

Company Introduction
**The Story Behind Our
Squirrel**

p 1.

Western Blot
General Protocol

p 4.

Immunoprecipitation
**How Not to Fail an IP
Experiment?**

p 17.

ChIP
Troubleshooting Guide

p 5-16.

No Fail Guide



For WB, IP, ChIP and ELISA

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“
We share our failure
experiences, so that
you can be successful.
”
arigo scientists



Antibodies are often the main ingredient to many widely-used laboratory applications, such as Western Blots (WB), Enzyme-Linked Immunosorbent Assays (ELISA), Chromatin Immunoprecipitation (ChIP), Immunohistochemistry (IHC), Immunocytochemistry (ICC), Immunoprecipitation (IP), and Flow Cytometry (FACS). In arigo, we mean quality and make it our first priority to provide high quality products to the research community. To this end, we screen our antibodies and validate them with scientific approaches to determine the specificity of our products.

Arigo scientists are dedicated to giving the best service and technical support to our customers. In this No-Fail Guide for WB, IP, ChIP and ELISA, we will discuss and share our protocols, technical hints, failure experiences and step-by-step troubleshooting guides to make sure that your experiments obtain a higher rate of success.

Through our products and service, we aim to provide our customers with the best scientific tools to get quality results and papers published.

The Story Behind Our Squirrel



arigo. biolaboratories

Excellence :

As smart as squirrels, arigo provides smart solutions allowing researchers to accelerate experiments with convincing results. Unlike many other antibody companies, our scientists are dedicated to developing the most comprehensive collection of Antibody Duos and Antibody Panels. Pathway Duos identifies protein-protein relationships, PTM Duos detect total and modified form of target proteins, Control Duos and ELISA Duos accelerate experimental procedures. Antibody Panels consist of several antibodies grouped together according to their scientific relevance.

Responsiveness :

The same as squirrels responsive to the environment, scientists in arigo thoroughly analyze the research frontier and cooperate with scientists worldwide. We bring out cutting edge research solutions in the timeliest manner, to meet the needs of unlimited possibilities in the emerging life science world.

Our Brand Story :

Have you noticed a squirrel hidden in the logo of arigo? Arigo Biolaboratories made itself synonymous with Smart Solutions using "squirrel" as a symbol.

Quality :

Arigo scientists precisely screen our products and validate them with strict control. As a result we are proud to offer customers quality products that generate the most valuable results.

Balance :

To pursue a balance in laboratory life, our staff builds out many interesting ideas, to let researchers have fun while performing experiments in the labs. Our cute squirrel will always be with you to brighten your day.

Best for the future :

Just as squirrels save the best nuts for the future, arigo is dedicated to providing our customers with the most up to date, reliable product range to get quality results and papers published. We know that your research determines the future of mankind.

About Us

WB General Protocol

IP General Protocol

ChIP General Protocol

ELISA General Protocol

Preface	01
The Story Behind Our Squirrel	02
Buffer Preparation	04
Procedures	05
How Not to Fail a Western Blot Experiment?	06
Internal Control Information	07
Protease Inhibitor Information	08
Western Blot Troubleshooting Guide	10
Buffer Preparation	14
Procedures	14
How Not to Fail an IP Experiment?	16
IP Troubleshooting Guide	18
Buffer Preparation	20
Procedures	21
How Not to Fail a ChIP Experiment?	24
ChIP Troubleshooting Guide	25
Buffer Preparation	27
Procedures	27
How Not to Fail an ELISA Experiment?	29
ELISA Troubleshooting Guide	30

WB General Protocol

Buffer Preparation

15 M Tris, pH 8.8

(stock buffer for separating gels)

Tris base : 181.65g

ddH₂O : 800ml

Dissolve well.

Adjust pH to 8.8 with concentrated HCl.

Bring up the volume to 1L with ddH₂O

(Make sure to let the solution cool down to room temperature before making the final pH adjustment)

Sterilize by autoclaving.

15 M Tris, pH 6.8

(stock buffer for stacking gels)

Tris base : 181.65g

ddH₂O : 700ml

Adjust pH to 6.8 with concentrated HCl.

Bring up the volume to 1L with ddH₂O

(Make sure to let the solution cool down to room temperature before making the final pH adjustment)

Sterilize by autoclaving.

10X PBS

NaCl : 80g

KCl : 2g

Na₂HPO₄ : 14.4g

KH₂PO₄ : 2.4g

ddH₂O : 800ml

Dissolve well.

Adjust pH to 7.4.

Bring up the volume to 1L with

ddH₂O

Sterilize by autoclaving.

20X TBS

Tris-base : 48.4g

NaCl : 160g

ddH₂O : 800ml

Dissolve well.

Adjust pH to 7.6 with HCl.

Bring up the volume to 1L with

ddH₂O

Sterilize by autoclaving.

2X SDS Loading Buffer

1M Tris pH6.8 : 2ml

50% Glycerol : 4.6ml

10% SDS : 1.6ml

0.5% Bromophenol Blue : 0.4ml

β-mercaptoethanol : 0.4ml

ddH₂O : 1ml

10X SDS Running Buffer

Tris-base : 30g

Glycine : 144g

SDS : 10g

ddH₂O : 1L

10X Transfer Buffer

Tris-base : 30g

Glycine : 144g

ddH₂O : 1L

1X Transfer Buffer

10X Transfer Buffer : 100ml

Cold ddH₂O : 800ml

Methanol : 100ml

Western Blotting - Work Flow -

Sample preparation

SDS-PAGE

Transfer

Blocking

1^o Ab incubation

Wash

2^o Ab incubation

Wash

Development

Imaging

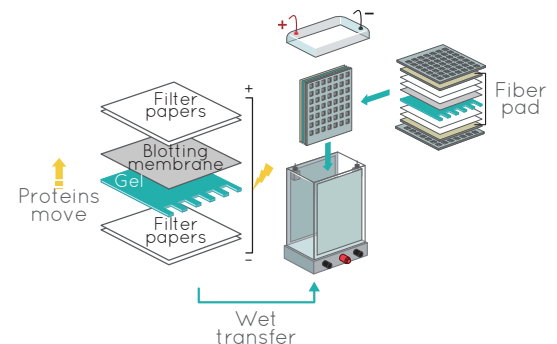
Procedures

Sample Preparation, SDS-PAGE and Transfer

1. Wash cells twice with ice-cold 1X PBS gently. Harvest cells by centrifugation.
2. Resuspend and incubate cell pellets in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Keep on ice for 20 mins. Sonicate for 10-15s to complete cell lysis if necessary.
3. Centrifuge and harvest the supernatant. Perform protein quantification with Bradford assay.
4. Add SDS loading buffer, boil the samples for 5 mins and load 30-50 μ g protein into each well of Polyacrylamide gel. Include a prestained protein marker.
5. Run gel at 25mA (2 gels run at 50mA) in 1X SDS-PAGE running buffer until dye front runs towards the end of glass plates.
6. Prepare 1X Transfer buffer. Wet PVDF membrane in methanol, or NC membrane in water. Soak membrane in transfer buffer for 10 mins.
7. Assemble the transfer sandwich and allow protein to be transferred from SDS-PAGE gel to NC or PVDF membrane for 1-2 hours in cold transfer buffer.

Blocking, Blotting and detection of proteins

1. Block blot by soaking in Blocking buffer (5% milk, 1X PBS/0.1% Tween20) for 1 hour in shaking.
2. Incubate blot in primary antibody for 1 hour at RT or 4 ° C overnight with shaking.
3. Wash blot 3-5 times, 5-10 minutes each in Wash buffer (PBST).
4. Incubate blot in secondary antibody for 1 hour at RT with shaking.
5. Wash blot 3-5 times, 5-10 minutes each in Wash buffer (PBST).
6. Detection by freshly prepared ECL.



How Not to Fail a Western Blot Experiment?

How Not to Fail a Western Blot Experiment?

1. Include appropriate positive and negative controls.

Always include appropriate positive and negative controls in your western blot experiment. These will help narrow down possible causes just in case the experiment didn't work out well. Internal controls such as GAPDH, Actin or Tubulin are good candidates for positive controls. Untreated or knocked down cell lysates serve as good negative controls. Refer to our internal control guide at [page 7](#) for more tips and advice!

2. Get a reliable antibody source and optimize it!

Make sure that your antibody is WB-validated. Some antibodies do not recognize denatured epitopes, hence not suitable for use in WB experiments. Always optimize the antibody dilution factor as inappropriate dilutions will give you high background or low/weak signals. Refer to the recommended dilution factor indicated in the datasheet and try a few more dilutions to find the optimal condition.

3. Add suitable protease/phosphatase inhibitors for each sample types

Protease inhibitors or phosphatase inhibitors can be added into the freshly prepared cell lysis buffer to prevent proteolytic degradation of target proteins. Each sample type requires different combination of protease inhibitors. Understand what your sample needs and choose the right protease inhibitors to protect them. Need help? Refer to our protease inhibitor guide at [page 8-9](#) for more details!

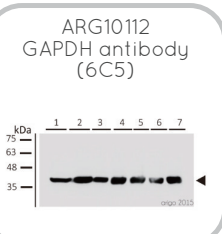
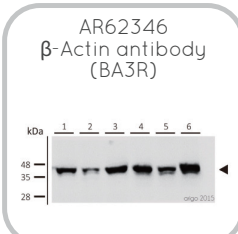
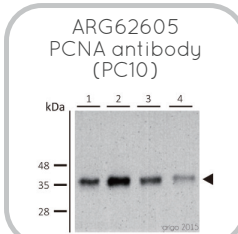
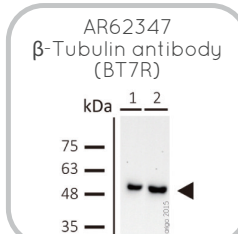
4. Keep things “cool” ~

Proteins are sensitive to heat and the whole western blot experiment can be destroyed if high heat is applied during SDS-PAGE, transfer, blocking or antibody incubation steps. If possible, run your SDS-PAGE gel or transfer your blot at 4 ° C. Compared to RT, incubating blot with primary antibodies overnight at 4 ° C gives better antibody-target binding and a cleaner background.

Internal Control Information

When Western Blot or other experiments are performed, loading controls are required to ensure that (1) the same amount of protein sample is loaded into each lane; (2) protein is transferred from gel to membrane with equal efficiency and (3) antibody incubation and detection is uniform. Loading control must fulfill certain criteria before they are chosen as normalization factor in various samples. Follow our guide below to choose the best internal controls for your experiments!

What is your sample type?

Whole Cell Lysate	Nuclear Extract	Mitochondrial Extract	Serum
Choose a control which its molecular weight is different from your protein of interest			
<p>116 kDa Vinculin (ARG53723)</p> <p>55 kDa Tubulin (ARG62347) (ARG53696, ARG53698)</p> <p>43 kDa β-Actin (ARG62346)</p> <p>34 kDa GAPDH (ARG10112)</p> <p>19 kDa Cofilin (ARG51149)</p>	<p>66 kDa Lamin B1 (ARG20013)</p> <p>29 kDa PCNA (ARG62605)</p>	<p>17 kDa COX IV (ARG54003)</p>	<p>77 kDa Transferrin (ARG63114)</p>
<p>ARG10112 GAPDH antibody (6C5)</p> 	<p>AR62346 β-Actin antibody (BA3R)</p> 	<p>ARG62605 PCNA antibody (PC10)</p> 	<p>AR62347 β-Tubulin antibody (BT7R)</p> 

How Not to Fail a Western Blot Experiment?

Protease Inhibitor Information

Within a few minutes, proteases can destroy the proteins you have spent days isolating. Inhibition of proteolytic activity is therefore becoming very important to prevent unwanted degradation of proteins during their isolation and characterization.

See the list below for commonly used inhibitors grouped into different classes of proteolytic enzymes.

Serine Protease inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
Leupeptin	Trypsin, chymotrypsin, pepsin, thrombin, calpain, cathepsinB,H, Papain etc	10-100 μ M	Water
PMSF	Broad spectrum serine protease	0.1-1.0 mM	Anhydrous ethanol or isopropanol
Aprotinin	Broad spectrum serine protease but not thrombin or factor Xa	0.3 μ M	Water
AEBSF	Broad spectrum serine protease	0.1-1.0 mM	Water

Aspartic Protease inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
Pepstatin A	Renin, Chymosin, Pepsin and other aspartic proteases	1 μ M	Methanol or DMSO

Cysteine Protease inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
E-64	Broad spectrum cysteine protease and trypsin	1-10 μ M	Water
Chymostatin	Chymase cathepsins A,B,D,G, Papain	10-100 μ M	DMSO

Metalloproteinase inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
EDTA	Broad spectrum metalloproteinase	1-10 mM	Water
Bestatin	Amino peptidase	1-10 μ M	Methanol

Western Blot Troubleshooting Guide

Weak or no Signal

Possible Causes	What can you do?
Protein not expressed in the sample used	Make sure that the protein of interest is sufficiently induced in the sample used. Fractionation might be necessary for some proteins expressed in particular organelles.
Inadequate/incomplete transfer	Be sure that the transfer is adequate, especially for high molecular weight proteins. Make sure that the transfer is complete by staining the membrane with Ponceau S solution or using a pre-stained marker as indicator.
Antibody issue	Make sure that primary antibody dilution and the incubation condition is optimal. Compatible secondary antibody should be used.
Poor activity of ECL	Prepare ECL solution freshly prior to detection.
Sodium Azide interference	Make sure that there is no Sodium Azide in the antibody dilution buffer. Wash the blot thoroughly before adding ECL.

Black Dots

Possible Causes	What can you do?
Reagent contaminated	Prepare all reagents freshly.
Blocking agent insufficiently dissolved	Make sure that the blocking agent such as milk or BSA is completely dissolved before use. Alternatively, filter the blocking solution with 0.45 μ m filter before use.

High Background

Possible Causes	What can you do?
Antibody concentration too high	Optimize the dilution factor of primary and secondary antibody.
Insufficient washing	Make sure that the blot is washed in sufficient washing buffer. Increase washing time or the percentage of Tween-20 if necessary.
Insufficient blocking	Make sure that the blot is sufficiently blocked. Increase the percentage of skimmed milk up to 5% if necessary.
Improper blocking buffer used	For the detection of phospho-proteins, use BSA instead of milk as blocking agent.
Membrane dried out	Make sure that the membrane is moist throughout the whole process of western blot.

Smeary Staining

Possible Causes	What can you do?
Poor gel preparation	Mix gel completely before pouring. If SDS-PAGE should be run the day after preparation, make sure that the gel is prepared at RT and stored in moist chamber at 4°C.
Protein overloaded	Make sure that total amount of protein loaded into each well is between 20-50µg.
High membrane protein concentration in samples	If membrane fraction is used, make sure that sample is sufficiently diluted before loading into SDS-PAGE.

Band Artifacts (White bands, smile effect, streaks)

Possible Causes	What can you do?
Poor gel preparation	Mix gel completely before pouring. If SDS-PAGE should be run the day after preparation, make sure that the gel is prepared at RT and store in moist chamber at 4°C.
Voltage, temperature too high, field effect	Keep the voltage and temperature low while running SDS-PAGE. If necessary, run gel in the cold room. Remove bubbles trapped at the bottom of gel to ensure even electrophoresis.
High salt concentration in samples	Make sure that the salt concentration of lysis buffer is kept between 0.15M to 0.5M.
Protein overloaded	Make sure that total amount of protein loaded into each well is between 20-50 μ g.
Antibody concentration too high	Optimize the dilution factor of primary and secondary antibody.

Molecular Weight Different from Predicted

Possible Causes	What can you do?
Protein post-translationally modified or alternatively spliced	Check if the protein of interest is post-translationally modified or alternatively spliced and produce other isoforms.
Incomplete protein denaturation	Freshly add DTT or β -Mercaptoethanol to sample buffer, or adequately boil samples to ensure complete bond breakage between peptides.
Membrane protein issue	If a membrane protein is to be detected, try low temperature (-65°C) or avoid boiling which might cause aggregation of membrane proteins.

Multiple bands

Possible Causes	What can you do?
Protein post-translationally modified or alternatively spliced	Check if the protein of interest is post-translationally modified or alternatively spliced and produce other isoforms.
Protein degradation	Add enough protease inhibitor to lysis buffer throughout all steps of sample preparation. Avoid frequent freeze-thawing of samples.
Protein Multimerization	Freshly add DTT or β -Mercaptoethanol to sample buffer, or adequately boil samples to ensure complete bond breakage between peptides.
Antibody concentration too high	Optimize the dilution factor of primary and secondary antibody.
Antibody issue	Some antibodies use common epitopes as immunogen. Blast the immunogen region of the antibody to make sure that the immunogen does not cross-react with epitopes from other proteins.
Interference from secondary antibody	While performing Immunoprecipitation (IP) experiment, make sure that the secondary antibody used to detect the protein of interest is derived from a species different from that of antibody used to pull down protein. Alternatively, use a secondary antibody that recognize only the native form of IgG to detect IP protein.

IP General Protocol

Buffer Preparation

Lysis Buffer (Non denaturing)

1M Tris-HCl, pH8: 0.5ml
 10% NP-40: 1ml
 5M NaCl: 0.3ml
 ddH₂O: 8.2ml

RIPA Buffer

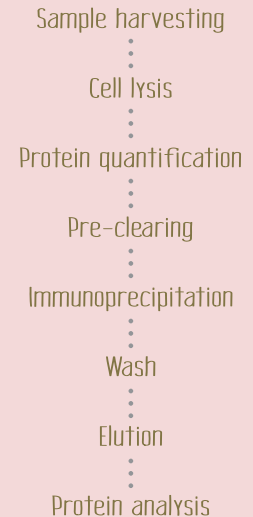
1M Tris-HCl, pH8: 0.5ml
 10% NP-40: 1ml
 5M NaCl: 0.3ml
 10% SDS: 0.1ml
 10% Sodium Deoxycholate: 0.5ml
 ddH₂O: 7.6ml

Procedures

Cell Lysate Preparation

1. Wash cells gently with ice-cold PBS.
2. Harvest cells (10^7) and transfer to conical tube. Wash cells with 10ml ice-cold PBS and centrifuge at 400xg for 10 mins at 4 ° C. (The number of cells used per IP should be optimized specifically for each protein and antibody)
3. Discard supernatant and repeat wash once.
4. Remove supernatant completely and resuspend cell pellet in 1ml ice-cold Lysis buffer (supplemented with protease inhibitors and phosphatase inhibitors).
5. Gently vortex and transfer to 1.5ml tube.
6. Place tube on ice for 30 mins with occasional mixing.

Western Blotting - Work Flow -



7. Centrifuge cell lysate at 10,000xg for 15-30 mins at 4°C.
8. Carefully collect supernatant into clean tube.
9. The protein concentration can be determined by Bradford assay. Samples can be diluted to 1µg/ul for further experiments.
10. Cell lysates can be frozen at -80°C or used immediately for IP procedures.

Preclearing

1. Add 50ul of prepared Protein A or G slurry to 500µl cell lysates.
2. Incubate on a rotator for 30 to 60 minutes at 4°C .
3. Centrifuge at 2500xg for 2-3 mins at 4°C . Transfer supernatant to clean tube.

Immunoprecipitation

1. Add 1-10µg of antibody to the pre-cleared lysates. (The concentration of antibody should be optimized)
2. Incubate at 4°C overnight on a rotator.
3. Add 50µl Protein A or G slurry to capture complexes. (Protein A or G should be equilibrated in the corresponding lysis buffer used).
4. Incubate for 1-2 hour at 4°C on a rotator.
5. Centrifuge the tube at 2500xg for 30s at 4°C.
6. Carefully discard the supernatant. Wash beads 3-5 times with 500µl ice-cold lysis buffer.
7. After last wash, carefully remove supernatant and add 50µl SDS Sample buffer to bead pellet.
8. Vortex and heat at 90-100°C for 10 minutes.
9. Centrifuge at 10,000xg for 5 mins. Collect the supernatant carefully and load onto SDS-PAGE for further analysis.

How Not to Fail an IP Experiment?

How Not to Fail an IP Experiment?

1. Lysis buffers

The denaturing ability of lysis buffer used during cell lysis can be critical. The ideal lysis buffer should protect proteins in their most native conformation while allowing adequate amounts of protein released for further analysis. Non-ionic detergents such as NP-40 or Triton X-100 are milder than ionic detergents such as SDS or Sodium Deoxycholate. Salt concentration or pH can also affect the binding capability of the antibodies to the protein of interest.

2. Elution method

There are various ways to perform elution step in IP experiments. The harshest method would be boiling of beads in a reducing SDS sample loading buffer. This method also elute non-covalently bound antibody fragments along with the protein of interest. Other methods such as using glycine buffer or applying pH shift are also applied to avoid disruption of antibody in order to obtain lower background.

3. Choice of Protein A or Protein G

Protein G is often considered a more universal IgG Binding Protein than Protein A, but different species, and subtypes of species, do vary in their binding to these proteins. Refer to the table at page 17 to find the best material that suit your antibody subclasses.

4. Secondary antibodies optimized for IP Western Blotting

Heavy chains or light chains contributed by the denatured primary antibodies during IP often cause problems for the Western Blot detection of protein of interest especially if it should be found to migrate around 50kDa or 35kDa. To overcome this issue, some secondary antibodies which only recognizes native form of IgG have been developed. These antibodies significantly eliminates the detection of denatured heavy or light chains during Western Blotting.

Species	Subclass	Protein A	Protein G	Protein L
Human	Total IgG	+++	+++	+++
	IgG1	+++	+++	+++
	IgG2	+++	+++	+++
	IgG3	-	+++	+++
	IgG4	+++	+++	+++
	IgA,M,D	-	-	+++
Mouse	Total IgG	+++	+++	+++
	IgG1	+++	+++	+++
	IgG2	+++	+++	+++
	IgG3	-	+++	+++
	IgG4	+++	+++	+++
	IgA,M,D	-	-	+++
Rat	Total IgG	-	++	+++
	IgG1	-	+	+++
	IgG2a	-	+++	+++
	IgG2b	-	+	+++
	IgG2c	+	+	+++
Chicken	IgY	-	-	-
Goat	Total IgG	-	++	-
Rabbit	Total IgG	+++	+++	-

+++ Strong binding ++ Medium binding + Weak binding - No binding

Protein A

Protein A is a cell wall component of *Staphylococcus aureus* which binds to the Fc region of immunoglobulins, especially IgG.

Protein G

Protein G is a cell wall component isolated from Group B streptococci which binds to most mammalian immunoglobulins through their Fc region.

Protein L

Protein L is a surface component of *Peptostreptococcus magnus* which binds immunoglobulins through their light chains. This protein is thus able to bind to antibody classes including IgA, IgD, IgE or IgM

IP Troubleshooting Guide

High Background

Possible Causes	What can you do?
Inadequate washing	Use a more stringent washing buffer. Try to use a high salt washing buffer or add 0.2% SDS or 1% Tween20 to washing buffer. Increase the number of washes.
Concentration of antibodies too high	Check the recommended amount of antibody as indicated in the datasheet. Titrate and optimize the optimal antibody amount used per IP experiment.
Non specific binding to Protein A,G or L	Pre-block beads with BSA. Incubate beads with 2%BSA in PBS for 1 hour wash in PBS before use.
Non specific binding to agarose beads	Include a pre-clear step by incubating lysate with Protein A/G/L agarose beads.
Antibody not specific enough	Use affinity purified and pre-absorbed antibody for IP experiments.
Sample degradation	Add adequate protease inhibitors and phosphatase inhibitors throughout sample preparation and Ip steps.

Interference from heavy or light chain

Possible Causes	What can you do?
Secondary antibody recognizes heavy / light chain denatured from primary antibody	Use secondary antibodies which only recognizes native form of IgG for immunoblotting.

No binding

Possible Causes	What can you do?
Insufficient antibody	Check the recommended amount of antibody as indicated in the datasheet. Titrate and optimize the optimal antibody amount used per IP experiment.
Washes too stringent	Reduce the number of washes. Reduce salt concentration in the wash buffer.
Incorrect Protein A/G/L used	Make sure that the Protein A/G/L beads are capable of binding to the antibody subclass being used.
Target protein not present in the sample used	Make sure that the target protein is expressed at a relatively high level in the sample used by including an Input sample in the WB.
Incorrect Lysis buffer used	Make sure that the lysis buffer used is not over-denaturing and destroy the native conformation of the target proteins.
Antibody not capable of immunoprecipitation	Try a different antibody. Try polyclonal antibody if monoclonal antibody does not work well.

ChIP General Protocol

Buffer Preparation

Glycine (25M)

Glycine: 93.8g
ddH₂O: 500ml
Gentle heating might be required

Lysis Buffer

1M HEPES-KOH (pH7.5): 10ml
5M NaCl: 5.6ml
0.5M EDTA (pH8): 0.4ml
Triton X-100: 2ml
10% SDS: 2ml
10% Sodium Deoxycholate: 2ml
ddH₂O: 178ml
Total: 200ml
Protease inhibitors (add fresh)

Low Salt Wash Buffer

10% SDS: 0.5ml
Triton X-100: 5ml
0.5M EDTA (pH8): 2ml
1M Tris-HCl (pH8): 10ml
5M NaCl: 15ml
ddH₂O: 467.5ml
Total: 500ml

High Salt Wash Buffer

10% SDS: 0.5ml
Triton X-100: 5ml
0.5M EDTA (pH8): 2ml
1M Tris-HCl (pH8): 10ml
5M NaCl: 50ml
ddH₂O: 432.5ml
Total: 500ml

LiCl Wash Buffer

1M LiCl: 125ml
NP-40: 5ml
10% Sodium Deoxycholate: 50ml
0.5M EDTA (pH8): 1ml
1M Tris-HCl (pH8): 5ml
ddH₂O: 314ml
Total: 500ml

TE Buffer

1M Tris-HCl (pH8): 10ml
0.5M EDTA (pH8): 1ml
ddH₂O: 489ml
Total: 500ml

Elution Buffer

10% SDS: 10ml
0.5M EDTA (pH8): 10ml
1M Tris-HCl (pH8): 5ml
ddH₂O: 75ml
Total: 100ml

Western Blotting - Work Flow -

Fixation

⋮

Quenching

⋮

Chromatin shearing

⋮

Check fragment size

⋮

Immunoprecipitation

⋮

Elution

⋮

DNA quantification

Procedures

Cross-linking and Chromatin Preparation

1. Add Formaldehyde to cell culture medium at a final concentration of 1%. (Start with 50-100 million cells per experiment)
2. Shake culture flasks for 10 minutes at RT.
3. Add glycine to quench the reaction at a final concentration of 125mM. Shake culture flasks for 5 minutes at RT.
4. Wash cells twice with 10-15ml of ice-cold PBS.
5. Add 5-10ml of cold PBS to flask, scrape cells and transfer to 50ml tube.
6. Centrifuge at 1000xg, 5 mins at 4°C.
7. Remove supernatant and resuspend pellet in lysis buffer (750µl for 10 million cells) (For suspension cultures, pellet cells after glycine treatment, wash twice with ice-cold PBS. Resuspend pellet in lysis buffer (750µl for 10 million cells))
8. Incubate pellet on ice for 10 minutes.
9. Proceed with sonication or MNase digestion to shear DNA.
10. Make sure that the sheared DNA give rise to fragment size of 200-1000bp. Optimization is needed for each cell types or tissues.
11. After sonication, centrifuge cell lysate at 8,000xg for 10 mins at 4°C.
12. Transfer supernatant to new tube. Remove 50µl to determine fragment size and DNA concentration, keep the rest at -80°C freezer for storage.

Determination of fragment size and DNA concentration

1. Add 70 μl of elution buffer to 50 μl of sheared DNA. Add 1 μg RNase A and 4 μg Proteinase K, and heat at 65 ° C overnight to reverse crosslink.
2. Purify DNA by phenol-chloroform or using a kit.
3. Run 5 μl of purified DNA sample on 1.5% agarose gel. Determine DNA concentration with spectrometer.

Immunoprecipitation and Washing

1. Use 25 μg of DNA per IP as a start. (The optimal starting material need to be optimized according to each sample type and antibody)
2. Prepare 1 tube of chromatin for antibody IP, and another tube for control (beads only). Remove 50 μl of chromatin as input sample, store temporarily at -20°C for later use.
3. To preclear the chromatin, add 50 μl Protein-A agarose/salmon sperm DNA or Protein-G agarose/salmon sperm DNA beads to chromatin and rotate for 1-2h at 4 ° C. Centrifuge chromatin samples at 2000xg for 5 mins at 4°C. Transfer supernatant to new tube.
4. Add primary antibody to tube (except for bead-only tube). The amount of antibody used per IP can found on the datasheet or determined by user.
5. Rotate overnight at 4°C.
6. Add 50 μl of blocked Protein A or G slurry to capture complexes.
7. Incubate for 2 hour at 4°C on a rotator.
8. Centrifuge the tube at 2000xg for 1min at 4°C.
9. Carefully discard the supernatant.
10. Wash beads once with low-salt buffer, once in high-salt buffer, once in LiCl wash buffer and once with TE buffer. Washing procedure: Resuspend beads in 1 ml wash buffer, rotate for 10 minutes at 4°C, centrifuge at 2000xg for 1 min at 4°C and remove supernatant.

Elution, reverse-crosslink and DNA purification

1. Elute DNA by adding 300 μ l of Elution buffer (supplemented with 1 μ l of Proteinase K (20 μ g/ μ l) to beads. Incubate samples for 2 hours at 55°C. Gently vortex tubes occasionally.
2. To reverse-crosslink, incubate overnight at 65 ° C. (Input should also be reverse cross-linked at the same time)
3. Centrifuge samples at 15,000xg for 5 mins at RT.
4. Transfer supernatant to a new tube.
5. Purify DNA using phenol-chloroform or DNA purification kit.
6. Resuspend or elute DNA in 50 μ l TE buffer and analyze by PCR or Real-Time PCR.

How Not to Fail a ChIP Experiment?

How Not to Fail a ChIP Experiment?

1. Cross-linking

Cross-linking is an important step to make sure that the protein is still bound to the DNA fragment during immunoprecipitation. However, it is a time-critical process and should be optimized for each sample type or antibody type. Excessive cross-linking might mask the antibody binding sites and reduce binding ability. Therefore, it is advisable to optimize the cross-linking steps by using different concentration of formaldehyde or changing cross-linking time.

2. Fragmentation method

Optimal sonication time course or concentration of microcococcus nucleus should be determined prior to performing a ChIP experiment. Make sure that the majority of fragmented DNA falls between 200-500bp. Load 5-10 μ l of sample on agarose gel to analyze DNA fragment size after each sonication course.

3. Choice of Protein A or Protein G

Protein G is often considered a more universal IgG Binding Protein than Protein A, but different species, and subtypes of species, do vary in their binding to these proteins. Refer to the table at page 17 to find the best material that suit your antibody subclasses.

4. Include positive and negative antibody controls

Histone H3K4me3 antibody is a popular positive control to use when studying active gene, while Histone H3K9me3 antibody is a negative control for studying inactive gene. An antibody that do not recognize chromatin epitope such as GFP antibody or IgG isotype control antibody should also be included in ChIP experiments to make sure that results came out to be valid.

ChIP Troubleshooting Guide

High Background observed in negative control

Possible Causes	What can you do?
Inadequate washing	Use a more stringent washing buffer. Try to use a high salt washing buffer or increase the number of washes.
Non specific binding to Protein A,G or L	Include a pre-clear step by incubating lysate with Protein A/G/L agarose beads.
Too much DNA template added to the PCR reaction, or too many cycles of amplification	Add less DNA template or reduce the number of cycles of amplification. Alternatively, real-time PCR can be used for the detection of ChIPed DNA products.
Buffers may be contaminated	Use freshly prepared lysis or wash buffers.

Positive signal seen in no template control

Possible Causes	What can you do?
PCR reagent might be contaminated	Prepare new solutions from stock

ChIP Troubleshooting Guide

Low /No Signal

Possible Causes	What can you do?
Not enough cells/chromatin	Add enough chromatin for each IP experiment. We suggest using at least 25 μ g of chromatin for each IP.
Incorrect Protein A/G/L used	Make sure that the Protein A/G/L beads are capable of binding to the antibody subclass being used.
Cross-linking process too long	Over Cross-linking with formaldehyde might mask the antibody binding sites and reduce antibody binding ability. It is advisable to optimize the cross-linking steps by using different concentration of formaldehyde or reducing cross-linking time.
Not enough antibody	Titre antibody amount used for each IP to determine the optimal condition. Up to 10ug of antibody can be used for each IP experiment.
Washes too stringent	Reduce the number of washes. Reduce salt concentration in the wash buffer.
Antibody not capable of immunoprecipitation	Try a different antibody. Try polyclonal antibody if monoclonal antibody does not work well.
The chromatin size might be too small	Make sure that the shearing condition is not too harsh which might results in fragments of DNA smaller than what the primers are able to amplify.
Incomplete elution from the Protein A/G/L beads	Incubate beads in elution buffer at 65°C with frequent mixing.

Buffer Preparation

10X PBS

NaCl: 80g
 KCl: 2g
 Na₂HPO₄: 14.4g
 KH₂PO₄: 2.4g
 ddH₂O: 800ml
 Dissolve well.
 Adjust pH to 7.4.
 Bring up the volume to 1 L with
 ddH₂O
 Sterilize by autoclaving.

1X Wash Buffer

10X PBS: 100ml
 ddH₂O: 900ml
 Tween-20: 50u

1X Blocking Buffer

10X PBS: 10ml
 ddH₂O: 90ml
 BSA: 1g
 Dissolve well.

Stop Solution

0.5M H₂SO₄

Procedures

1. Coat 96-well plate with capture antibody overnight at room temperature or 4 ° C for 1-2 days. (Antibody should be coated with the concentration of 1-10 μg/ml, 50 μl/well in 1X PBS)
2. Wash antibody-coated plate 3X with Wash buffer.
3. Block with 200 μl/well of blocking buffer for 30 minutes at RT.
4. Prepare serial dilutions of standards in PBS.
5. Add samples, standards and blank into antibody-coated microtiter plate (50 μl/well, duplicates) and incubate for 2 hours at RT.

Western Blotting - Work Flow -

Plate coating

⋮

Blocking

⋮

Standard preparation

⋮

Sample incubation

⋮

Wash

⋮

Antibody incubation

⋮

Wash

⋮

Streptavidin-HRP

⋮

Wash

⋮

Substrate incubation

⋮

Stop reaction

ELISA General Protocol

6. Wash 3X with wash buffer.
7. Add biotinylated Capture antibody and incubate for 1 hour at RT.
(Concentration of 1-10 $\mu\text{g/ml}$, 50 $\mu\text{l/well}$ in 1X PBS)
8. Wash 5X with Wash buffer.
9. Add Streptavidin-HRP in Wash buffer (50 $\mu\text{l/well}$) and incubate for 45 mins at RT.
10. Wash 5X with Wash buffer.
11. Add TMB substrate (50 $\mu\text{l/well}$) and watch for color change.
12. Stop reaction with Stop solution (50 $\mu\text{l/well}$).
13. Measure OD at 450nm in an ELISA plate reader. (Reference wavelength 620nm)

How Not to Fail an ELISA Experiment?

1. Ensure consistency between wells

Use multiwell plates, multichannel pipettes and plate washers for a more consistent result. Make sure that all pipettes are accurately calibrated on a regular basis. In the initial stage of assay development, test a range of parameters to optimize ELISA conditions.

2. Prevent sample degradation

Protease inhibitors or phosphatase inhibitors can be added into the freshly prepared cell lysis buffer to prevent proteolytic degradation of target proteins. It is important to test all samples in duplicate or triplicate in conjunction with a known standard to ensure the accuracy of results.

3. Optimize coating condition

Coating of antibodies or antigens onto plastic surface is a passive absorption process which depend highly on time, temperature, pH and the concentration of coating agents. Typical coating conditions fall within the range of 1-10 µg/ml in 50-100 µl buffer, incubating overnight at 4 ° C or 1-3 hours at RT. Optimization for each assay need to be performed individually.

4. Choose the right antibodies

Antibodies of high specificity, affinity and avidity need to be used for ELISA assays. Monoclonal antibodies offer better homogeneity by targeting a single epitope while polyclonal antibodies consist of complex antibody pools that target various epitopes. For sandwich ELISA, arigo offers ELISA antibody duos for the optimized performance of ELISA assays.



Not all standard curves are straight lines. It is advisable to use program capable of generating a four parameter logistic (4-PL) for a better fitting curve.



ELISA Troubleshooting Guide

ELISA Troubleshooting Guide **No signal**

Possible Causes	What can you do?
Assay set up incorrectly	Make sure that the instructions in the protocol is followed carefully.
Incorrect secondary antibody used	Check if the correct secondary antibody is used.
Insufficient antibodies used	Increase concentration of primary or secondary antibody.
Substrate reagents not fresh	Use fresh substrate reagents
Wrong settings of plate reader	Check the settings (wavelength, filters, gain etc) of plate reader.
Insufficient incubation	Follow the incubation time as indicated in the protocol booklet.
Sample concentration falls below detection limits of kit	Decrease dilution factor or concentrate samples.
Plate washing too vigorous	Check the setting of plate washer. Pipette wash buffer into wells gently.
Wells dried out	Cover plate with adhesive film or incubate in humidified chamber throughout experiment.
Enzyme inhibitor present in buffers or reagents	Inhibitors such as Sodium Azide can affect enzyme and assay performance. Ensure that there is no enzyme inhibitor in any buffers.

High background

Possible Causes	What can you do?
Too much antibodies was used	Reduce the concentration of primary or secondary antibodies.
Antibodies bind nonspecifically	Use blocking buffer or choose another affinity-purified antibody.
Too much substrate reagent used	Use substrate with higher dilution.
Insufficient washing	Increase washing cycles.
Wrong concentration of blocking reagent	Check the recommended concentration of blocking buffer.
Reaction not stopped	Stop reactions with STOP buffer before reading.
Plate left too long before reading	Take measurements shortly after addition of substrate and STOP buffer.
Insufficient Tween in wash buffer	Use PBS+0.05% Tween as wash buffer.
Incubation temperature too high	Optimize incubation temperature for each experiment.
Plate stacking during incubation lead to uneven temperature throughout the plate	Avoid stacking plates together during incubation.
Pipetting error	Calibrate pipettes to make sure that the correct volume is dispensed.
Reagents not mixed properly	Make sure that all reagents are mixed properly and equilibrated to room temperature before assay

Continued ►►►

ELISA Troubleshooting Guide

High background

Possible Causes	What can you do?
Salt concentration of incubation and wash buffer	Increase salt concentration to reduce nonspecific interaction.
Substrate incubation carried out in light	Perform substrate incubation in dark.
Dirty plate	Make sure that the bottom of plate is clean.

Poor standard Curve

Possible Causes	What can you do?
Improper standard dilution	Use appropriate diluent as blank. Make sure that the dilution is performed as according to protocol.
Standard improperly reconstituted	Briefly spin standard vial before opening. Make sure that there is no undissolved material after reconstituting.
Standard degraded	Store standards as according to protocol.
Curve doesn't fit the scale	Try plotting log-log or 5 parameter logistic curve fit.
Pipetting error	Calibrate pipettes to make sure that the correct volume is dispensed.
Incomplete washing	Increase washing cycles.

Weak signal

Possible Causes	What can you do?
Insufficient coating	Use more antigens or antibodies for coating.
Substrate reagents have expired or prepared at a wrong pH	Use fresh substrate reagents.

Variation among replicates

Possible Causes	What can you do?
Improper washing	Make sure that the washing is done as according to protocol.
Poor mixing of samples	Mix samples gently and evenly.
Dirty plate	Make sure that the bottom of plate is clean.
Reagents too old	Make sure that the reagents are not expired. Use freshly prepared reagents.
Bubbles in wells	Make sure that there is no bubble in wells before reading.
Inconsistent pipetting	Calibrate pipettes to make sure that the correct volume is dispensed.
Edge effects	Make sure that the plate and reagents are equilibrated to room temperature before starting assay.

nuts about antibodies

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