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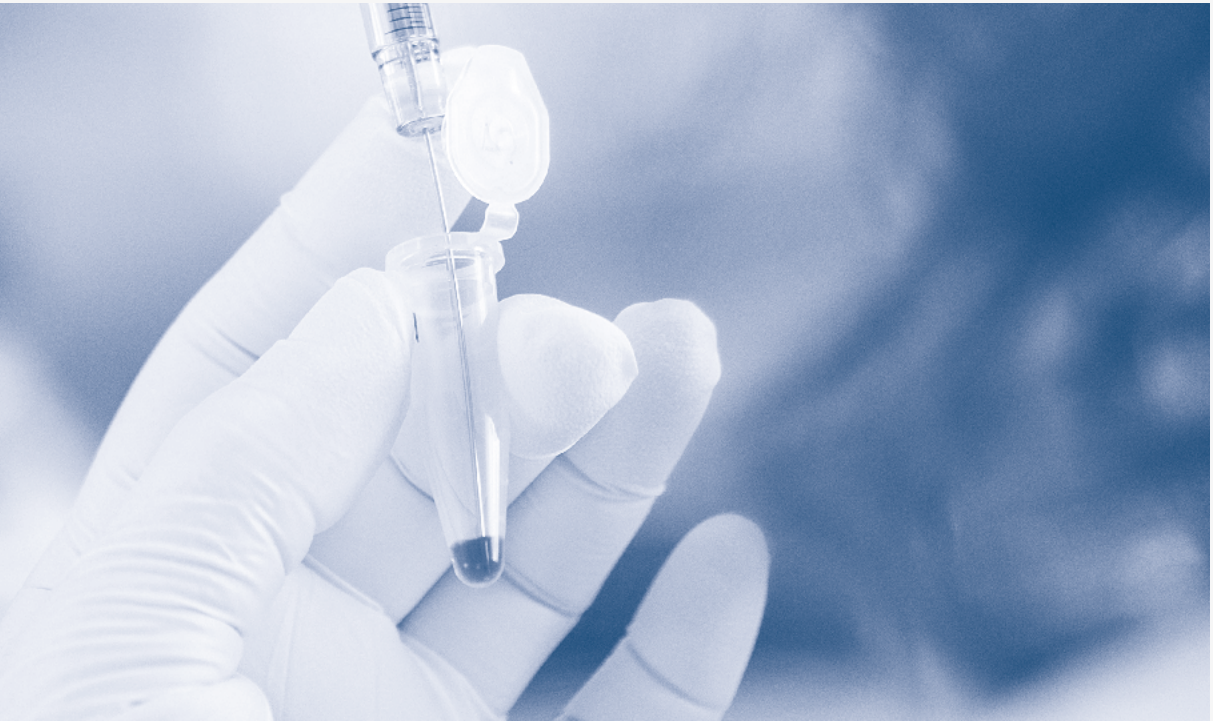
IMMUNOPRECIPITATION (IP)

Overview and Technical Tips



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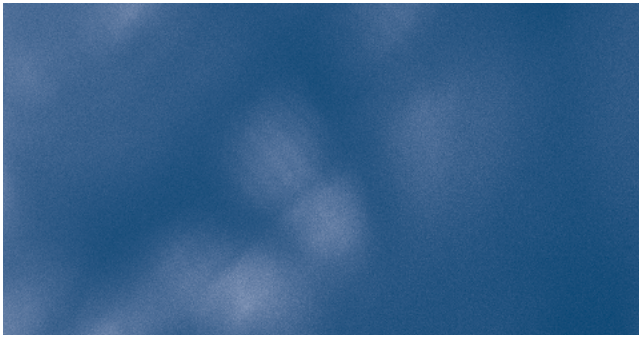

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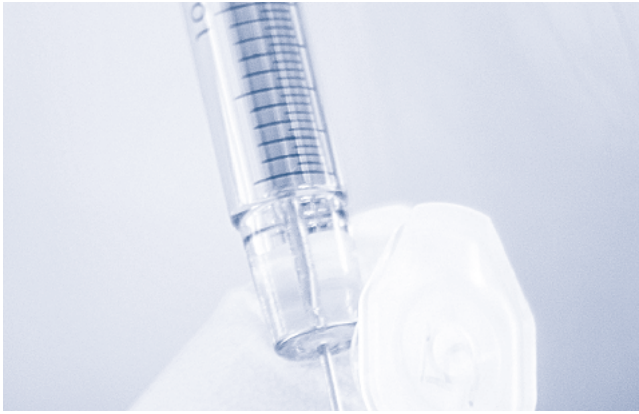
INTRODUCTION

- **Immunoprecipitation (IP)** is a precipitation technique.
- IP is a technique used to purify and enrich the protein-of-interest out of a **protein mixture.**
- IP isolated proteins can then further be analysed by **Western blotting, ELISA, and mass spectrometry.**
- IP helps to identify: **presence, up/down regulation, size, stability, and interactions of the protein-of-interest.**



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APPLICATIONS

- Isolation and detection of protein-of-interest
- Enrichment of low expressed proteins
- Investigation of protein-protein interactions
- Identification of proteins as part of a protein complex

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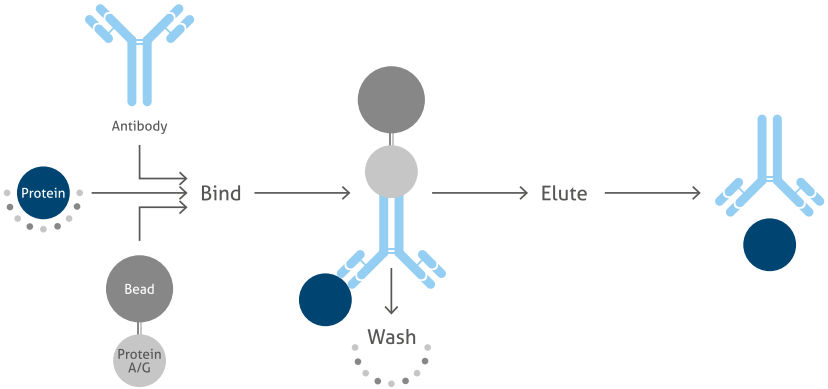
IMMUNOPRECIPITATION (IP)

- **IP is based on a solid phase (bead) that contains a binding protein.**
- **IP requires a specific antibody to purify a single antigen.**
- **The sample containing the protein-of-interest is incubated with the beads and antibody.**
- **The antibody binds to the protein and bead.**
- **The beads get washed.**
- **The protein is eluted.**



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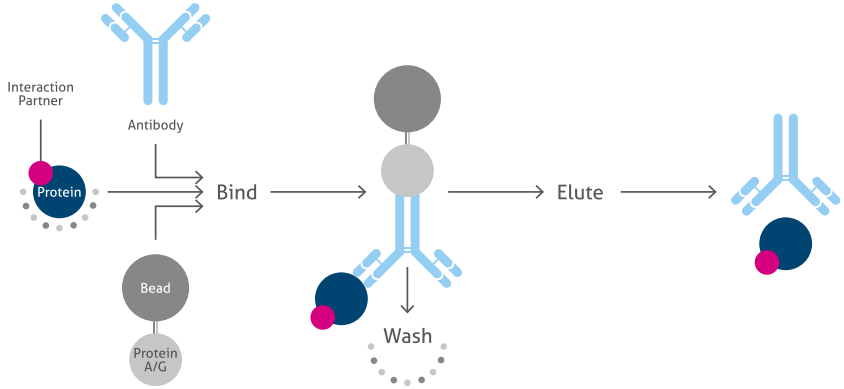
CO-IMMUNOPRECIPITATION (Co-IP)

- **Co-IP contains similar steps to that of an IP experiment.**
- **A specific antibody and a solid phase (bead) is needed.**
- **Co-IPs do not just isolate one protein-of-interest but also its binding partners (proteins, ligands, co-factors, signaling, molecules etc).**
- **When optimizing a Co-IP experiment, additional factors have to be considered.**



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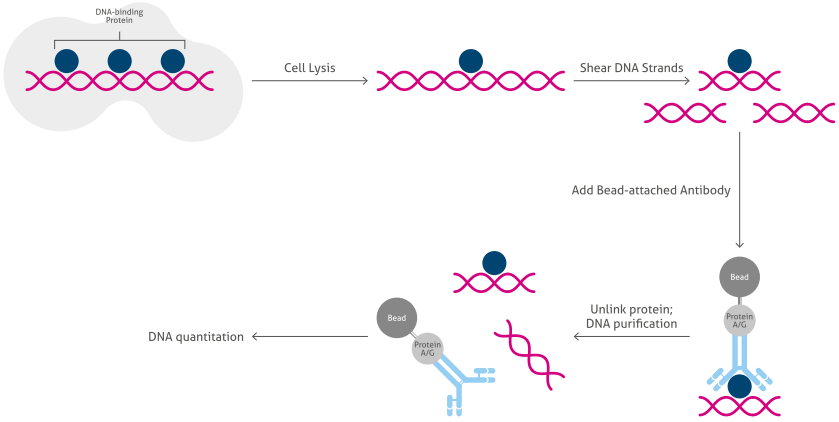
CHROMATIN IMMUNOPRECIPITATION (ChIP)

- ChIP is a powerful tool used in epigenetics.
- ChIP helps to detect special protein-DNA interactions.
- ChIP monitors transcriptional regulation via modifications of histones.
- The results are further analysed by qPCR.
- A ChIP is more complex then a simple IP.
- Main steps: Crosslinking, cell lysis, chromatin preparation, IP, reverse crosslinking, DNA clearing, DNA quantitation.



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FACTORS INFLUENCING IP

- **Performing an IP experiment is relatively simple.**
- **Variable factors depend on the protein-of-interest and the antibody.**
- **The core of an IP experiment is the purification of a specific protein with specific binding to an antibody.**

Main Factors	Possible Solution
Wash, elution, binding buffer	Composition, volume
Type of solid support	Physical characteristics
Antibody	Amount, specificity
Pre-clearing of lysate	Non-specific binding

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GENERAL PROTOCOL

Sample Preparation

- Prepare lysate from cells according to your routine protocol.
- Commonly used amounts: 0.3–0.5 ml lysate containing 1–4 mg total protein.
- Use protease inhibitors when working with RIPA lysate buffer.

Tips

- **High concentrations of detergent interfere immunoprecipitation. Try to lysis cells with a small volume RIPA and then dilute the lysates with PBS to the final volume.**
- **Use sufficient lysates for the first trial. Typically 1–3 mg total protein is needed for each IP.**
- **Concentration of proteinase inhibitor should be 1.5–2 times of that for Western blotting lysates.**

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GENERAL PROTOCOL

Lysate Pre-cleaning

- Resuspend Protein A or G sepharose beads slurry by gentle vortex, then add 50 µl of 50% beads slurry per 0.5–1 mg of cell lysate.
- Incubate at 4°C for 30 min on a rotator.
- Centrifuge at 1000 rpm for 3 min at 4°C and transfer the supernatant to a fresh tube.

Tip

- Tissues with abundant IgG are suggested to be pre-cleared with Protein A or G sepharose.

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GENERAL PROTOCOL

Immunoprecipitation

- Add appropriate amount of primary antibody to the whole (or pre-cleared) lysate. Optimal antibody concentration should be determined by titration. Gently rock the mixture at 4°C for 2–4 h or overnight. Set up a negative control with control IgG corresponding to the primary antibody source.
- Add Protein A or G sepharose beads slurry to capture the immunocomplex. Gently rock the mixture at 4°C for 1–4 h.
- Centrifuge the mixture at 500–1000 rpm for 30 s at 4°C and discard the supernatant.
- Wash the beads 3–4 times with 1 ml RIPA lysis buffer or 1X PBS with 0.2% Tween 20 (less stringent), centrifuge and discard the supernatant.

Tip

- Do not use too much Protein A or G sepharose. Protein A or G will bind IgG in Western blotting and cause intense non-specific bands.

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GENERAL PROTOCOL

Elution

- Elute the pellet twice with 40 µl 0.10 M Glycine, 0.05M Tris-HCl (pH 1.5–2.5) elution buffer containing 500 mM NaCl. Pool elutions and neutralize by Alkali neutralization Buffer or 10X PBS buffer (pH 6.8–7.2) to a final of 1X.
- Add 5X SDS sample buffer to the elutions. Heat at 95°C for 5 min.

Tip

- **Increasing the salt concentration in elution buffer will help to elute protein.**

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MODIFICATIONS OF IP PROTOCOLS

Binding Proteins

- Protein A, Protein G or Protein A/G are used as immunoglobulins binding proteins in IPs.
- Protein A/G binds to all subclasses of immunoglobulins.
- Protein A or Protein G bind to multiple subclasses of immunoglobulins.

Binding capacity of immunoglobulins to Protein A and G

Species	Subclass	Protein A	Protein G	Protein A/G
Human	IgG1	+++	+++	+++
Human	IgG2	+++	+++	+++
Human	IgM	+	–	+
Mouse	IgG1	+	++	++
Mouse	IgG2a	+++	+++	+++
Mouse	IgG2b	+++	+++	+++
Rat	IgG1	+	++	++

Binding Capacity:
+++ High
++ Good
+ Low
– None

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BUFFER OPTIMIZATION

Binding Buffer

- Most bindings to protein A or G work well under physiological conditions.
- Some bindings to protein A or G can be enhanced by adapting the pH value (e.g. Protein G binds best to IgG at pH 5.0).

Washing Buffer

- The washing step should not interfere with the desired protein bindings.
- The washing step should remove all unwanted protein bindings.
- If the observed background signal is too high in an IP experiment, buffer type and additives can be varied. Commonly used buffers are: PBS or TBS.
- Reagents: NP40, Triton-X, CHAPS.
- Additives: DTT (reduction of disulfid bonds).
- Increased ionic strength reduces non-specific electrostatic interactions

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BUFFER OPTIMIZATION

Elution Buffer

- If the IP sample is further used for Western blotting, the sample can be directly diluted in SDS-PAGE sample buffer containing reducing agents.
- Most common used elution buffer: glycine 0.1M at pH 2.5–3.5.
- If the antibody-protein binding does not dissociate or if the protein gets denaturated the pH can be changed.

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PRE CLEARING OF SAMPLES

- **Pre-clearing is a carried out before the actual IP experiment.**
- **Pre-clearing of the sample helps to get rid of unwanted proteins to the solid support.**
- **Therefore, the sample gets incubated with the plain beads.**
- **Non-specific components of the sample that bind to the solid phase will be removed.**

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CONTROLS

Isotype Control

- An isotype control helps to understand the specificity of the obtained signal.
- The isotype control should always be run in parallel to the sample.

Negative Control

- Plain beads (without antibody) can be used as negative controls. They help to distinguish between specific and non-specific bindings.

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TROUBLESHOOTING

High Background

Issue	Possible Solution
Insufficient washing.	Increase washing volume/time.
Non-specific antibody.	Pre-test the antibody for its specifity.
Antibody amount.	A too high amount of antibody leads to non-specific binding.
Too high amount of sample.	Decrease the amount of cells/lysate.
Proteins bind non-specific to the antibody.	Reduce sample amount, pre-clear samples.
Antigen degradations.	Add fresh protease inhibitors.

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TROUBLESHOOTING

Insufficient Elution

Issue	Possible Solution
Wrong lysis buffer.	Change lysis buffer.
No antibody binding to beads.	Make sure, isotype specific beads were used.
Protein of interest cannot be eluted from the beads.	Change elution buffer (components, pH etc).
Insufficient antibody amount for binding properly.	Too low antibody amount, titrate antibody concentration.
Protein-of-interest is low expressed.	Increase amount of lysis volume. Pre-clear the sample to decrease non-specific binding.

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CONTACT US

Proteintech Group
US Head Office
proteintech@ptglab.com

Proteintech Europe
United Kingdom
europe@ptglab.com

Proteintech
China Office
service@ptglab.com

Support
Available 24 hours via Live Chat and 9–5 (CDT) via phone.

Please visit us at www.ptglab.com for more information about our antibodies and technical tips.