ChIP and optimization techniques for western blots.

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Scientific Support Specialist
Abcam KK (Japan)
PART 1

• Introduction
• ChIP protocols
• Optimization
• Choosing a good antibody
• ChIP at Abcam
• ChIP resources

PART 2

• Introduction
• WB protocols
• Troubleshooting
• Summary
INTRODUCTION
Chromatin Immunoprecipitation (ChIP)

Allows quantitative analysis of Protein-DNA interactions *in vivo*.

- Localisation of proteins associated with DNA
- Follow changes at single promoter
- Map distribution of factor over entire genome
Basic structural unit of chromatin:
Chromatin is the complex of DNA and protein that makes up chromosomes.

nucleosome = DNA + 2x(histone H2A + H2B + H3 + H4)

- **Histones:**
  - core histones – H2A, H2B, H3 and H4
  - linker histones – H1 and H5
Histone Modifications

Types of modifications:
- Methylation
- Phosphorylation
- Acetylation

Histone modifications play role in:
- Gene expression
- Chromatin assembly
- Replication
- DNA repair

Gene activation: methylation of H3K4, acetylation of H3 & H4

Cell death: phosphorylation of H2B S14

Aurora B histone kinase activity – colorectal cancer
CHIP PROTOCOL
1. Cross-linking & sonication

2. Immunoprecipitation

3. Reverse cross-link & purify DNA

4. DNA analysis

Brief overview
1. Cross-linking

Living Cells (HeLa) → Cross-link DNA-bound proteins to chromatin in the living cells (n.b. X-ChiP) → Lyse cells and sonicate → Resolve DNA fragments on agarose gel. Check size 200bp – 1000bp → Sheared, soluble chromatin ready to be used for immunoprecipitation. Can be stored at -80C for 3 months.
2. Immunoprecipitation

Add antibody of interest to chromatin

Antibody binds to a specific protein-DNA complexes

Add Protein A/G sepharose beads

Block beads to avoid non-specific binding

Antibody also binds to Protein A/G (beads) via Fc fragment

Matrix (beads)

Protein A/G

Therefore specific complexes are isolated out of solution

BSA ssDNA

Protein A/G coupled to sepharose beads
3. Reverse cross-links & purify DNA

Wash beads with buffer of high salt concentration

Protein–DNA complex broken up

Elute protein–DNA complex (from beads)

Reverse cross-links

65 degree C, 4-5 hr

DNA binds to gel membrane of column

Mix with Qiaquick binding buffer (high salt conc) and add to Qiaquick column (or equivalent DNA purification kit)

Elution buffer, mix, collect supernatant

Elute DNA from column

Qiaquick elution buffer

Purified DNA fragments
4. DNA analysis

Quantitative Real-time PCR: Detection method based on changes in fluorescence proportional to increase of product.

1. Initialization – Heat at high temp to denature DNA template and primers

2. Annealing – Primers anneal to single stranded DNA template

3. Extension/Elongation – DNA polymerase copies DNA fragment starting at primers annealed to both of its strands. Two resulting strands make up template DNA for next cycle……etc

DNA Template (purified DNA)

2 x Primer (complementary to both ends of the DNA fragment to be amplified)

DNA polymerase + dNTPs

Measurement - Real-time PCR measures quantity of product throughout this exponential stage
Controls

**Negative**

- No antibody control (IP)
- No template control (PCR)
- Region where antigen is absent

**Positive**

- Well defined locus where antigen is present
- Antibody of interest (tested antibody)
Histone modifications play a role in gene expression. This is important when interpreting ChIP results.

**ab8580 Histone H3 tri methyl K4**

Enrichment on highly active loci

We should see high enrichment compared to background (beads, i.e. no antibody control).

**ab8898 Histone H3 tri methyl K9**

Enrichment on heterochromatin
OPTIMIZATION
Optimise sonication

Generally 200-1000 bp is sufficient

![Sonication Results](image)
Western blot can be used to test whether the target has been immunoprecipitated

*Picture from Abreview (ab17721)
ChIP using standard conditions

- Requirement: Control loci (+/-)

![Graphs showing TBP relative to Input for different conditions](https://example.com/graphs)
Antibody concentration should be optimised

- 1–10ug of antibody for every 25ug of chromatin

Antibody: 2ug

Antibody: 10ug
Test different washing conditions

- Salt concentration of last wash: 150-500mM NaCl

[NaCl]:

500mM

250mM

- Washes should be as stringent as possible
CHOOSING A GOOD ANTIBODY
1. Antibody should be fully characterised and specific

- Affinity purified
- Specificity tested in:
  - ELISA
  - Peptide inhibition western blot

Peptide inhibition western blot

H3 tri-methyl K4 (ab8580)

Peptide competitor:

- none
- Mono me K4
- Di me K4
- Tri me K4
- Di me K9
- Tri me K9
- Tri me K27
- unmodified

H3 tri-methyl K4

Does not cross react with other modifications
2. IHC is a good indicator for success in ChIP

Protein conformation: Native  Denatured

Technique: ChIP/IP/IHC  Western blot

Immunohistochemistry – TAF1 antibody (ab28450)
3. Polyclonal antibodies have higher success rate in ChIP

**Polyclonal antibodies**

+ recognise several epitopes
- batch to batch variation

**Monoclonal antibodies**

- recognise single epitope
+ minimal batch to batch variation
CHIP AT ABCAM
• >230 ChIP grade antibodies

• >40 ChIP batch tested antibodies:
  - Histones
  - Histone modifications
  - Chromatin binding proteins
  - Other modified proteins

ChIP: Histone H3 (tri methyl K4) ab8580

Every new batch of this antibody is tested in ChIP.
The Abcam ChIP Kit

ab500 – the Abcam ChIP kit

- All critical components for:
  - chromatin preparation (lysis buffer)
  - Immunoprecipitation (agarose beads)
  - DNA purification

- H3 antibody ab1791 (positive control)

- Detailed protocol
CHIP RESOURCES
**PCR primers used to test ab8580 in ChIP at Abcam**

The following primers were used to test ab8580 by chromatin immunoprecipitation (ChIP).

<table>
<thead>
<tr>
<th>Primer Accession</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0312995</td>
<td>TCC ACA GTC ACC CCC ATG T</td>
<td>CTA GCC TCC CCG GTG TCT CT</td>
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<tr>
<td>hsGAPDH exon 1 F1</td>
<td>TGG CCA GGC GAT GAC GGG CG</td>
<td></td>
</tr>
<tr>
<td>hsGAPDH intron 1 R1</td>
<td>GCC GGT TCA ATG CCT GCA ATT</td>
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<td>hsGAPDH exon 1</td>
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<td>CAA GGC AAA GGC AAA ATG GT</td>
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<td>hsRPL30 first exon F1</td>
<td>GCC GAT TGC ATG TTG ATT</td>
<td></td>
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<td>hsRPL30 first exon R1</td>
<td>GCC GAT TGC ATG TTG ATT</td>
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<tr>
<td>hsRPL30 exon 1</td>
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<tr>
<td>B0010677 (accession number)</td>
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<td>hsALDOA second intron F</td>
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<td>hsALDOA second intron R</td>
<td>AGA CAC GAT AGC CTT AGG AAT CTC</td>
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<td>hsALDOA exon 2</td>
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<td>B0014033 (accession number)</td>
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<td>hsMYO-D exon 1 F1</td>
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<tr>
<td>hsMYO-D exon 1 R1</td>
<td>GCC AGC AAC TGG TGG TTT GG</td>
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<tr>
<td>hsMYO-D exon 1</td>
<td>GCC AGC AAC TGG TGG TTT GG</td>
<td></td>
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<tr>
<td>AF113275 (accession number)</td>
<td>GCC GTA AGC TGG CAT TCC T</td>
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<tr>
<td>hsSPP1 (anti-trypsin) intron 1 F1</td>
<td>GCC TCA AGC TGG CAT TCC T</td>
<td></td>
</tr>
<tr>
<td>hsSPP1 (anti-trypsin) intron 1 R1</td>
<td>GCC TCA AGC TGG CAT TCC T</td>
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<tr>
<td>hsSPP1 (anti-trypsin) intron 1</td>
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<td>L32140 (accession number)</td>
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<td>hsAFM test intron F</td>
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<tr>
<td>hsAFM test intron R</td>
<td>GCC GTA AGC TGG CAT TCC T</td>
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<tr>
<td>hsAFM exon 1</td>
<td>GCC GTA AGC TGG CAT TCC T</td>
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<tr>
<td>The PCR is performed with the TaqMan Universal PCR Master Mix from ABI.</td>
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<td></td>
</tr>
<tr>
<td>Amplification:</td>
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<td></td>
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<tr>
<td>Stage 1 - 2 min at 95°C 1 repetition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 2 - 10 min at 95°C 1 repetition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 3 - 30 sec at 95°C, 1 min at 60°C 35 repetitions</td>
<td></td>
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</tbody>
</table>
Protocols and troubleshooting tips

Protocols and troubleshooting tips on how to get the best from our antibodies

- Antibody storage guide
- Need help? Contact Abcam's Technical Team

- Chromatin
- Developmental Biology
- Immunology
- Microbiology
- Neuroscience
- Nuclear Signaling
- Signal Transduction
- Stem Cells

Research areas

- Cancer
- Cardiovascular
- Cell Biology
- Chromatin
- Developmental Biology
- Immunology
- Microbiology
- Neuroscience
- Nuclear Signaling
- Signal Transduction
- Stem Cells

Buying and contact info

United States

Scientific support

Protocols - by application

- Antibody conjugation (1)
- Cell culture (4)
- Cell isolation (2)

Protocols - by research area

- Apoptosis protocols (5)
- Cardiovascular protocols (5)
- Molecular biology protocols (4)
- Neuroscience protocols (12)

General help

- Fluorescence
- Information (2)
- Frequently asked questions (6)
- Troubleshooting tips (7)

ChIP tips

ChIP troubleshooting tips

- Chromatin preparation from tissues for chromatin immunoprecipitation (ChIP)
- Cross-linking chromatin immunoprecipitation (X-ChIP) protocol
- Native chromatin immunoprecipitation protocol
### Histone Modifications & Variants

<table>
<thead>
<tr>
<th>Regulation of modifications</th>
<th>Yeast</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td>[Diagram]</td>
<td>[Diagram]</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>[Diagram]</td>
<td>[Diagram]</td>
</tr>
<tr>
<td>Methylation</td>
<td>[Diagram]</td>
<td>[Diagram]</td>
</tr>
<tr>
<td>Ubiquitination</td>
<td>[Diagram]</td>
<td>[Diagram]</td>
</tr>
</tbody>
</table>

#### Yeast

#### Human

Chromatin antibodies, new data and poster downloads - www.abcam.com/chromatin/posters
Abcam Products

- Datasheets
  - Comprehensive, current, honest information
  - Include species reactivity, tested applications, references

- Abreviews
  - Customer product reviews
  - Positive and negative reviews
  - Abpoints (more for images)
  - www.abcam.com/abreviews
New Chromatin Antibodies

Modified histones:
- Histone H3 (phospho T118) ab33310
- Histone H2B (acetyl K11) ab40975

Mediator subunits:
- Med4 ab54028, Med12 ab49053, Med19 ab49271

Histone modifying enzymes and regulators:
- KDM6B/JMJD3 ab38113
- KMT2C/MLL3 (acetyl K1869) ab40973
- MSL1v1 ab35160
- Ring6a ab48010
Upcoming Events

Chromatin:
Histones, Nucleosomes, Chromosomes & Genomes
Singapore,
February 9-11 2009

INVITED SPEAKERS:
Sung Hee Baek | Laurie Boyer | Xing Wang Deng |
Anne Ferguson-Smith | Xiang-Dong Fu | Shiv Grewal |
Kristian Helin | Huck Hui Ng | Takashi Ito | Tony Kouzarides |
Wouter De Laat | En Li | Jason Lieb | John Lis | Azim Surani |
Bryan Turner | Jonathan Widom |

ORGANIZERS:
Neil Clarke, Edwin Cheung and Abcam

REGISTER AT:
www.abcam.com/chromatin/singapore
The chromatin team

Candy Smelly
Marketing coordinator

Dr Abarna Thiru
Business Development Associate

Dr Rachel Imoberdorf
Senior Development Scientist

Dr Karen Halls
Senior Scientific Support
Questions?
PART 1

• Introduction
• ChIP protocols
• Optimization
• Choosing a good antibody
• ChIP at Abcam
• ChIP resources

PART 2

• Introduction
• WB protocols
• Troubleshooting
• Summary
INTRODUCTION
- Proteins separated based on size using electric current.
- Antibody to protein of interest used to determine presence of protein in sample.

The dark bands show where the antibody has bound to the protein of interest (~45kDa)
WESTERN BLOT PROTOCOLS
Step by step guide to procedure

- 1. Sample preparation
- 2. Loading the gel
- 3. Running the gel
- 4. Transfer to the membrane
- 5. Blocking
- 6. Antibody incubation and detection
### 1. Sample preparation

Choose the correct lysis buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell</td>
<td>NP-40 or RIPA</td>
</tr>
<tr>
<td>Cytoplasmic (soluble)</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td>Cytoplasmic (cytoskeletal bound)</td>
<td>Tris-Triton</td>
</tr>
<tr>
<td>Membrane bound</td>
<td>NP-40 or RIPA</td>
</tr>
<tr>
<td>Nuclear</td>
<td>RIPA</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>RIPA</td>
</tr>
</tbody>
</table>
Reducing and denaturing

- Check datasheet for advice

- SDS detergent, mercaptoethanol and heat breaks disulphide bonds - disrupts secondary and tertiary structure. Linear protein will then run at the correct molecular weight.

- May also make antibody epitope more accessible.
2. Loading the gel

Loading

- 20 – 30 ug whole lysate
  100 ng purified protein

- Optimize amount depending on expression level of the protein

- Load evenly – same amount of protein in each well
3. Running the gel

Proteins negatively charged, move towards the positive cathode

**Anode -**

- A negative charge is applied to the buffer in the inner chamber, to which the top of the gel is exposed

**Cathode +**

- Proteins (which are negatively charged) travel down toward the bottom (positive) electrode

Samples are pipetted into the wells

- Smaller proteins move more quickly through the gel. Proteins separate out according to size

A positive charge is applied to the buffer in the outer chamber, to which the bottom of the gel is exposed

The progress can be monitored by watching the dye travel toward the bottom of the gel.
- Low molecular weight use high % gel (40kDa, 20%)
  High molecular weight use low % gel (200kDa, 8% gel)

- Optimize the running time to ensure proteins well separated but not run off the gel
4. Transfer to the membrane

The gel is placed against a PVDF membrane that binds proteins

Transfer Stack

Negatively charged proteins move up towards + cathode and onto the filter paper
- Choice of membrane:
  - PVDF membrane less background than nitrocellulose
  - Larger molecular weight = both types of filters
  - Less than 10 kDa = nitrocellulose (higher binding capacity)

- Check the transfer: use dyes

(A) Transillumination,
(B) Coomassie™ Brilliant Blue, (irreversible)
(C) Ponceau-S red,
(D) Amido black (irreversible)
(E) CPTS total protein stains.
5. Blocking

- Milk or BSA 5% for 1 to 2 hours

Membrane incubated with BSA or milk to occupy the spaces on the membrane that are not yet occupied by protein. This prevents the antibody from simply sticking to the membrane.
6. Antibody incubations and detection

Primary antibody:
- 1-2 hr RT or 4°C overnight

Secondary antibody:
- 1 -3 hours RT

Substrate
eg Hydrogen peroxide + luminol
3-aminophtalate (light sensitive)

Antigen on membrane
Primary
Secondary
HRP
TROUBLESHOOTING
Troubleshooting

- No signal
- High background
- Non specific bands
- Incorrect band size
- White bands on black/grey blot
- Patchy staining
- ‘Smear’
Troubleshooting: NO SIGNAL

Sample preparation
- Untested species? Positive control. Check alignment.
- Not enough antigen in the sample or loaded onto the gel.

Transfer to the membrane
- Poor transfer to membrane.
- Excessive washing.

Blocking
- Too much blocking. Optimize blocking agent, concentration and time
- Cross-reaction blocking agent / antibody.

Antibody incubation and detection
- Not enough antibody / Incorrect secondary antibody.
- Detection kit is old - substrate inactive.
Troubleshooting: HIGH BACKGROUND

Blocking
- Need to block for longer.
- Optimize blocking agent, concentration.
- Cross-reaction between blocking agent and primary or secondary.
- Phospho-specific proteins don’t use Casein to block.

Antibody incubation and detection
- Primary antibody concentration too high.
- Incubation temperature too high.

Other
- Ensure membrane washed adequately
- Membrane has dried out.
- Run a no primary control
Troubleshooting: NON SPECIFIC BANDS

Sample preparation
- Multimers (dimers) of protein (reduce and denature).
- Splice variants / isoforms or possibly proteins from the same family.
- Target protein is in fragments – degradation by proteases or cleaved.
- Bands may be non-specific. Run a no primary control
- Too much sample protein

MML4 30 kDa
Troubleshooting: NON SPECIFIC BANDS

Blocking
- Insufficient blocking. Optimizing concentration, time.

Antibody incubation and detection
- Primary or secondary antibody concentration too high.
- Antibody has not been purified.

Other
- Ensure sufficient membrane washes.
Troubleshooting: INCORRECT BAND SIZE

Sample preparation

- Ensure sample is reduced and denatured unless stated otherwise on the datasheet.
- Check for isoforms.
- Is the sample a recombinant fragment? These will run at a different size.

Eg. ab48197
Troubleshooting: WHITE BANDS/ BLACK BLOT

- Too much primary and/or too much secondary antibody.

Note – this is an extreme case!
Troubleshooting: PATCHY STAINING

- Not enough antibody
  Incubate with agitation. Do not let membrane dry out

- Contamination from bacteria
  Store antibodies at correct temperature and use fresh buffers
Troubleshooting: ‘Smear’

- Overloading

Recommendation:
20 – 30 ug whole lystate
100 ng purified protein

- Protein degradation

Use protease inhibitors
Summary

1. Sample preparation

- Ensure sample will have detectable level of protein.
- Endogenous positive and negative control?
- Is species tested? Predicted cross-reactivity?
- Check if reduced and denatured, possible isoforms.
- Use correct lysis buffer (use protease inhibitors)

Always check!
2. Loading the gel
   - Ensure the right amount of sample is loaded onto the gel (20 – 30 ug)

3. Running the gel
   - Run on the correct % gel?
   - Run on a reducing gel unless specified on datasheet.

4. Transfer to the membrane
   - Check the transfer to the membrane
   - (ponceau red or loading control)
5. Blocking
   - Ensure the correct blocking agent used and the blocking time optimized

6. Antibody incubation and detection
   - Ensure the antibodies have been used at the correct concentration and the incubation time is optimized.
   - Ensure correct secondary antibody has been used – no primary control

Always check!
Questions?
Thank you!
ERROR:
syntaxerror
OFFENDING COMMAND:
--nostringval--
STACK:
Title ( )
Subject ( D:20081201110205+08'00' )
ModDate ( )
Keywords ( PDFCreator Version 0.9.5 )
Creator ( D:20081201110205+08'00' )
CreationDate ( ringol )
Author -mark-

OFFENDING COMMAND: --nostringval--
ERROR: syntaxerror