UHN Microarray Centre

Chromatin Immunoprecipitation Protocol for Microarray Analysis – Protein A/G Bead Method

This is the UHNMAC Chromatin Immunoprecipitation (ChIP) protocol using Protein A/G beads for the recovery of the protein-DNA complex. This four-day protocol also includes a reagent/solution list, including most catalogue numbers, at the end of the protocol.

Protocol Timeline (all times are approximate):

Day 1 (3.5 hr): Crosslink sample cells: 0.75 hr
Sonication/chromatin shearing: 0.5 hr + 0.25 hr per sample
Preclearing/Immunoprecipitation with antibody: 1 hr; incubate overnight

Day 2 (8 hr): Antibody-protein-DNA complex recovery/washing: 3 hr
Elution of bound protein-DNA complex: 1 hr
De-crosslink protein-DNA complex/RNA digest: 5 hr; precipitate overnight

Day 3 (9 hr): Proteinase K digest: 3 hr
DNA recovery/purification: 0.4 hr
DNA amplification/purification of PCR product: 3.5 hr
Dye conjugation: 1 hr
Purification of fluor-labelled DNA: 0.4 hr
Hybridisation: 0.5 hr (set-up); overnight

Day 4 (1 hr): Washing arrays: 1 hr

Prepare Solutions

Prepare necessary volumes of 1x PBS + 0.5 mM EDTA and RIPA Lysis Buffer by adding 100x Protease Inhibitor Cocktail as required. The Protein A Sepharose/1x TE (pH 8.0) – 50% Slurry may also be prepared at this time.

Each + or – antibody IP reaction will require the combined sonicated cell lysate from two 150 mm dishes, each dish having 5 – 15 x 10^6 cells depending on confluence.

Cross-Linking Reaction

1. To reduce any variability in fixation, pool the medium from all like-treated dishes, mix, and re-aliquot so that each 150 mm dish has a final volume of 13 mL.

2. Cross link protein to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% (351 µL of 37% formaldehyde (Sigma, catalogue # F-8775)) into 15 mL growth medium). Incubate at 37°C for 10 minutes.

3. Stop the cross-linking reaction by adding glycine (Sigma, catalogue # G7126) to a final concentration of 125 mM (650 µL of 2.5 M stock in 15 mL medium). Incubate at 37°C for 5 minutes.

4. Aspirate away medium and rinse twice with 13 mL of ice-cold 1x PBS + 0.5 mM EDTA. If collecting cells from a number of dishes at once, allow them to sit on ice in the second wash as you work through them.

5. Aspirate away wash, add 1.5 mL 1x PBS + 0.5 mM EDTA + protease inhibitors and scrape cells into 2 mL microfuge tube (Sarstedt, catalogue # 72.695). Pellet cells by centrifuging at 380 x g at 4°C for 5 minutes and carefully remove supernatant. *At this point cell pellets may be snap frozen in liquid nitrogen and stored at -70°C for subsequent processing.*

Sonication/Chromatin Shearing Efficiency

1. Resuspend the cell pellets in 200 µL RIPA Lysis Buffer + protease inhibitors and transfer to a 1.5 mL microfuge tube (conical bottom improves sonication efficiency). Incubate samples on ice for 10 minutes.

2. Keeping samples ice cold, sonicate the cell lysates in order to shear chromatin into lengths of approximately 1000 base pairs. (6 intervals of 25 seconds, 2 minute 'rests' between intervals, power setting '3', Branson 150 cell disruptor).

3. Remove cell debris by centrifuging 16,060 x g at 4°C for 20 minutes. Combine the cleared supernatants from the two sonicated cell pellets/condition to a fresh 2 mL Sarstedt tube (catalogue # 72.695).

4. Following sonication, a 3 µL aliquot of lysate may be run on a 1% agarose gel to confirm shearing efficiency. To get a truly accurate determination of fragment size, samples should be de-crosslinked at 65°C for 5 hours and the DNA purified before running.

5. Typical lysate volume for two sheared cell pellets should be about 600 µL. Bring IP reaction up to 2 mL by adding RIPA Lysis Buffer with protease inhibitors as required.

Preclearing/Immunoprecipitation with Antibody

1. Reduce nonspecific background by pre-clearing the 2 mL sample with 80 µL of Protein A Sepharose/1x TE (pH 8.0) – 50% Slurry. Allow samples to incubate at 4°C for 1 hour with rocking.

2. Centrifuge samples at 380 x g for 2 minutes to pellet the beads and carefully transfer supernatant to a new 2 mL Sarstedt tube avoiding any carry-over of beads. Add 20 µL (1% volume) of 10 mg/mL purified BSA (New England Biolabs, catalogue # B900IS) to each IP sample as a blocking agent.

3. Add 1 µg of antibody against the protein of interest to each '+ antibody' IP reaction and incubate at 4°C overnight with rocking. No-antibody negative control samples are also incubated at 4°C overnight with rocking before continuing with Day Two protocol.

Day Two:

Antibody – Protein – DNA Complex Recovery and Washing

1. Add 20 µL of 100 mg/mL yeast tRNA (Invitrogen, catalogue # 15401-029) to each 2 mL IP reaction (1000 µg/mL final concentration) as a blocking agent before adding 60 µL of Protein A Sepharose/ 1x TE (pH 8.0) – 50% slurry. Incubate at 4°C for 1 hour with rocking.

2. Pellet beads by centrifuging at 380 x g for 2 minutes. Discard the supernatant from the antibody treated samples as this contains unbound, non-specific DNA. A 100 µL aliquot of the supernatant from the 'no-antibody' samples may be taken as 'Total Input Chromatin' positive control for the PCR reaction. This sample is considered to be the input/starting material and needs to have the Protein – DNA crosslinks reversed by heating at 65°C for 5 hours.

3. Wash beads with 1 mL of each of the wash buffers listed below, rocking at room temperature for 10 minutes.

Note: It is recommended that the pelleted beads be gently resuspended in the Low Salt wash buffer and carefully transferred to a conical bottom 1.5 mL microfuge tube as this greatly facilitates spinning the beads down during subsequent wash steps. Remove as much buffer as possible between washes without loss of beads.

- one wash: Low Salt Immune Complex Wash Buffer
- four washes: High Salt Immune Complex Wash Buffer
- one wash: LiCl Immune Complex Wash Buffer
- two washes: 1x TE (pH 8.0)

Elution of bound Protein – DNA complex

1. Prepare fresh Elution Buffer (1% SDS, 0.1 M $NaHCO_3$).

For each IP reaction: 2x 250 µL elutions → prepare 510 µL:

$NaHCO_3$:	51 µL 1 M stock (Sigma, catalogue # S-6297)
SDS:	51 µL 10% stock (BioRad, catalogue # 161-0302)
H_2O:	408 µL (Sigma, catalogue # W-9502)
	510 µL

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2. Elute the Protein–DNA complex by adding 250 µL of elution buffer to the pelleted beads. Incubate at room temperature for 15 minutes with rocking. Spin down and transfer supernatant to a fresh tube avoiding any bead carry-over. Repeat elution and combine the supernatants, centrifuging and transferring supernatant to a fresh tube if necessary to remove all beads. Add 200 µL elution buffer to 100 µL Total Input Chromatin control samples to bring volume up to 300 µL.

De-crosslinking Protein – DNA complex/RNA Digest

De-crosslinking and Proteinase K digest is based on the Farnham Lab Protocol:
http://www.genomecenter.ucdavis.edu/farnham/protocol.html
(previously found at: http://mcardle.oncology.wisc.edu/farnham/protocols/chips.html)

1. Add 20 µL of 5 M NaCl to the combined eluates (200 mM final concentration) and 1µL of 10 mg/mL RNAse A (Qiagen, from DNeasy Tissue Kit, catalogue # 69504). Incubate at 65°C for 5 hours to reverse the formaldehyde crosslinks.

2. Add 1 mL 100% ethanol, mix by inversion, and allow samples to precipitate at – 20 °C overnight.

<u>Day Three:</u>

Proteinase K Digest

1. Centrifuge samples at 16,060 x g for 15-20 minutes at 4°C. Carefully remove supernatant and resuspend pellet in 70% ethanol. Spin again at 16,060 x g at 4°C for 15-20 minutes. Remove supernatant and allow pellet to air dry completely.

2. Resuspend pellet in 100 µL 1x TE (pH 7.5). Add 25 µL 5x PK Buffer and 1.5 µL 22 mg/mL Proteinase K (Roche, catalogue # 745723). Incubate at 42°C for 2 hours.

DNA Recovery – QiaQuick PCR Purification Columns

DNA may be recovered from the 126.5 µL Proteinase K digested sample using QiaQuick PCR Purification columns following the manufacturer's protocol, but eluting with 100 µL Sigma water. Sample is then dried down to 7 µL using the SpeedVac.

Immunoprecipitated DNA Amplification

DNA amplification is based on the Brown Lab Protocol (previously found at: http://www.microarrays.org/pdfs/BeadBeat_IP.pdf)

- wear gloves throughout protocol as you will amplify any DNA that is present/introduced
- enzyme is available from US Biochemical, Sequenase Kit Ver. 2.0 (USB cat # US 70770)

- Primer A: GTT TCC CAG TCA CGA TCN NNN NNN NN
- Primer B: GTT TCC CAG TCA CGA TC

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Round A :

1. Prepare Round A Setup and Reaction Mix.

Arabidopsis Control DNA

During slide scanning, signals from the experimental (+antibody) and reference (– antibody) samples may be normalised using the Arabidopsis Control Spots printed on the array. To do this it is necessary to add a quantity of Arabidopsis Control DNA directly to the Round A Setup master mix for all samples to be amplified and labelled. A stock of Arabidopsis Control DNA is created by amplification of the Arabidopsis Chlorophyll Synthetase insert from a vector produced in-house. The product is size confirmed on an agarose gel, excised and purified using the GFX PCR DNA and Gel Band Purification Kit following manufacturer's protocol.

- Arabidopsis Control Primer sense: GAG CCA TAT CGT CCA ATT CC
- Arabidopsis Control Primer antisense: GTT TCG GTG CCA AAA GCT AC

The amount of Arabidopsis Control DNA to be added to the Round A Setup master mix is determined by the number of amplification cycles needed to give a good signal from the experimental samples while avoiding saturating the Control Spots. Typically, for amplifications of 22 cycles or so, 1 ng of Arabidopsis Control DNA added to the Round A Setup mix (0.25 ng per Round B amplification reaction) is sufficient.

Round A Setup (per IP):	µL
DNA (can dry down more)	7
5X Sequenase buffer	2
Primer A (100 pmol/ µL)	0.6
Arabidopsis Control DNA (optional)	1 ng
Water (if necessary)	to a final volume of 10 µL
Total Volume	10

Round A Reaction Mix	1 rxn (µL)	2.5 rxn (µL)
5x Sequenase Buffer	1	2.5
DNTP's	1.5	3.75
DTT	0.75	1.875
BSA	1.5	3.75
Sequenase	0.3	0.75
Total volume	5.05	5.05 µL aliquots

2. Incubate Round A Setup at 94°C for 2 minutes. Go to 10°C and hold for 5 minutes while adding Reaction Mix.

3. Following the 10°C hold, ramp up to 37°C over 8 minutes.

4. Hold at 37°C for 8 minutes; go to 94°C for 2 minutes.

5. Go to 10°C and hold for 5 minutes while adding 1.2 µL of diluted Sequenase (dilute 4-fold with Sequenase Dilution Buffer).

6. Following the 10°C hold, ramp up to 37°C over 8 minutes.

7. Remove Round A and add 43.75 µL RNAse/DNAse-free water (Sigma, catalogue # W4502). This brings the template volume up to 60 µL.

Round B:

1. Prepare Round B Setup in PCR tube.

Round B Setup:	1 rxn (μL)	2.5 rxn (μL)
Round A Template	15	--
MgCl2	8	20
10x PCR Buffer	10	25
50x aa-dUTP/dNTP's	2	5
Primer B (100 pm/μL)	1	2.5
Taq	1	2.5
Water	63	157.5
Total volume	100	85 μL aliquots + 15 μL sample

2. Amplification/Nucleotide Incorporation Program: 92°C for 30 seconds → 40°C for 30 seconds → 50°C for 30 seconds → 72°C for 1 minute x optimal cycle number determined by the researcher.

Round B Purification Using CyScribe™ GFX™ Purification Kit
PCR Amplified DNA is purified using the CyScribe™ GFX™ Purification Kit (Amersham, catalogue # 27-9602-02). The Wash Buffer and Elution Buffer provided with the CyScribe™ kit contain tris. Since tris contains primary amines that will interfere with the dye-coupling reaction, 80% ethanol is substituted for the Wash Buffer and DNA must be eluted with 0.1 M sodium bicarbonate (pH 9.0), NOT water!

1. Place GFX column in a collection tube (one column per sample).

2. Add 500 μL Capture Buffer to the GFX column.

3. Transfer the 100 μL PCR amplification reaction to the GFX column.

4. Mix thoroughly by pipetting sample 4 – 6 times.

5. Centrifuge at 13, 800 x g for 30 seconds.

6. Discard flow-through by emptying collection tube. Place the GFX column back into the collection tube.

7. Add 600 µL of 80% ethanol to the column. Centrifuge at 13, 800 x g for 30 seconds. Repeat this wash step twice more for a total of three washes. Empty the collection tube and spin column for an additional minute to remove all traces of 80% ethanol.

8. Discard the collection tube and transfer GFX column to a fresh 1.5 mL microfuge tube.

9. Apply 60 µL of 0.1 M sodium bicarbonate (pH 9.0) directly to the top of the glass fiber matrix in the GFX column, ensuring that it is completely covered.

10. Incubate sample at room temperature for 5 minutes.

11. Centrifuge sample at 13, 800 x g for 1 minute to recover the purified DNA.

12. To reduce the sample volume to 8 µL for the labelling reaction, SpeedVac for approximately 20 minutes at medium heat.

Should it be necessary, the purified aminoallyl-labelled DNA may be frozen at – 20°C before concentration and the protocol continued the next day.

Labelling Reaction with Alexa Fluor Reactive Dyes

1. Just prior to use, resuspend one vial of Alexa 647 or Alexa 555 dye (Molecular Probes catalogue # A-32756) in 2 µL 100% DMSO (Sigma, catalogue # D-5879). Vortex for 10 seconds to ensure that dye is completely dissolved.

2. Add 8 µL aminoallyl-labelled DNA to the resuspended fluor and vortex briefly to ensure that reaction is well mixed. DO NOT spin sample down to bottom of tube, but let it settle by gravity instead.

3. Allow the reaction to incubate in the dark at room temperature for 1 hour.

Purification of Fluorescently Labelled Probe Using CyScribe™ GFX™ Purification Kit

Following dye-coupling, samples are purified separately using the CyScribe™ GFX™ Purification Kit (Amersham catalogue # 27-9606-02)

1. Add 90 µL RNAse/DNAse-free water (Sigma, catalogue # W4502) to each labelling reaction to bring volume up to 100 µL.

2. Place GFX column in a collection tube (one column per sample).

3. Add 500 µL Capture Buffer to the GFX column.

4. Transfer the 100 µL labelled sample to the GFX column.

5. Mix thoroughly by pipetting sample 4 – 6 times.

6. Centrifuge at 13, 800 x g for 30 seconds.

7. Discard flow-through by emptying collection tube. Place the GFX column back into the collection tube.

8. Add 600 µL of 80% ethanol to the column. Centrifuge at 13, 800 x g for 30 seconds. Repeat this wash step twice more for a total of 3 washes. Empty the collection tube and spin column for an additional minute to remove all traces of 80% ethanol.

9. Discard the collection tube and transfer GFX column to a fresh 1.5 mL microfuge tube.

10. Apply 60 µL of 0.1 M sodium bicarbonate (pH 9.0) directly to the top of the glass fiber matrix in the GFX column, ensuring that it is completely covered.

11. Incubate the GFX column at room temperature for 5 minutes. Centrifuge at 13, 800 x g for 1 minute to collect the purified labelled DNA. At this point paired reactions may be pooled together.

12. To reduce the 120 µL combined eluent volume to 5 µL for hybridisation, SpeedVac for approximately 35 minutes at medium heat.

Hybridisation

1. Prepare 100 µL of hybridisation solution per slide. To each 100 µL of DIG Easy Hyb solution (Roche, catalogue # 1 603 558) add 5 µL of 10 mg/mL calf thymus DNA (Sigma, catalogue # D8661) and 5 µL of 10 mg/mL yeast tRNA (Invitrogen, catalogue # 15401-029). Mix thoroughly avoiding formation of bubbles and incubate mixture at 65 °C for two minutes. Cool to room temperature (at least 2 minutes).

2. Add 85 µL of hybridisation solution to the pooled fluor-labelled DNA. Mix and incubate the solution at 65 °C for two minutes. Cool to room temperature (at least 2 minutes).

3. Pipette the labelled-DNA/hybridisation solution onto an array. Place a 24 x 60 mm glass coverslip (Corning, catalogue # 48396-160) onto the droplet, angling it in such a way that any bubbles are pushed out from underneath as the coverslip settles. Place the loaded array into a hybridisation chamber (microscope boxes using slides as rails to support the arrays). Hybridisation chambers contain a small amount of DIG Easy Hyb solution in the bottom to maintain a humid environment.

4. Incubate on a level surface in a 37°C incubator for 8-18 hours.

Day Four:

Washing CpG arrays

Use MilliQ water to prepare wash and rinse solutions.

1. Preheat three slide staining boxes containing a wash solution of 1x SSC and 0.1% SDS (50 mL of 20x SSC stock, 10 mL of 10% SDS, bring to 1L with milliQ water) by allowing to incubate 15 minutes

in 50 °C water bath. Also, prepare one staining box containing a staining rack filled with 1x SSC, and two other boxes containing 0.1x SSC for the final rinses. These boxes are kept at room temperature.

2. Open the hybridisation chamber and remove the coverslip by gently but quickly dipping the array into the slide box containing 1x SSC, allowing the coverslip to slide off on its own. Place slide into the staining rack (up to four slides) and agitate several times before transferring to box containing incubating wash solution. Agitate, then incubate for 8 minutes. Agitate, then incubate for an additional 8 minutes, agitate and transfer to the next wash box. Repeat with the next two wash boxes.

3. Transfer the staining rack into a staining box containing 0.1x SSC at room temperature. Rinse the slides by agitating briskly about 10 times in each of the rinse boxes and quickly remove rack.

4. Remove slide from rack, tap off excess rinse solution and quickly transfer to a microscope box lined on the bottom with blotting paper. Spin slides dry at 80 x g for 15 minutes. Store arrays in the dark until scanned. It is recommended that arrays be scanned as soon as possible after they are washed (at least within two days).

Reagents & Solutions for Chromatin Immunoprecipitation Protocol for Microarray Analysis

Protease Inhibitor Cocktail (100x):
50 mM AEBSF (Sigma, catalogue # A-8456) dissolved in DMSO
100 µg/mL aprotinin (Sigma, catalogue # A-1153) stored at - 20°C,
100 mM benzamidine (Sigma, catalogue # B-6506) store at 4°C once thawed
1 mg/mL leupeptin (Sigma, catalogue # L-2884)
1 mg/mL pepstatin (Sigma, catalogue # P-5318)

1x PBS + 0.5 mM EDTA: 2 x 15 mL washes required per 150 mm dish:
Add 500 µL 0.5M EDTA to 500 mL 1x PBS to yield approximately 0.5 mM final concentration of stock for washing adherent cells.

1x PBS + 0.5 mM EDTA with protease inhibitors:1.5 mL per dish to harvest cells:
Add 15 µL 100x protease inhibitor cocktail to 1485 µL 1x PBS + 0.5 mM EDTA

RIPA Lysis Buffer:
0.1% SDS (Biorad, catalogue # 161-0302)
1% sodium deoxycholate (Sigma, catalogue # D-5670)
150 mM NaCl (Sigma, catalogue # S-7653)
10 mM NaPO4 (pH 7.2) (Sigma, catalogue # S-9638)
2 mM EDTA
0.2 mM NaVO3 (Sigma, catalogue # S-6508)
1% IGEPAL CA-630 (Sigma, catalogue # I-3021)

Prepare 210 µL per dish/cell pellet by adding 2.1 µL 100x protease inhibitor cocktail to 207.9 µL RIPA Lysis Buffer

Protein A Sepharose/1x TE (pH 8.0) – 50% Slurry:
Each IP reaction will require 40 µL (preclearing) + 30 µL (recovery) of Protein A Sepharose beads (Amersham, catalogue # 17-0974-01)

Determine total required amount and add a little extra to allow for pipeting error. Wash up to 1 mL of beads three times in 1 mL 1x TE (pH 8.0) by vortexing briefly and then spinning down at 2000 rpm for 2 min. Discard the supernatant. Following last wash add an equal volume of 1x TE to yield a 50% final slurry. Prepare fresh for each experiment as slurry lasts 4 days at 4 °C.

Low Salt Wash Buffer: 0.1% SDS
1% Triton X-100 (Sigma, catalogue # T-9284)
2 mM EDTA
150 mM NaCl
20 mM Tris-HCl (pH 8.0)

High Salt Wash Buffer: 0.1% SDS
1% Triton X-100
2 mM EDTA
500 mM NaCl
20 mM Tris-HCl (pH 8.0)

LiCl Wash Buffer: 0.25 M LiCl (Sigma, catalogue # L-4408)
1% IGEPAL CA-630
1% sodium deoxycholate
1 mM EDTA
10 mM Tris-HCl (pH 8.0)

Elution Buffer: 1% SDS
0.1 M NaHCO3 (Sigma, catalogue # S-6297)

Round A Stocks:
dNTP's : dATP, dGTP, dCTP, dTTP = 3 mM (Invitrogen, catalogue # 10297-018)
DTT: 0.1 M (Invitrogen, Part # Y00147)
BSA: 500 µg/mL (New England Biolabs, catalogue # B900IS)
Sequenase: 13 U/µL (USB, catalogue # 70775Z)

Round B Stocks:
10x PCR Buffer: 500 mM KCl (Sigma, catalogue # P-9333)
100 mM Tris (pH 8.3)
MgCl2: 25 mM (Sigma, catalogue # M-2670)
Taq: 5 U/µL

50x aa-dUTP/dNTP: dATP, dGTP, dCTP = 25 mM each
dTTP = 10 mM
aa-dUTP = 15 mM (Sigma, catalogue # A-0410 or Enzo cat. # 42861)