kit for detection of only native IgG.

4. The Worst Case Scenario: Co-IP is Not Successful

In some cases, co-IP is not successful for finding and/or confirming protein interactions. Co-IP is most useful for high-affinity interactions. If low-affinity or transient interactions shall be detected, co-IP often fails without further optimization such as crosslinking.

In some cases, lysis conditions simply cannot be optimized in a way to allow efficient extraction of proteins while retaining their binding properties. However, there are a number of alternate techniques that you can use, such as Pull-Down assays [similar to co-IP, but bait protein of interacting pair is immobilized instead of antibody (bait is typically present in significantly larger amounts than its native counterpart in an IP)], label-transfer[17] (especially useful for transient interactions), far Westerns[19], protein arrays, etc.

Contact Thermo Scientific Technical Support for more details on these alternative methods.

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Thermo Scientific Pierce Protein Research Products Related to Co-IP

IP Products

21230	Clean-Blot IP Detection Reagent (HRP)	2.5 mL
21232	Clean-Blot IP Detection Kit (HRP)	Kit
21233	Clean-Blot IP Detection Reagent (AP)	2.5 mL
23600	Pierce Co-Immunoprecipitation Kit	Kit
23605	Pierce Mammalian Co-Immunoprecipitation Kit	Kit
45210	Seize X Protein G Immunoprecipitation Kit	Kit
45213	Pierce Classic (A) Classic Protein A IP Kit	Kit
45215	Seize X Protein A Immunoprecipitation Kit	Kit
45217	Pierce Classic Mammalian IP Kit	Kit
45218	Pierce Classic Protein G IP Kit	Kit
45225	Seize X Mammalian Immunoprecipitation Kit	Kit

Resins

Agarose-based Resins		
20333	Immobilized Protein A	5 mL
20394	AminoLink® Plus Trial Kit	Trial Kit
20398	Immobilized Protein G	2 mL
20421	Pierce Protein A/G Agarose	3 mL
Magnetic Beads		
21348	MagnaBind Protein A Beads	5 mL
21349	MagnaBind Protein G Beads	5 mL
21354	MagnaBind Goat Anti-Mouse IgG Beads	50 mL
21356	MagnaBind Goat Anti-Rabbit IgG Beads	50 mL
UltraLink (polyacrylamide-based) Resins		
53125	Protein G UltraLink Resin	2 mL
53132	Protein A/G UltraLink Resin	2 mL
53139	Protein A UltraLink Resin	5 mL

Cell Lysis Reagents, Detergents & Inhibitors

28314	Surfact-Amps® X-100	6 x 10 mL
28320	Surfact-Amps 20	6 x 10 mL
28324	Surfact-Amps NP-40	6 x 10 mL
78420	Halt™ Phosphatase Inhibitor Cocktail	1 mL
78430	Halt Protease Inhibitor Single-Use Cocktail	24 x 100µl
78501	M-PER Mammalian Protein Extraction Reagent	250 mL
78833	NE-PER Nuclear & Cytoplasmic Extraction Reagent	Kit
89826	Mem-PER Eukaryotic Membrane Protein Extraction Kit	Kit
89839	Lysosome Enrichment Kit for Tissues and Cultured Cells	Kit
89874	Mitochondria Isolation Kit	Kit
89901	RIPA Buffer	250 mL

Crosslinkers & Labeling Reagents

20593	DSG	50 mg
21555	DSS	1 gm
21565	EGS	1 gm
22610	L-Photo-Leucine	100 mg
22615	L-Photo-Methionine	100 mg
33034	No-Weigh™ Sulfo-SBED Label Transfer Reagent	8 x 1 mg
89881	Pierce Cell Surface Protein Isolation Kit	Kit

Other Products

34095	SuperSignal West Femto Maximum Sensitivity Substrate	100 mL
69725	Pierce Spin Column – Snap Cap	50 /Pkg
89888	Pierce SDS-PAGE Sample Prep Kit	Kit

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PB 2008 42

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