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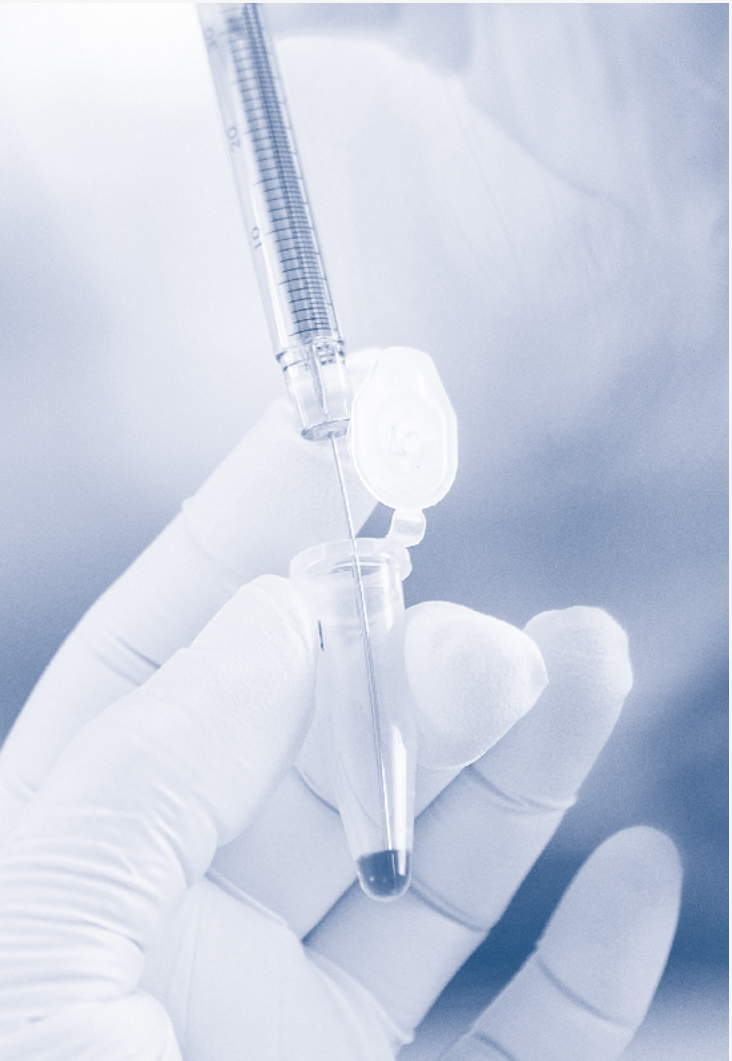
Antibodies | ELISA kits | Proteins

Overview & Technical Tips:

IMMUNOPRECIPITATION

Including detailed Chromatin Immunoprecipitation (ChIP) protocol



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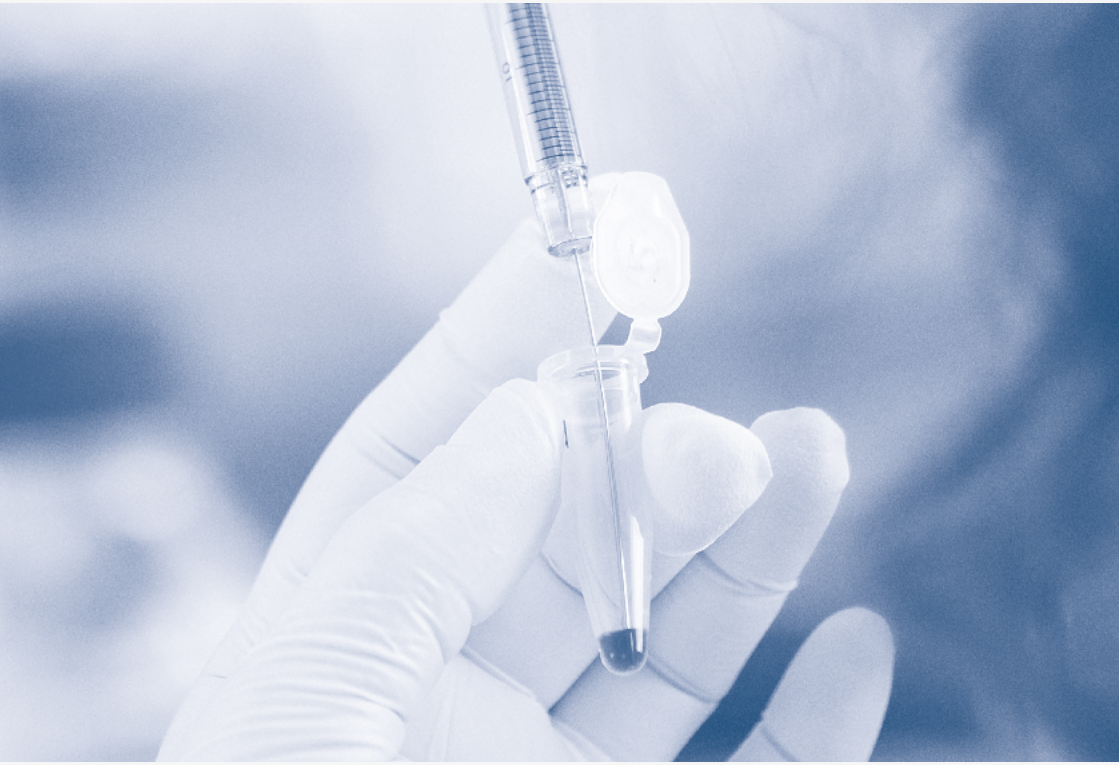


CONTENTS

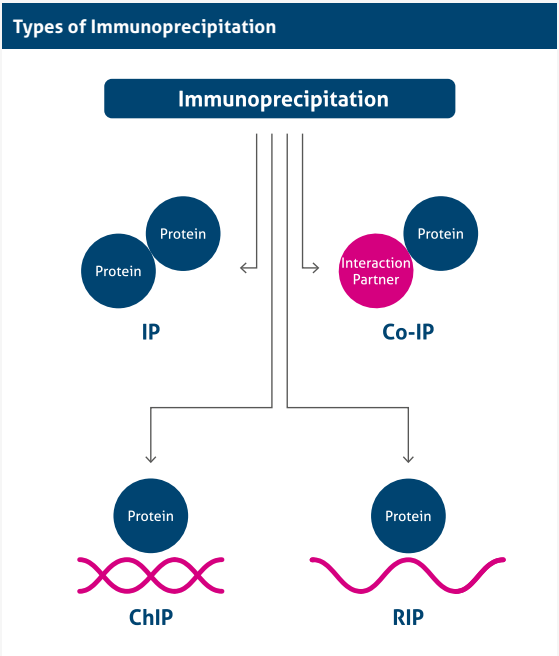
Introduction To IMMUNOPRECIPITATION

Immunoprecipitation (IP) is a precipitation technique that purifies and enriches a protein of interest, allowing the identification of protein-protein interactions in proteomics workflows. IP is an important technique used to investigate the presence, relative abundance, size, up-regulation or down-regulation, stability, post-translational modifications (PTMs), and interactions between proteins.

There are different types of immunoprecipitation:

IP is used to isolate a single protein (individual protein immunoprecipitation, the target antigen of the antibody). Variations of IP (e.g., Co-Immunoprecipitation, Co-IP) are used to study the interactions between the primary antigen protein and other proteins or nucleic acids (e.g., Chromatin Immunoprecipitation, ChIP, or RNA Immunoprecipitation, RIP) *(Figure 1)*.

Figure 1

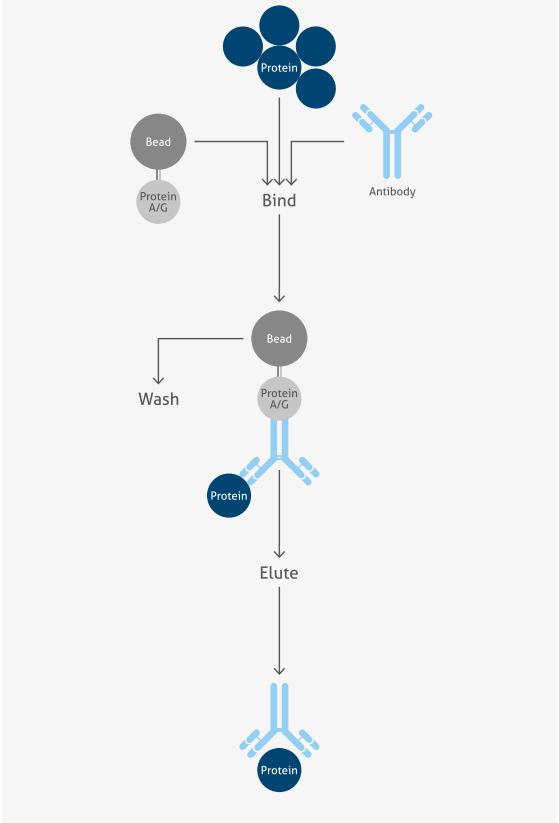


Individual Protein Immunoprecipitation (IP)

Individual Protein Immunoprecipitation uses an antibody to isolate a selected protein of interest from cell lysate. The antibody binds to the protein and the antibody/antigen complex is pulled out of the sample using protein A/G-coupled agarose or magnetic beads. The beads are washed and the protein is eluted. Purified antigen obtained by IP is verified by a variety of molecular techniques (e.g., ELISA and Western blot), and isolated proteins are quantified and identified by mass spectrometry using enzymatic digestion patterns based on the primary sequence *(Figure 2)*.

Figure 2

Overview of the main steps of Individual Protein Immunoprecipitation (IP)

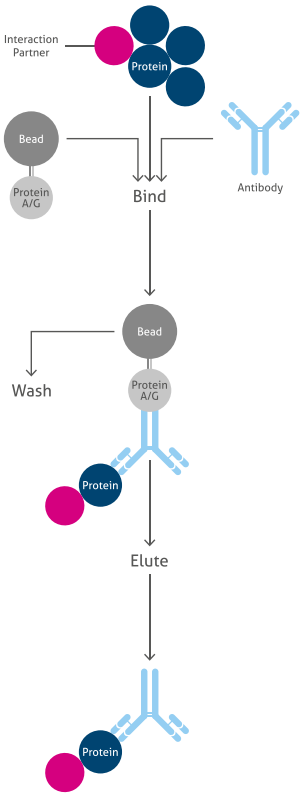


Co-Immunoprecipitation (Co-IP)

Co-Immunoprecipitation (Co-IP) is a powerful tool used to analyze protein–protein interactions. The main purpose of Co-IP is the identification of interaction partners (other proteins, ligands, co-factors, or signaling molecules) to the protein of interest. It is an effective process used to separate proteins from serum, cell lysate, homogenized tissue, or conditioned media *(Figure 3)*.

Figure 3

Overview of the main steps of Co-Immunoprecipitation (Co-IP)



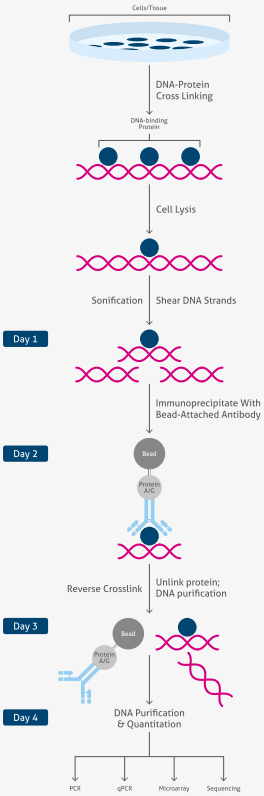
Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) is a type of immunoprecipitation used to investigate regions of the genome associated with a target DNA-binding protein, or conversely to identify specific proteins associated with a particular region of the genome. It is commonly used in epigenetics research (e.g., ChIP monitors transcriptional regulation via modifications of histones) *(Figure 4)*.

For the full Proteintech®* ChIP protocol, see pages 20–23.

Figure 4

Overview of the main steps of Chromatin Immunoprecipitation (ChIP)



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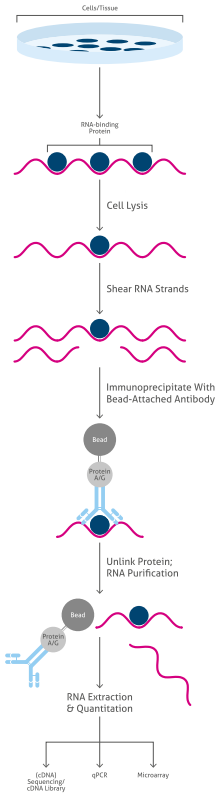
RNA Immunoprecipitation (RIP)

RNA Immunoprecipitation (RIP) targets RNA-binding proteins (ribonucleoproteins). It is performed using an antibody that targets a specific RNA-binding protein. The RNA-protein complexes are separated by RNA extraction. Some variants of RIP, such as photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) include cross-linking steps, which then require less careful lysis conditions *(Figure 5)*.

Figure 5

Overview of the main steps of RNA Immunoprecipitation (RIP).



Considerations Before Starting Your IP EXPERIMENT

Binding Proteins & Beads

Protein A and Protein G are suited for detection of IP proteins as they work by binding to the intact Fc regions of IgGs. However, they are not interchangeable detection reagents; their use is determined by the isotype of the capture antibody as they exhibit differing isotype selectivity. Protein A/G binds to all subclasses of immunoglobulins *(Summary in Table 1)*.

There are two types of beads available:

- Agarose (Sepharose) beads
- Magnetic beads (commonly used)

Table 1

++++ Strong Binding
+++/++ Medium Binding
Variable/– Weak or No Binding

Affinity of Protein A and Protein G to different immunoglobin subclasses of human, mouse, rat, and guinea pig species.

Species	Ig Subclass	Protein A binding	Protein G binding
Human	IgA	Variable	–
	IgD	+	–
	IgE	+	–
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	–	++++
	IgG_4	++++	++++
	IgM	Variable	–
Mouse	IgG_1	+	++++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++
	IgM	Variable	–
Rat	IgG_1	–	+
	IgG_{2a}	–	++++
	IgG_{2b}	–	++
	IgG_{2c}	Variable	++
	IgG_3	+	++
Guinea Pig	IgG	++++	++

Choosing The Right Primary Antibody

In IP, the target protein is usually in the native conformation. For this reason, antibodies used for IP need to recognize and have high affinity to the epitope on the surface of the protein.

What to consider before choosing the right antibody:

- The immunogen is on the exposed surface of the protein (it can be checked if the structure of the protein and immunogen are known).
- The antibody works for ChIP and/or IHC. These techniques, similar to IP, require antibodies that recognize surface epitopes in the native form.
- Monoclonal antibodies recognize a single epitope and have minimal batch-to-batch variation.
- Polyclonal antibodies recognize several epitopes and different affinities. Batch-to-batch variation is more likely compared to monoclonal antibodies.

Tip: Where possible, use a polyclonal antibody to capture the target protein. Polyclonals bind to multiple epitopes on the target protein, meaning a greater amount of protein is retained.

Antibody Pairs

The use of antibody pairs, i.e., a capture antibody from one species, and an antibody for WB detection from another, is an additional factor to consider when planning an IP experiment. The antibody selection process should ensure that both antibodies recognize different epitopes of the target protein, in addition to originating from different species. The antibody type (i.e., polyclonal or monoclonal) is important. The best option is a combination of a polyclonal capture antibody and a monoclonal antibody for detection. This combination ensures maximum capture efficiency with high detection specificity.

Antibody Titration For IP

A titration experiment should be performed with multiple antibody concentrations in order to determine the optimal signal-to-noise ratio. In general, this titration should range from 1 to 10.0 µg for ~5000 µg of protein extract.

Example

As an example, if a datasheet suggests a 1:100 dilution, the titration experiment should include dilutions of 1:25, 1:50, 1:100, 1:200, and 1:250. To keep the same experimental conditions, after selecting the incubation time, all dilutions should be performed with the same type of sample.

Isotype & Negative Control Bands

Isotype Control

An isotype control is used to determine which bands in the experimental sample are specific versus non-specific signal due to the isotype. 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 specific and non-specific bindings. Any products obtained with these control conditions can be attributed to non-specific (off-target) interactions.

Protein Isoforms, Post-Translation Modification & Protein Interaction

(STRING 10.0; PUBMED; UNITPROT)

The STRING database *(http://string-db.org)* aims to provide a critical assessment and integration of protein–protein interactions, including direct (physical) and indirect (functional) associations. It covers more than 2000 organisms, which necessitates novel, scalable algorithms for transferring interaction information between organisms; based on genomic data, and high throughput analyses including co-expression experiments.

(UnitProt database; (http://www.uniprot.org/), PubMed https://www.ncbi.nlm.nih.gov/pubmed).

Phosphatase, Methylation & Deubiquitination Inhibitors; Why They Are Important

Preservation of protein post-translational modifications (PTMs) such as phosphorylation, ubiquitylation, or methylation can be required to maintain protein–protein interaction. They play essential roles in regulating chromatin dynamics. Maintaining PTMs during cell lysis with phosphatase, methyltransferase, or deubiquitinase inhibitors can be important for the maintenance of *in vivo* interactions *in vitro*. As an example, adding phosphatase inhibitors to the lysis buffer will maintain the phosphorylated stage of the protein of interest. *Table 2* shows a few examples of phosphatase, methylation, and deubiquitination inhibitors.

Table 2

Phosphatase Inhibitors	Action
Sodium Orthovandate with the correct pH	Tyrosine phosphatase inhibitor
β-Glycerophosphate	Serine and threonine phosphatase inhibitor
Okadaic Acid	Protein phosphatase 1/2A inhibitor
Sodium Fluoride	Serine and threonine phosphatase inhibitor
Deubiquitination Inhibitors	**Action**
TAME	Ubiquitin ligase inhibitor
USP14 Inhibitor, IU1	USP14 deubiquitinase (DUB) inhibitor
DUB Inhibitor IV, b-AP15	19S DUB inhibitor
DUB Inhibitor VI, P22077	USP7 and USP47 DUB inhibitor
Methylation Inhibitors	**Action**
RG108	DNA methyltransferase (DNMT) inhibitor
5-Azacytidine	DNMT inhibitor

Immunoprecipitation PROTOCOL

Sample Preparation

Cell lysis is the breaking down of the cell membrane and the separation of proteins from the non-soluble parts of the cell. Lysate buffers contain different detergents that help to release soluble proteins. Dependent on the location of the protein of interest, a different lysate buffer is needed to obtain a high yield and purity of the protein.

Cell and tissue lysate preparation tips

- **Use protease inhibitors in the lysis buffer**
  The concentration of proteinase inhibitor(s) should be 1.5–2 times that used for Western blotting lysates. Proteintech usually uses RIPA buffer *(Table I, page 24)*, which enables efficient cell lysis and protein solubilization, while avoiding protein degradation and interference with the protein's immunoreactivity and biological activity.
- **Optimize your lysate concentration**
  Commonly used amounts for IP: 0.2–0.5 ml lysate containing 1–4 mg total protein. Measure the total protein amount by protein assay, such as Bradford or BCA assay.
- **High concentrations of detergent interfere with IP**
  Try to lyse cells with a small volume of RIPA and then dilute the lysates with PBS *(Table II, page 24)* to the final volume. Depending on the experiment, the detergent strength is crucial for the IP results (e.g., Triton-X, Tween, SDS, CHAPS).
- **Keep cells/tissue on on ice and use ice-cold buffers.**

Cultured cells

- Pre-cool a refrigerated centrifuge to 4°C. Pellet the cultured cells by centrifugation at 4°C, for 5 minutes at 2000 rpm.
- Wash 3 times with ice-cold 1X PBS and then add cold RIPA buffer with protease inhibitors.
- Add 100 µl RIPA buffer for approximately every 10^6 cells present in the pellet (count cells before centrifugation).
- Reduce the volume of RIPA buffer accordingly if a higher protein concentration is required.
- Vortex to mix and keep on ice for 30 minutes. Vortex occasionally.

Tissues

- Dissect the tissue of interest and wash briefly with cold 1X PBS.
- Cut the tissue into smaller pieces while keeping it on ice.

- Transfer the tissue to a homogenizer and add RIPA buffer with protease inhibitors.
- Add 500 µl RIPA buffer for approximately every 20–50 mg of tissue.
- Homogenize thoroughly and keep the sample on ice for 30 minutes. Vortex occasionally.

Lysis & Storage

- Sonication: high power, 1–2 minutes, then keep the sample on ice for 20 minutes.
- Centrifuge at 10,000 rpm for 20 minutes at 4°C to pellet cell debris.
- Transfer the supernatant to a fresh microfuge tube without disturbing the pellet. Determine protein concentration by protein assay, such as Bradford or BCA assay.
- Samples can be frozen at -80°C for long-term storage, or be used for immediate immunoprecipitation.

Pre-Clear The Lysate (Optional)

Pre-clearing is a step that decreases binding of non-specific proteins, lipids, carbohydrates, or nucleic acids. This step is performed by incubation of the lysate with the solid support (e.g., agarose or magnetic beads) in the absence of the capture antibody.

Pre-clearing with Protein A or G agarose beads is recommended for tissues abundant with IgG.

- Resuspend Protein A or G agarose bead slurry by gently vortexing the storage bottle.
- Transfer beads and wash with 1X PBS 4~5 times before use. Quickly add 50 µl of 50% bead slurry per 1–4 mg of total protein to the microfuge tube containing the lysate.
- Carefully cut the end of your pipette tip to facilitate pipetting and homogenization.
- Incubate on a rotary mixer for 30 minutes at 4°C.
- Centrifuge at 1000 rpm for 3 minutes at 4°C and transfer the supernatant to a fresh tube.

Immunoprecipitation Workflow

- Add 300 µl incubation buffer *(Table III, page 24)* and an appropriate amount of primary antibody to the whole (or pre-cleared) lysate. Optimal antibody concentration should be determined by a previous titration experiment *(please see page 10: Titration of antibodies for IP)*.
- Gently rock the mixture at 4°C for 4 hours or overnight.
- Set up a negative control with control IgG corresponding to the primary antibody source *(please see page 11: Isotype and negative control bands)*.

- Add Protein A or G agarose slurry (50 µl) to capture the immunocomplex. Gently rock the mixture at 4°C for 4 h.
- Centrifuge the mixture at 500–1000 rpm for 30 seconds at 4°C and discard the supernatant.
- Wash the beads 3–4 times with 1X TBS or 1X PBS with 0.2% Tween 20 (or another detergent depending on its stringent and protein of interest).
- Centrifuge and discard the supernatant. Keep about 60 µl of supernatant after the last centrifuge.
- Resuspend the pellet with 20 µl 4X SDS Sample Buffer *(Table IV, page 25)*, gently vortex for several seconds, and load on the gel.

Buffer Optimization

Binding Buffer

The majority of bindings to protein A or G work well in 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 and should remove all unwanted protein bindings and debris.

Perform washing and obtain elutions using gravitational flow through filter columns loaded in microfuge tubes.

Most commonly used buffers:

– PBS

– TBS

Most commonly used detergents:

– NP40

– Triton-X

– CHAPS

Most commonly used additive:

– DTT (reduction of disulfid bonds)

Elution Buffer (EB)

If the IP sample is further used for Western blotting, the sample can be directly diluted in a SDS-PAGE sample buffer containing reducing agents.

The most commonly used EB is glycine 0.1 M at pH 2.5–3.5 and 1% of SDS to disturb the bead-antibody-antigen interactions.

If the antibody-protein binding does not dissociate, or if the protein becomes denaturated, the pH can be changed.

Immunoprecipitation TROUBLESHOOTING

How To Improve Elution Conditions

Issue	Possible Solution
Wrong lysis buffer	Change lysis buffer (depends on the protein of interest; nuclear or cytoplasmic protein).
No antibody binding to beads	Make sure that the isotype-specific beads were used.
Protein of interest cannot be eluted from the beads	Change elution buffer (components, salt concentration, pH, etc).
Insufficient antibody amount for binding properly	Optimize the antibody concentration (titration experiment).
Protein of interest is low expressed	Increase amount of lysis volume. Pre-clean the sample to decrease non-specific binding and remove debris.

High Background Or Unwanted Protein Precipitation

Issue	Possible Solution
Substances in sample bind non-specifically to either agarose/magnetic beads or antibodies	Include a pre-cleaning step by incubating the lysate with Protein A/G conjugate without the antibody.
Non-specific binding to Protein A or G agarose/ magnetic beads	Add saturating amounts of competitor proteins, e.g., 2% BSA to agarose/magnetic beads protein-A/G.
Concentration of antibodies too high	Determine the optimal concentration of antibody by titration.
Unsuited washing	Use more stringent washes, e.g., 1.0 M NaCl, 0.5 M LiCl, 1 M KSCN, 0.2% SDS, or Tween 20. Consider using distilled water. Increase the number of washes. Transfer the pellet to a new tube before last washing step.

Weak/No Signal

Issue	Possible Solution
Antibody not capable of IP	Try a different antibody. Polyclonal antibodies usually perform better than monoclonal antibodies.
Insufficient amount of primary antibody used	Determine optimal concentration of primary antibody by titration experiment.
Too many competing proteins in sample	Spin the lysate for 30 minutes before adding the antibody in order to remove insoluble proteins, membrane fragments, debris, etc.
Antigen of interest not present	Make sure sample is suitable/appropriate for the experiment.
Antigen of interest lost or destroyed in sample	Try fresh lysates. Use appropriate inhibitors for each sample preparation.
Washes were too stringent	Reduce the number of washes and/or salt and detergent concentration or use a different antibody.
Problems with incubation times	Usually the primary antibody and antigen of interest are incubated for 4 hours to overnight at 4°C.
Used protein A or G may not bind species or subclass of selected primary antibody	See Table IV, page 25: Affinity of Protein A and G.
Interfering substances present in sample	Lysates containing dithiothreitol, 2-mercaptoethanol, or other reducing agents can destroy antibody function and should be avoided. Extremes in pH and excessive detergent concentrations may also interfere with the antibody-antigen interaction.

Considerations Before Starting Your ChIP EXPERIMENT

Always Keep Cells/ Tissues On Ice

- Temperature is critical. Perform cell lysis at 4°C. Keep the samples ice-cold and use ice-cold buffers.

Formaldehyde Cross-Linking

Please Note: Both cross-linking time and formaldehyde concentration are important.

- When using paraformaldehyde, ensure that it is freshly prepared (final concentration of 1%–1.5%).
- Usually, a 10-minute incubation at room temperature in agitation is sufficient.
- Under cross-linking can prevent the dissociation of protein-DNA complexes and can result in poor yield.
- Over cross-linking can mask epitope sites crucial for antibody binding, prevent proper chromatin shearing, and inhibit the successful uncross-linking of the complex in subsequent steps.

Chromatin Shearing & Sufficient Sonication

- Make sure the sonicator probe is not in contact with the tube wall.
- Increase the number of sonication steps; however try to avoid increasing the time (or the power) of each step as this may overheat the sample and lead to the loss of antigenicity.
- Add ice to the sonicator to avoid the sample overheating.
- Preferably do not sonicate chromatin to a fragment size of less than 500 bp (perform the size-testing step).
- Different cell types may have different optimal DNA fragmentation. Determine appropriate sonication times to get your optimal DNA fragmentation.

IP Fraction, The Right Choice Of Beads & Primary Antibody

Beads

- Always fully resuspend beads by vortexing before pipetting.
- Always store at 4°C and never allow beads to dry out.
- Check the subclass of your antibody is compatible with Protein A/G.

Antibody

- Insufficient antibody can result in too little material for successful PCR analysis.
- Too much antibody can increase PCR background.

Reverse Cross-Linking

Usually a 15-minute incubation at 95°C is sufficient. However, with some samples, Proteinase K treatment for 4 or more hours at 65°C may be necessary.

DNA Elution & Purification

- Use different washing buffers (low & high salt, LiCl, and TE buffers).
- While using a commercial purification column, check the column is completely dry after the wash step as any leftover wash will inhibit elution.
- Make sure the elution buffer is placed directly onto the silica membrane and allowed to adsorb for at least 1 minute.

ChIP PROTOCOL

Day 1

Reagents & Buffers:

- Formaldehyde 37%, molecular grade
- PBS
- Glycine 2.5 M
- Protease Inhibitors
- Cell Lysis Buffer *(Table V, see page 25)*
- Nuclear Lysis Buffer *(Table VI, see page 25)*

ChIP & Cell Line Samples

For suspension cells, ensure the appropriate number are resuspended in 10 ml of fresh media to which 37% of formaldehyde solution is subsequently added until a final concentration of 1% is reached. Vortex for a few seconds to displace from the bottom and incubate for 10 minutes at room temperature in agitation.

ChIP & Tissue Samples

Grind frozen tissue into powder with a pestle and mortar. Pour the resulting powder into a 15 or 50 ml falcon. Add 10 ml PBS + 270 µl formaldehyde 37% (final concentration 1%) to the frozen powder. Vortex for a few seconds to displace from the bottom, next incubate for 10 minutes at room temperature in agitation.

Protocol

1. Block the reaction with 500 µl Glycine 2.5 M (final concentration 0.125 M). Incubate for 5 minutes at room temperature.
2. Transfer the cells to a 50 ml falcon and centrifuge at 2500 rpm for 5 minutes at 4ºC.
3. Discard the supernatant and wash twice with ice-cold PBS ph 7.4 and centrifuge at 2500 rpm for 5 minutes at 4ºC after each washing.
4. Resuspend cells in 5 ml Cell Lysis Buffer supplemented with protease inhibitor. To facilitate the cell membrane breaking, pass the lysate 3 times to a douncer. Incubate for 15 minutes at 4ºC.
5. Centrifuge at 4000 rpm for 5 minutes at 4ºC. Discard the supernatant.
6. Resuspend nuclei in Cell Lysis Buffer.
   Pipette with cut tips to homogenize better.

7. Divide the sample into small aliquots and sonicate for 15 minutes (high power; 30 seconds sonication, 30 seconds rest).
   Put ice into the sonicator to avoid sample overheat.
8. Centrifuge at 12000 rpm for 10 minutes at room temperature to remove nuclear debris. Discard the pellet. Repeat this passage until the pellet cannot be detected. Store the samples at -20°C.
   If SDS precipitates, dissolve it prior to centrifuge.
   Take an aliquot of chromatin to quantify (2–3 µl) and to assess the size (30–40 µl).

DNA Fragment Testing

1. De-crosslink chromatin by incubating samples at 65°C for 4 hours (results may improve with an overnight incubation).
2. Incubate for 30 minutes with Proteinase K 50 µg/ml final concentration at 42°C.
3. Add 1 volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1). Mix with vortex and let samples stand at room temperature for 2–3 minutes.
4. Centrifuge at 12000 rpm for 5 minutes and transfer the aqueous phase to a new tube.
   (If the interphase is dirty repeat steps 3 and 4).
5. Add 1/5 volume of AcNH4 10 M and 2.5 volumes of EtOH 100%. Mix and let the DNA precipitate for at least 30 minutes at -20°C.
6. Centrifuge at max speed for 15 minutes at 4°C. Discard the supernatant.
7. Wash with 70% ethanol and centrifuge at max speed for 15 minutes at 4°C. (Try to discard as much supernatant as you can without touching the pellet.)
8. Resuspend with TE buffer and pipette until complete dissolution.
9. Incubate for 30 minutes at 37°C with RNAse A at a final concentration of 50 µg/µl.
10. Prepare a 1.5% agarose gel.

Day 2

Reagents & Buffers:

- Beads
- 5% BSA/PBS
- Dilution Buffer *(Table VII, see page 25)*

Protocol

1. Take 70 µl of Magnetic Beads for each sample to be immunoprecipitated. (Take the extra volume in excess: 0.5–1 times more.)
2. After precipitation with a magnet, discard the supernatant and wash twice with 600 µl at 5% BSA/PBS.
3. After the second wash reconstitute the initial volume (70*N° of samples µl). (Take the extra volume in excess: 0.5–1 times more.)
4. Take 20 µg (dependant on the tissue/cell type) for each sample, dilute the chromatin 1:10, and bring to a final volume of 1 ml with dilution buffer.
5. Take 25 µl of beads for each sample and add them to the chromatin for the pre-clearing step.
6. Divide the remaining beads into 45 µl aliquots. Add the corresponding antibody to each tube, plus a negative control (specific IgG). Incubate overnight at 4°C in a rotating wheel.

Day 3

Reagents & Buffers:

- 5% PBS/BSA
- Low Salt Buffer *(Table VIII, see page 26)*
- High Salt Buffer *(Table IX, see page 26)*
- LiCl Buffer *(Table X, see page 26)*
- TE Buffer *(Table XI, see page 26)*
- Elution Buffer (EB) *(Table XII, see page 26)*

Protocol

Please Note: From this stage it is better to work with siliconized tubes.

1. Discard the beads from the chromatin samples by putting the tubes in the magnet.
2. Wash the Ab-Bead complexes twice with ice-cold 300 µl at 5% PBS/BSA. Spin after the second wash and remove the supernatant.
3. Add 1 ml of the chromatin to each sample and resuspend with the tip. Incubate for 2 hours at 4°C in a rotating wheel. *Store the exceeded chromatin for the input sample.*
4. Spin the samples and put them on the magnet.
5. Wash twice with 1 ml low salt buffer.
6. Wash twice with 1 ml high salt buffer.
7. Wash twice with 1 ml LiCl buffer.
8. Wash twice with 1 ml TE when adding the second washes. Change tubes for new ones.

9. Remove last wash almost completely with the pipette.
10. Prepare Elution Buffer (EB) and set the thermomixer to 65°C.
11. Add 100 µl of Elution Buffer to each sample. Incubate for 10 minutes at 65°C in the thermomixer.
12. Put the supernatant in a new tube and repeat step 11 to obtain a final volume of 200 µl.
13. Take 50 µl of the exceeded chromatin from the pre-clearing as a 5% input. Add 150 µl Elution Buffer to reach a 200 µl final volume.
14. Incubate samples and inputs at 65°C overnight to de-crosslink.

Day 4

Reagents:

- Proteinase K
- 5 M Betaine

Protocol

1. Add 1 µl of Proteinase K to reach 50 µg/ml final concentration. Incubate at 42°C for 1 hour.
2. Elute samples twice with 30 µl of TE/EB/water until a final volume of 60 µl is reached.

BUFFER SOLUTIONS

Table I

RIPA Buffer
50 mM Tris•HCl, pH 7.4 (1 M stock)
150 mM NaCl
1% Triton X-100 or NP-40
0.5% Sodium deoxylcholate
0.1% SDS
1 mM EDTA (0.5 M stock)
10 mM NaF
Add ddH_2O to the final volume
Add PMSF to a final concentration of 1 mM and any other protease inhibitors immediately before use

Table II

1X PBS
10 mM Na_2HPO
1.8 mM KH_2PO_4
137 mM NaCl
2.7 mM KCl
Adjust pH to 7.4
Add ddH_2O to the final volume

Table III

Incubation Buffer
2.7 mM KCl
1.5 mM KH_2PO_4
3.2 mM $Na_2HPO_4•12H_2O$
137 mM NaCl
5 mM EDTA•2Na
10 mM NaF
Add ddH_2O to 1000 ml, adjust to pH 7.4

Table IV

4X SDS Sample Buffer
12% SDS
25% Glycerol
150 mM Tris•HCl (pH 7.0•1M stock)
0.03% Bromophenol Blue
20% β-mercaptoethanol
Add ddH_2O to the final volume, aliquot, and store at -20°C
20% β-mercaptoethanol (or 500 mM DTT replace) should be freshly added before use

Table V

Cell Lysis Buffer
5 mM HEPES
85 mM KCl
0.5% NP40, pH 8.0

Table VI

Nuclear Lysis Buffer
50 mM Tris•HCl
10 mM EDTA
1% SDS, pH 8.1

Table VII

Dilution Buffer
0.1% SDS (protein interaction depend)
1.1% Triton X-100
1.2 mM EDTA
165 mM NaCl
16.7 mM Tris•HCl, pH 8.1

Table VIII

Low Salt Buffer
Tris•HCl 50 mM, pH 8.0
150 mM NaCl
0.1% SDS
1% NP40
1 mM EDTA
0.5% Deoxycholate Na

Table IX

High Salt Buffer
Tris•HCl 50 mM, pH 8.0
500 mM NaCl
0.1% SDS
1% NP40
1 mM EDTA
0.5% Deoxycholate Na

Table X

LiCl Buffer
Tris•HCl 50 mM, pH 8.0
250 mM LiCl
0.1% SDS
1% NP40
1 mM EDTA
0.5% Deoxycholate Na

Table XI

TE Buffer
Tris•HCl 10 mM, pH 8.0
0.25 mM EDTA

Table XII

Elution Buffer (EB)
100 mM $NaHCO_3$
1% SDS

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