Chromatin Immunoprecipitation Assay Protocol

User Guide

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Chapter 1 Overview

Introduction

The Chromatin Immunoprecipitation (ChIP) Assay is designed to generate double-stranded labeled DNA targets that identify sites of protein-DNA interactions or chromatin modifications on a genome-wide scale. This assay has been designed specifically for use with GeneChip™ Tiling Arrays for ChIP-on-chip studies in order to study transcription factor binding sites, histone protein modifications, and other chromatin-protein interactions.

ChIP experiments can be used as a powerful tool to complement RNA transcription studies because they enable researchers to study the DNA-protein interactions that regulate gene expression. Following the protocol, cells are first fixed with formaldehyde to crosslink DNA to any associated proteins. The cells are then lysed and DNA is sheared into smaller fragments using sonication. Protein-DNA complexes are then immunoprecipitated with an antibody directed against the specific protein of interest. Following the immunoprecipitation, crosslinking is reversed, samples are protease-treated and the purified DNA sample is amplified using a random-primed PCR method. Subsequently, targets are fragmented and labeled to hybridize onto GeneChip™ Tiling Arrays. By comparing the hybridization signals generated by an immunoprecipitated sample versus an antibody-negative or non-specific antibody control, the regions of chromatin-protein interaction can be identified.

Studies were performed to evaluate the robustness and sensitivity of the ChIP assay; however, because of the variability associated with the quality and affinity of various antibodies against their intended targets, results may vary from one antibody to the next. The procedure outlined in this protocol describes all the necessary steps and reagents for fixing cells, fragmenting chromatin, immunoprecipitating sheared chromatin, amplifying and labeling precipitated DNA.

We would like to acknowledge Mark Biggin and Xiao-Yong Li of the Lawrence Berkeley National Lab for sharing their modifications to the ChIP protocol. We have incorporated their improvements to the amplification step with their approval.

Chromatin Immunoprecipitation Assay Protocol Optimization

This protocol has been developed for use with GeneChip™ Tiling Arrays. Exact protocol conditions will require optimization by each user due to the variability inherent in:

- Experimental Biology: cell types, proteins of interest, antibodies
- Assay conditions: DNA fragmentation, PCR conditions

To ensure success with this protocol, it is critical that users optimize the following steps in the ChIP protocol prior to performing microarray hybridizations. Additional information on the optimization steps are available throughout this protocol.

1. Sonication conditions of fixed cells.

Some cells are resistant to sonication treatment. Micrococcal nuclease treatment may improve DNA shearing for some cell lines.

2. Antibody Qualification.

Antibodies should be qualified for use with chromatin immunoprecipitation experiments. ChIP qualification information is available from www.chiponchip.org or directly from antibody vendors.

3. Antibody Titration.

Antibody affinities and avidities can vary, so the amount of antibody may need to be titrated to achieve optimal sample enrichment.

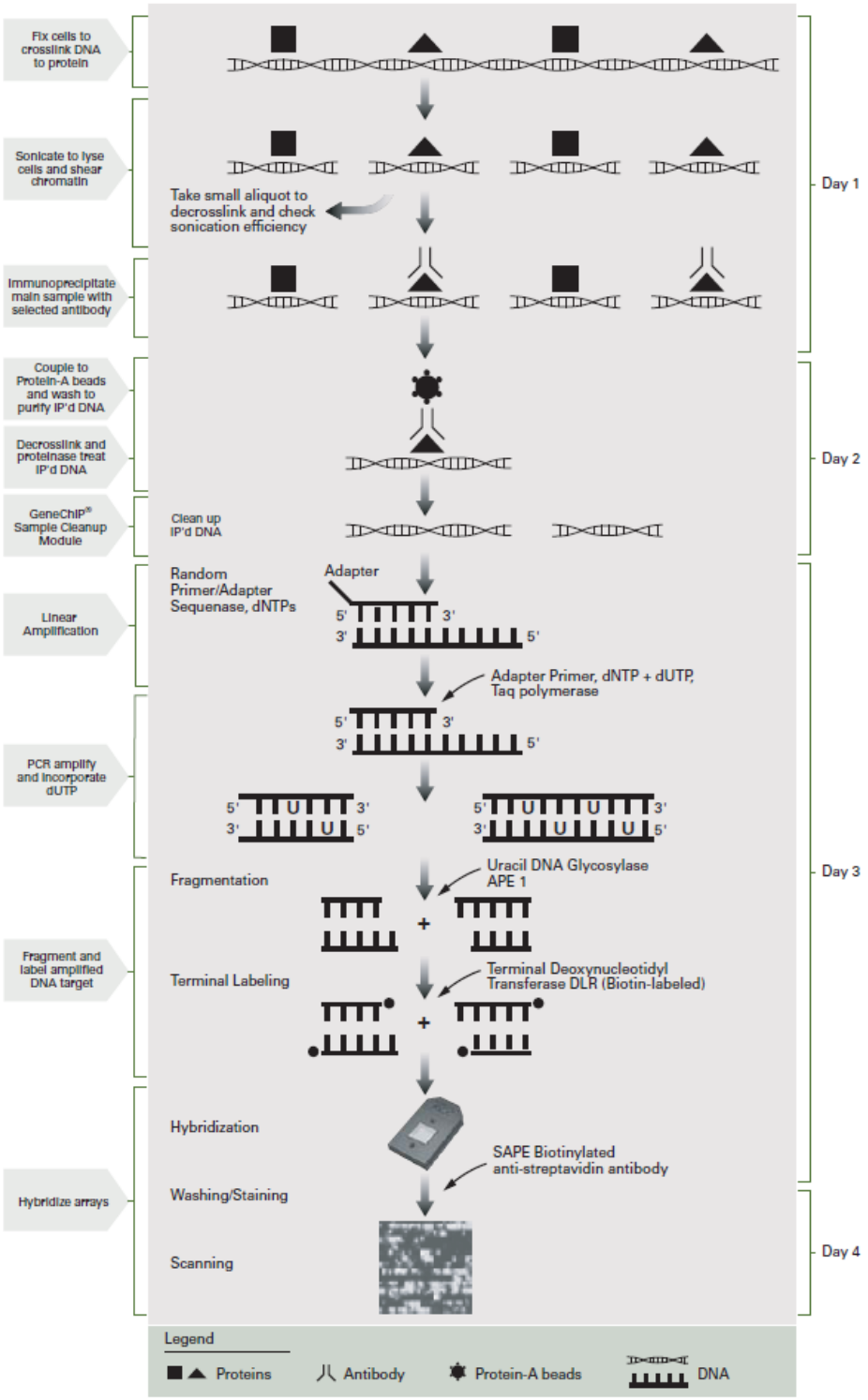
4. PCR amplification of immunoprecipitated DNA.

The optimal number of PCR cycles may require optimization to avoid saturation and ensure that the IP enrichment is maintained.

5. QPCR Positive Control.

A QPCR control is recommended to test ChIP conditions. This test requires a known protein binding to a known DNA sequence. After performing ChIP with an antibody to the known protein, QPCR is used to verify that the known DNA binding elements are enriched in experimental vs. negative control samples. This QPCR test can also be used to ensure that the enrichment of experimental samples vs. control samples is maintained after IP column clean up.

Figure 1.1 Chromatin Immunoprecipitation Assay Schematic



Materials

Table 1.1 Materials Required

Material	Source	Part Number
Formaldehyde Solution (37%), 500 mL	Sigma-Aldrich	F8775
Glycine, 1 kg	Sigma-Aldrich	50046
Phosphate Buffered Saline (PBS) pH 7.4 (1X), liquid	Various	
IGEPAL™ CA-630	Sigma-Aldrich	9036-19-5
Phenylmethanesulfonyl Fluoride Solution (PMSF), 250 mL	Sigma-Aldrich	93482
Microccocal Nuclease (MNase) (Optional)	USB	70196Y
EGTA (optional)	Sigma-Aldrich	E3889-100G
Protease Inhibitor Tablet	Roche	11873580001
Decrosslink and Check Sonication Efficiency		
Proteinase K	New England BioLabs	P8102S
LiCl (8M), 500 mL	Sigma-Aldrich	L7026
Glycogen	Roche	10901393001
Immunoprecipitation		
Triton-X100 (non-ionic viscous liquid)	Roche	10789704001
Protein A Sepharose™ CL-4B	Amersham	17-0963-03
Antibody NOTE: Antibody should be qualified for chromatin immunoprecipitation. See www.chiponchip.org for a list of qualified antibodies.	Various	
PCR Amplification		
Sequenase™ Version 2.0 DNA Polymerase	USB	70775Y
Primer A: 200 µM GTTTCCCAGTCACGGTC(N)9	Various	HPLC purified
Primer B: 100 µM GTTTCCCAGTCACGGTC	Various	HPLC purified
Taq Polymerase 5 U/µL	Various	

Material	Source	Part Number
10X PCR Buffer	Various	
dATP 100 mM	Various	
dCTP 100 mM	Various	
dGTP 100 mM	Various	
dTTP 100 mM	Various	
dUTP 100 mM	Various	
BSA 20 mg/mL	Various	
DTT 0.1M	Various	
Material	**Source**	**Part Number**
Wash Buffer		
Tris-HCl	Various	
EDTA	Various	
SDS, 100g	Sigma-Aldrich	71725
NaCl	Various	
Deoxycholate (sodium salt), 100g	Sigma-Aldrich	D6750
$MgCl_2$, 1M	Various	
$CaCl_2$, 1M	Sigma-Aldrich	21115
Fragmentation and Labeling		
Uracil-DNA Glycosylase (UDG) (2 U/µL)	USB	71960
Human Apurinic/Apyrimidinic Endonuclease 1 (APE 1) (Includes 10X APE 1 Reaction Buffer)	USB	78454
Terminal Deoxynucleotidyl Transferase (rTdT), Recombinant, (30 U/µL) (5X TdT buffer included)	USB	72033
DNA Labeling Reagent, DLR, 10 mM	USB	79015

Material	Source	Part Number
DNA Cleanup		
PrepEase DNA Clean-Up Kit	USB	78758
Hybridization, Stain and Wash		
GeneChip™ Hybridization, Wash, and Stain Kit	Thermo Fisher	900720
Control Oligonucleotide B2, 3nM	Thermo Fisher	900301

Buffers

Table 1.2 Buffers

Lysis Buffer (Store at 4°C)
10 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5) 10 mM NaCl 3 mM $MgCl_2$ 0.5% IGEPAL 1 mM PMSF (add fresh)
Pre-IP Dilution Buffer (Store at RT)
10 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5) 10 mM NaCl 3 mM $MgCl_2$ 1 mM $CaCl_2$ 4% IGEPAL 1 mM PMSF (add fresh)
IP Dilution Buffer (Store at RT without protease inhibitors)
20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 2 mM EDTA 1% Triton X-100 150 mM NaCl Protease Inhibitor Stock (add fresh)
Protease Inhibitor Stock
Prepare a 25X stock by dissolving 1 protease inhibitor tablet in 2 mL of nuclease-free water
ChIP Wash 1 (Store at RT)
20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 2 mM EDTA 1% Triton X-100 150 mM NaCl 1 mM PMSF (add fresh)

ChIP Wash 2 (Store at RT)
20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 2 mM EDTA 1% Triton X-100 0.1% SDS 500 mM NaCl 1 mM PMSF (add fresh)
ChIP Wash 3 (Store at RT)
10 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 1 mM EDTA 0.25M LiCl 0.5% IGEPAL 0.5% Deoxycholate (sodium salt)
Elution Buffer
25 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5) 10 mM EDTA 0.5% SDS

Miscellaneous Reagents and Supplies

Table 1.3 Miscellaneous Reagents and Supplies

Material	Supplier	Part Number
Miscellaneous Reagents		
Absolute ethanol	Gold Shield Chemical Co.	
RNA-6000 Nano LabChip Kit	Agilent	5065-4476
Gel-Shift Assay (Optional)		
Novex XCell SureLock™ Mini-Cell*	Invitrogen	EI0001
TBE Gel, 4-20%,10 mm, 12 well*	Invitrogen	EC62252
5X Sucrose Gel Loading Dye	Amresco	E-274
10X TBE Buffer	Cambrex	50843
SYBR™ Gold	Invitrogen	S-11494
10 bp DNA ladder and 100 bp DNA ladder	Invitrogen	10821-015 and 15628-019
ImmunoPure NeutrAvidin	Pierce	31000
PBS, pH 7.2	Invitrogen	20012-027
Miscellaneous Supplies		
1-2% Agarose Gells	Various	
1.5 mL RNase-free Microfuge Tubes*	Ambion	12400

Material	Supplier	Part Number
1.5 mL Non-stick RNase-free Microfuge Tubes*	Ambion	12450
0.2 mL MicroAmp Reaction Tubes (8 tubes/strip)*	Applied Biosystems	N801-0580
MicroAmp Caps for 8 Strip Tubes	Applied Biosystems	N801-0535
Pipette for 25 mL*	VWR	53283-710
Pipet-Aid*	VWR	53498-103
Dolphin-nose Tubes	Costar (Corning)	3213
SpinX Columns	Costar (Corning)	8163
MicroSpin™ S-300 HR Columns	GE Healthcare	27-5130-01
Instruments		
Rotating Benchtop Platforms	Various	
Branson Sonifier™S-450D	Branson Ultrasonics	101-063-590
Double Step Micro Tip Assembly	Branson Ultrasonics	101-063-212
NanoDrop™ND-1000*	Nanodrop Technologies	ND-1000
GeneChip™Hybridization Oven 640	Thermo Fisher	8001318
Eppendorf Centrifuge*	Eppendorf	5417C
Refrigerated Centrifuge with swing bucket rotor	Various	
Tube-Strip Picofuge™	Stratagene	400540
GeneChip™Fluidics Station 450 or 400	Thermo Fisher	00-0079
GeneChip™ Scanner 3000 7G	Thermo Fisher	00-0073
GeneChip™Autoloader (Optional)	Thermo Fisher	90-0351
ABI GeneAmp PCR System 9700*	Applied Biosystems	N/A
Bioanalyzer 2100	Agilent	G2940CA
Heating Block*	VWR	13259-030
Pipette for 0.1 to 2 µL*	Rainin	L-2
Pipette for 2 to 20 µL*	Rainin	L-20
Pipette for 20 to 200 µL*	Rainin	L-200
Pipette for 100 to 1,000 µL*	Rainin	L-1000

** Or equivalent instrument/supplies.*

Safety Information

WARNING: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

CAUTION: All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.

Chapter 2
Chromatin Immunoprecipitation Assay

Procedure A: Prepare Cells

1. Grow enough cells for the number of immunoprecipitation (IP) reactions to be performed (usually 5 x 10^7 cells per IP for suspension cells, depending on IP efficiency). Prepare enough cells for two IP reactions. An antibody-minus (Ab- or mock IP) or non-specific IgG is recommended as a negative control using the same number of cells as the IP condition. The Ab- target would be treated identically to the experimental sample to serve as the "Control" group in the downstream two-sample analysis.
2. Use ~ 0.5 - 2 x 10^8 cells per IP. For example, grow 200 mL of 1 x 10^6 cells/mL for a total of 2 x 10^8 cells.

Procedure B: Fix Cells, Lyse, and Sonicate Whole Cell Extracts

DAY 1

NOTE: Centrifugation steps involving cells are best performed with a swing-bucket type rotor.

Adherent Cells

NOTE: End users may optimize the sequence of fixing and harvesting cells to minimize the degree to which cell physiology is disrupted.

1. Add formaldehyde to the culture flask to a final concentration of 1% and incubate in a fume hood for 10 minutes.
2. Add 1/20 volume of 2.5 M glycine and incubate at room temperature (RT) for 5 minutes with gentle mixing.
3. Pour off formaldehyde media into an appropriate waste container and add enough ice-cold 1X PBS to cover the bottom of the flask to wash cells. Pour off PBS into a formaldehyde waste container and add enough PBS to cover bottom of flask.
4. Using a cell scraper, scrape off cells to re-suspend and check flask with microscope to ensure that most cells are re-suspended.
5. From here, go to Step 1 of the *Wash Cell Pellet* section below.

Suspension Cells

1. Fix cells by adding formaldehyde to a final concentration of 1% (add 5.5 mL of 37% formaldehyde to 200 mL of culture medium).
2. Incubate at room temperature (RT) in fume hood for 10 minutes, gently swirl 200 mL culture or invert tube containing 20 mL of adherent cells occasionally to mix cells.
3. Add 1/20 volume 2.5 M glycine and incubate at RT 5 minutes with gentle mixing to quench formaldehyde reaction. Perform remaining steps on ice.
4. Pellet cells at 4°C, (300-500g), 4 minutes and discard supernatant in formaldehyde waste.

Wash Cell Pellet

1. Wash pellet with 10 mL ice-cold 1X PBS to resuspend cells, and transfer to 15 mL tube.
2. Pellet cells at 4°C, (300-500g), 4 minutes and discard supernatant and repeat wash with ice-cold 1X PBS once.
3. Wash the pellet 3 times with 10 mL Lysis Buffer with fresh PMSF. Pellet cells at (300-500g) 5 minutes between washes.
4. Discard supernatant and proceed to the next step or flash freeze pellet and store at –80°C.
5. Resuspend the pellet in 1 mL pre-IP dilution buffer (add 60 µL PMSF) and bring final reaction volume to 1.5 mL with pre-IP dilution buffer.
6. Add to the tube:

Table 2.1

Component	Volume for 1 Rxn
100 mM PMSF	40 µL
25X Protease Inhibitor Stock	100 µL
Pre-IP Dilution Buffer	460 µL
20% SDS	100 µL
5 M NaCl	80 µL
Nuclease-free Water	220 µL*
Final Sample Volume Before Sonication	2.5 mL

** if using optional MNase, see details in the next page.*

7. Sonicate sample to lyse cells and shear DNA to 100-1000 bp fragments. Some cell types (e.g., Jurkat) may require optional MNase treatment.

NOTE: Optimized shearing conditions are cell-type and instrument dependent. It is recommended that conditions are optimized with a single sample prior to scaling up procedures to multiple samples. Best sonication conditions at Thermo Fisher were achieved with a Branson Sonifier 450D (using a double-step microtip) set at 60% duty, 50% amplitude, 1 minute pulses with 1 minute rest. Both pulsing and resting steps were performed in an ice bath, 8 to 10 pulses total for HL-60 cells. Number of pulses may be dependent on cell density as well as cell type.

8. Aliquot the sonicated samples into two 1.5 mL microcentrifuge tubes, then microcentrifuge 14,000 rpm 10 minutes at 4°C to remove cellular debris.

9. Pool supernatants (from Step 8) in a 15 mL conical tube.

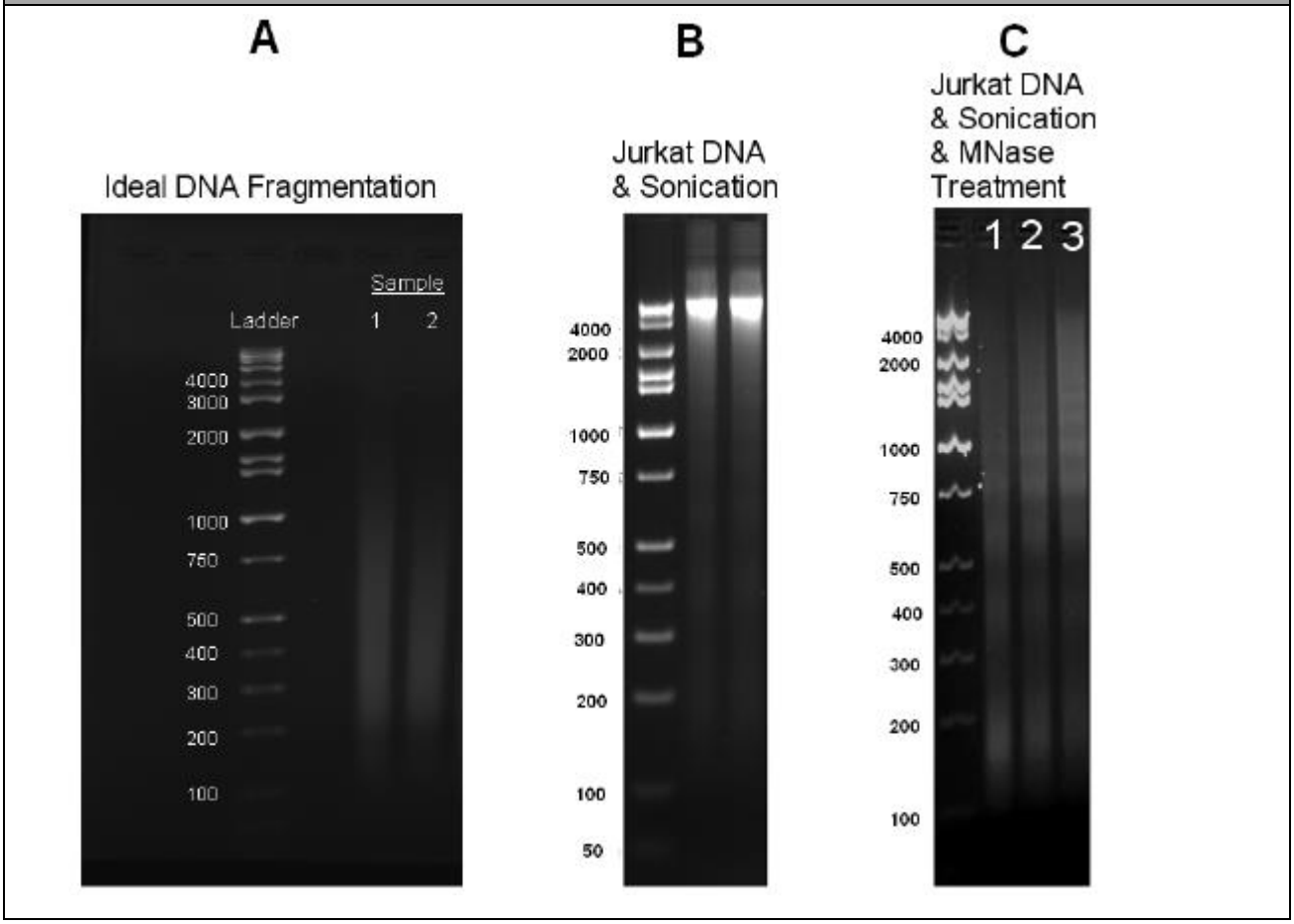
10. The sonication efficiency can be checked by taking an aliquot (100 µL) of this supernatant, de- crosslinking it (see Procedure C, below), and running the de-crosslinked DNA on a 1-2% agarose gel.

11. Divide the samples into aliquots equivalent to ~ 5 x 10^7 cells (1 IP), flash freeze and store at –80°C for later use or take straight through the IP.

Procedure C: Check Sonication Efficiency

1. Add 100 µL 10 mM Tris pH 8.0 to the 100 µL aliquot taken from the sonicated samples.
2. Add 2 µL Proteinase K (20 mg/mL) and mix well by vortexing.
3. Incubate 42°C for 2 hours, then 65°C for 6 hours to overnight (This step can be performed in a thermocycler.)
4. Clean-up using USB PrepEase DNA Clean-Up Kit (see *Cleanup of Double-Stranded DNA*).
5. Load 100-500 ng of purified DNA sample on an agarose gel to check sonication efficiency. Typically, sheared DNA size ranges from 100-4000 bp, with the average size fragment between 200-1000 bp.

Figure 2.1 (A) Sheared DNA from HL-60 cells following 8 sonication pulses show the optimal size range for immunoprecipitation (~200-1000 bp with the majority of DNA fragments between 300-500 bp). Certain cell types may be more resistant to shearing by sonication and would require treatment with Micrococcal nuclease (MNase) to fragment chromatin. (B) Jurkat cells after 15 pulses of sonication show little fragmentation of crosslinked chromatin. (C) Fragmentation of Jurkat chromatin is achieved with MNase treatment. MNase enzyme concentration may have to be titrated based on cell type and density, lane1: 200U, lane2: 100U, lane3: 25U. The 'laddering' phenomenon seen with MNase treatment is common due to the specific cleavage of DNA by MNase between nucleosomes.



NOTE: Optional DNA Shearing Method Micrococcal Nuclease Treatment

1. **Add appropriate units of MNase based on prior optimization of MNase to effectively shear crosslinked chromatin. This can range from 25U to 200U or more for each IP performed.**
2. **Incubate at 37°C, 10 minutes.**
3. **Add 30 µL 200 mM EGTA to stop the reaction.**

Procedure D: Incubate With Specific Antibody

1. If the sample (from Procedure B Step 11) was frozen, thaw.
2. Transfer supernatant to a 15 mL tube and add 5 volumes of IP dilution buffer containing protease inhibitors (tablet from Roche, add before use).
3. Pre-equilibriate protein A Sepharose™ beads by washing 100 µL beads with 1 mL IP dilution buffer, pellet cells by centrifuging for 2 minutes at 2,000 rpm at 4°C in a microcentrifuge. Remove ~ 800 µL supernatant.
4. Pre-clear chromatin by adding 200 µL pre-equilibrated Protein A Sepharose beads.
5. Incubate on a rotating platform at 4°C for 30 minutes.
6. Centrifuge at 2,000 rpm for 2 minutes at 4°C in a swinging bucket rotor.
7. Transfer supernatant to a new 15 mL tube and discard beads.
8. Add 10 to 15 µg of antibody per IP. Usually, a negative control is performed using the same number of cells with a non-specific IgG or no antibody (mock IP) control.

NOTE: The amount of antibody to be added is dependent on quality, affinity, specificity, and type of antibody used. Users may have to titrate the amount of antibody used for each IP.

9. Incubate on rotating platform at 4°C overnight (or for at least 3 hours at RT).

DAY 2

Procedure E: Immunoprecipitate and Wash

1. Pre-equilibrate protein A Sepharose™ beads by adding 1 mL IP Dilution Buffer and 200 µL beads for each IP'd sample. Centrifuge 2,000 rpm 2 minutes at 4°C.
2. Discard around 800 µL supernatant: save ~ 400 µL of beads in buffer at the bottom of the tube.
3. Transfer 400 µL beads to each sample.
4. Add PMSF to each tube sample (final concentration 1mM PMSF in final volume).
5. Incubate on rotating platform at RT for 1 to 3 hours.
6. Centrifuge at 2,000 rpm at 4°C for 4 minutes, and then discard supernatant.
7. Resuspend the pellet with 700 µL ChIP wash 1 (containing 1 mM PMSF added fresh), mix and transfer to spin-X column.
8. Incubate on rotating platform at RT for 1 minute.
9. Centrifuge at 2,000 rpm at RT for 2 minutes and discard flow-through.
10. Repeat steps 7–9.
11. Wash the beads with 700 µL ChIP wash 2 (containing 1 mM fresh PMSF).
12. Incubate on rotating platform at RT for 5 minutes.
13. Centrifuge at 2,000 rpm at RT and discard flow-through.
14. Wash the beads with 700 µL ChIP wash 3.
15. Incubate on rotating platform at RT for 5 minutes.
16. Centrifuge at 2,000 rpm at RT and discard flow-through.
17. Wash the beads with 700 µL TE (10 mM Tris-HCl pH 8, 1 mM EDTA).
18. Incubate on rotating platform at RT for 1 minute.
19. Centrifuge at 2,000 rpm at RT and discard flow-through.
20. Repeat steps 17 through 19.
21. Transfer the spin-X column with beads to a dolphin-nose tube.
22. Add 200 µL Elution Buffer to the column.
23. Incubate at 65°C for 30 minutes.
24. Centrifuge at 3,000 rpm at RT for 2 minutes.
25. Add 200 µL Elution Buffer to the column.
26. Centrifuge at 3,000 rpm at RT for 2 minutes. This 400 µL eluted sample is the "enriched" or "IP'd" sample.

Procedure F: Reverse Crosslinks

1. Add 5 µL Proteinase K (20mg/mL) per 100 µL of negative control or IP sample, mix well. (20 µL for 400 µL of eluted sample.)
2. Incubate in incubator at 65°C overnight.

DAY 3

Procedure G: Cleanup De-crosslinked Samples

1. USB PrepEase DNA Clean-Up Kit (see *Cleanup of Double-Stranded DNA*).

NOTE: 2. IP efficiency can be checked at this stage in the protocol using polymerase chain reaction (PCR) and designing primer sets against regions that are known to be bound by the protein of interest and immunoprecipitated using the antibody being investigated. A significant increase or enrichment for the specific target should be observed for the IP condition compared to the Ab- control. Using quantitative real-time PCR, Thermo Fisher has routinely obtained >8-fold enrichment for IP samples compared to the Ab- samples.

Procedure H: PCR Amplify Immunoprecipitated DNA Targets

NOTE: Dilute Sequenase™ stock with Sequenase Dilution Buffer (included with enzyme) to 1.3 U/µL. Four microliters of this 1.3 U/µL working stock will be needed for each sample being amplified.

1. Use 10 µL of IP'd or negative control sample for initial round of linear amplification.
2. Set up first round reaction. Set up 1 reaction for single array products (e.g., Human Promoter 1.0R Array). Setup 3 reactions for multi-array sets (e.g., Human Tiling 2.0R Array Set).

Table 2.2

Component	Volume for 1 Rxn
Purified DNA	10 µL
5X Sequenase™ Reaction Buffer*	4 µL
Primer A (200 µM)†	4 µL
Total Volume	18 µL

** Included with enzyme.*
† Primer A: GTTTCCCAGTCACGGTC(N) (HPLC purified)

3. Cycle conditions: Random priming.
    1. 95°C for 4 minutes.
    2. Snap cool samples on ice.
    3. 10°C hold.
    4. Prepare first cocktail (Table 2.3).

Table 2.3 First Cocktail

Component	Volume for 1 Rxn
20 mg/mL BSA	0.1 µL
0.1 M DTT	1 µL
25 mM dNTPs	0.5 µL
Diluted Sequenase™ (1/10 from 13 U/µL stock)	1 µL
Total Volume	2.6 µL

5. Add 2.6 µL per sample.
6. Mix well by pipetting, and put the sample back in thermocycler block.
7. 10°C for 5 minutes.
8. Ramp from 10°C to 37°C over 9 minutes.
9. 37°C for 8 minutes.
10. 95°C for 4 minutes.
11. Snap cool on ice.
12. 10°C hold.
13. Add 1.0 µL of 1.3U/µL Sequenase™ to each sample.
14. 10°C for 5 minutes.
15. Ramp from 10°C to 37°C over 9 minutes.
16. 37°C for 8 minutes.
17. Repeat from J) to P) for 2 more cycles.
18. 4°C hold.

4. For each IP, purify with Microspin S-300 HR (GE Healthcare) columns (2 columns per reaction) as follows:
    1. Add 20 µL of 10 mM TE pH 8.0 to each reaction.
    2. Spin 2 columns (A & B) at 3,000 rpm for 1 minute, discard flow-through.
    3. Transfer reaction volume (~ 43 µL) to column A, while equilibrating column B with 300 µL of 10 mM Tris pH 8.0.
    4. Spin both columns at 3,000 rpm for 1 minute, keep flow-through from column A (sample) and discard flow-through of column B (Tris buffer).
    5. Transfer flow-through of column A to column B with new collection tube.
    6. Spin at 3,000 rpm for 2 minutes.
    7. Collect ~ 56 µL of first round purified DNA per reaction.
5. Prepare dNTP/dUTP mix.

Prior to proceeding with the PCR amplification of immunoprecipitated DNA targets, prepare a dNTP mixture containing dUTP at the concentrations indicated below. Please note that this dNTP + dUTP mixture is only required for the PCR amplification reaction outlined in Table 2.4 and not in the Sequenase™ reaction setup in Table 2.3.

dCTP – 10 mM
dATP – 10 mM
dGTP – 10 mM
dTTP – 8 mM
dUTP – 2 mM

Store at –20°C.

6. PCR Mix Setup:

Table 2.4

Component	Volume for 1 Rxn
First-round DNA from Step 4	20 µL
10X PCR Buffer	10 µL
25 mM MgCl2*	3 µL
10 mM dNTPs + dUTP	3.75 µL
100 µM Primer B†	4 µL
5 U/µL Taq Polymerase	2 µL
Nuclease-free Water	57.25 µL
Total Volume	100 µL

** Add $MgCl_2$ if using magnesium-free 10X PCR Buffer.*
† Primer B (GTTTCCCAGTCACGGTC)

7. Cycle conditions:

1. 15 cycles[1]

1) 95°C 30 seconds.

2) 45°C 30 seconds.

3) 55°C 30 seconds.

4) 72°C 1 minute.

2. 15 cycles1

1) 95°C 30 seconds.

2) 45°C 30 seconds.

3) 55°C 30 seconds.

4) 72°C 1 minute.

For every subsequent cycle add 5 seconds.

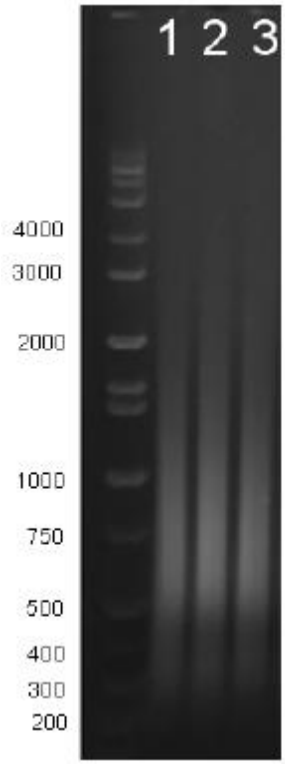
E.g., cycle 1: 60 seconds, cycle 2: 65 seconds, etc...

3. 4°C hold.

8. Check amplified DNA on 1% agarose gel.

[1] *Number of PCR amplification cycles may require optimization. QPCR can be used to evaluate enrichment of immunoprecipitated sample.*

Figure 2.2 PCR-amplified ChIP targets from HL- 60 cells immunoprecipitated with an Sp1 antibody. Replicate PCR reactions (lanes 1 to 3) were performed on the same IP sample and product sizes ranged from 200 bp to over 2 Kb but the actual product sizes may vary depending on original size of sheared chromatin.



9. Purify PCR samples using USB PrepEase DNA Clean-Up kit (see *Cleanup of Double-Stranded DNA*).

10. Measure DNA using a NanoDrop or other UV-vis spectrophotometer. Normally, greater than 9 µg of amplified DNA is obtained from each reaction.

NOTE: Maintenance of IP enrichment post-amplification is crucial in obtaining good array results. QPCR should be performed to post-amplified samples to ensure that differences between the IP and Ab- samples are maintained. Primer sets can be designed for DNA regions that are known to be specifically immunoprecipitated using the antibody of interest.

Procedure I: Fragment Amplified Targets

1. Fragment the samples using the appropriate table below depending on what array type the target will be hybridized to.

Table 2.5 Fragmentation Mix for single arrays (e.g., Human Promoter 1.0R Array) Fragmentation of ds cDNA

Component	Volume/Amount in 1 Rxn
ds cDNA	7.5 µg
Nuclease-free Water	up to 32.2 µL
10X APE 1 Reaction Buffer	4.8 µL
Uracil-DNA Glycosylase (UDG) (2U/ µL)	4.0 µL
Human Apurinic/Apyrimidinic Endonuclease 1 (APE 1) (10 U/µL)	7.0 µL
Total Volume	48.0 µL

Table 2.6 Fragmentation Mix for multi-array sets (e.g., Human Tiling 2.0R Array Set) Fragmentation of ds cDNA

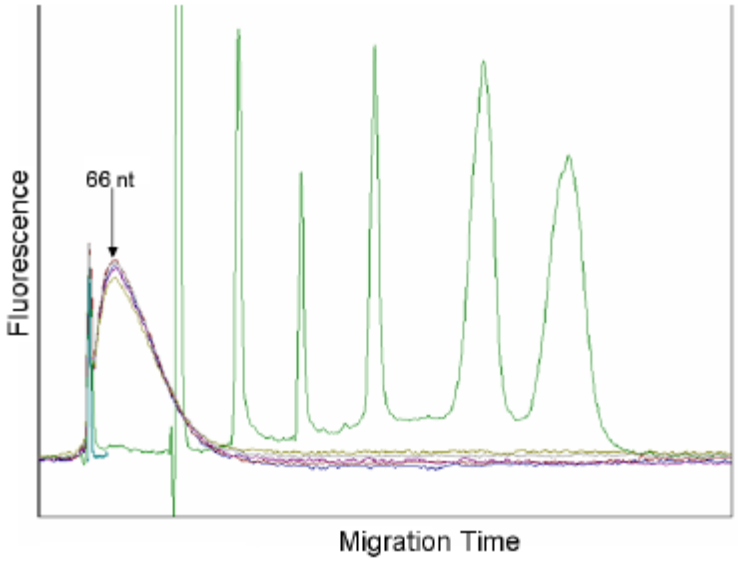
Component	Volume/Amount in 1 Rxn
ds cDNA	9.0 µg
Nuclease-free Water	up to 32.2 µL
10X APE 1 Reaction Buffer	4.8 µL
Uracil-DNA Glycosylase (UDG) (2U/ µL)	4.0 µL
Human Apurinic/Apyrimidinic Endonuclease 1 (APE 1) (10 U/µL)	7.0 µL
Total Volume	48.0 µL

2. Set up fragmentation mix according to either Table 2.5 or Table 2.6) Flick-mix and spin down the tubes.
3. Incubate the reactions at:

- 37°C for 1 hour.
- 93°C for 2 minutes.
- 4°C for at least 2 minutes.

4. Flick-mix, spin down the tubes, and transfer 45 µL of the sample to a new tube.
5. The remainder of the sample is to be used for fragmentation analysis using a Bioanalyzer or agarose gel. Please see the Reagent Kit Guide that comes with the RNA 6000 LabChip Kit for instructions. If not labeling the samples immediately, store the fragmented DNA at –20°C.

Figure 2.3 Bioanalyzer trace of fragmentation products following treatment of amplified ChIP targets with UDG and APE 1. Independently amplified Sp1 IP or Ab- samples from HL-60 cells were fragmented according to the protocol and products were analyzed on an Agilent Bioanalyzer with the RNA 6000 Nano LabChip Kit. Analyzing fragmented DNA on the RNA 6000 LabChip is recommended because it quickly assesses the degree and uniformity of the fragmented products.



Procedure J: Label Fragmented Double-Stranded DNA

1. Prepare the Double-Stranded DNA Labeling Mix as described in Table 2.7.

Table 2.7 Double-Stranded DNA Labeling Mix

Component	Volume in 1 Rxn
5x TdT Reaction Buffer	12 µL
Terminal Deoxynucleotidyl Transferase (rTdT), Recombinant, (30 U/uL)	2 µL
DNA Labeling Reagent, DLR, 10 mM	1 µL
Total Volume	15 µL

2. Add 15 µL of the Double-Stranded DNA Labeling Mix to the DNA samples, flick-mix, and spin them down.

3. Incubate the reactions at:

- 37°C for 60 minutes.
- 70°C for 10 minutes.
- 4°C for at least 2 minutes.

4. Remove 2 µL of each sample for gel-shift analysis (refer to the *GeneChip™ Whole Transcript (WT) Sense Target Labeling Assay Manual*).

Chapter 3
Hybridization and Array Processing

Procedure A: Hybridize Labeled Target on the Arrays

This Procedure requires the use of the GeneChip™ Hybridization, Wash, and Stain Kit (P/N 900720).

1. Prepare the Hybridization Cocktail in a 1.5 mL RNase-free microfuge tube as shown in Table 3.1 and Table 3.2, below depending on what array type the target will be hybridized to.

Table 3.1 Hybridization Cocktail for single tiling arrays (e.g., GeneChip™ Human Promoter 1.0R Array)

Component	Volume in 1 Rxn	Final Concentration or Amount
Fragmented and Labeled DNA Target	~ 60.0 µL*	~ 7.5 µg
Control Oligonucleotide B2	3.3 µL	50 pM
2X Hybridization Mix†	100 µL	1X
DMSO	14.0 µL	7%
Nuclease-free Water	up to 200.0 µL	
Total Volume	200.0 µL	

** This volume is 56 µL if a portion of the sample was set aside for gel-shift analysis.*
† Available in the GeneChip™ Hybridization, Wash, and Stain Kit.

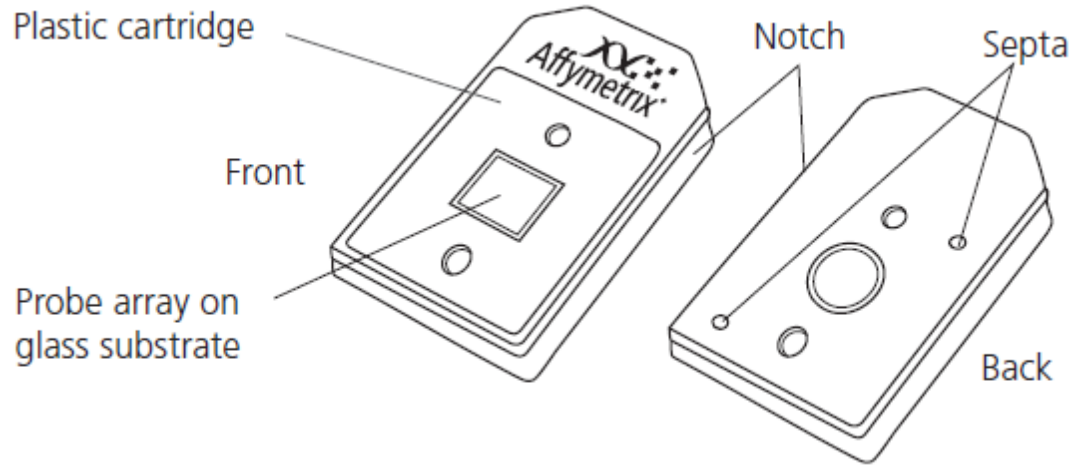
Table 3.2 Hybridization Cocktail for use with serial hybridizations (e.g., GeneChip™ Human Tiling 2.0R Array Set and GeneChip™ Mouse Tiling 2.0R Array Set)

Component	Volume in 1 Rxn	Final Concentration or Amount
Fragmented and Labeled DNA Target	~ 60.0 µL*	~ 9.0 µg
Control Oligonucleotide B2	4 µL	50 pM
2X Hybridization Mix†	120 µL	1X
DMSO	16.8 µL	7%
Nuclease-free Water	up to 240.0 µL	
Total Volume	240.0 µL	

** This volume is 58 µL if a portion of the sample was set aside for gel-shift analysis.*
† Available in the GeneChip™ Hybridization, Wash, and Stain Kit.

2. Flick-mix, and centrifuge the tube.
3. Heat the Hybridization Cocktail at 99°C for 5 minutes. Cool to 45°C for 5 minutes, and centrifuge at maximum speed for 1 minute.
4. Inject ~ 200 µL of the specific sample into the array through one of the septa (see Figure 3.1 for location of the septa on the array). Save the remaining hybridization cocktail in –20°C for future use.
5. Place array in 45°C hybridization oven, at 60 rpm, and incubate for 16 hours.
6. After hybridization, remove the hybridization cocktail for future use.

Figure 3.1 GeneChip™ Probe Array



Procedure B: Array Wash, Stain and Scan

For instructions on array washing, staining and scanning please refer to the *GeneChip™ Expression Wash, Stain and Scan User Manual* (P/N 702731).

Appendix A
Cleanup of Double-Stranded DNA

Cleanup of Double-Stranded DNA

This Step requires the use of the PrepEase™ DNA Clean-Up Kit (PrepEase™ DNA Clean-Up Kit, P/Ns 78758, 78759).

Brief Protocol for Concentration, Desalination and/or Removal of Enzymes

	IMPORTANT: Check that ethanol was added to NT3 Buffer before starting.

1. Adjust DNA binding conditions
    1. Add 5 volumes of N2P Buffer to 1 volume of sample (e.g., 500 µL N2P Buffer and 100 µL sample).
    2. Mix well.
2. Bind DNA sample to column
    1. Place PrepEase™ Clean-Up Column into a 2 mL PrepEase™ Collecting Tube.
    2. Pipet the sample directly into the center of the column.
    3. Centrifuge 1 min at 11,000 x g.
    4. Discard flow-through.
3. Wash column
    1. Add 600 µL NT3 Buffer to column.
    2. Centrifuge 1 min at 11,000 x g.
    3. Discard flow-through. Place column back into collecting tube.
4. Dry column

    Centrifuge 2 min at 11,000 x g.
5. Elute DNA
    1. Place the column into a clean 1.5 ml microcentrifuge tube.
    2. Add 15-50 µL NE Buffer to column.
    3. Incubate at room temperature for 1 min.
    4. Centrifuge 1 min at 11,000 x g.

Brief Protocol for PCR Purification

	IMPORTANT: Check that ethanol was added to NT3 Buffer before starting.

1. Adjust DNA binding conditions
    1. Add 5 volumes of N2P Buffer to 1 volume of sample (e.g., 250 µL N2P Buffer and 50 µL sample).
    2. Mix well.
2. Continue with Step 2 to Step 5 of the *Brief Protocol for Concentration, Desalination and/or Removal of Enzymes*.

Brief Protocol for DNA Purification from Chromatin Immunoprecipitation (ChIP) Assay

!	IMPORTANT: Check that ethanol was added to NT3 Buffer before starting.

1. Adjust DNA binding conditions
    1. Add 5 volumes of N2P Buffer to 1 volume of sample (e.g. 1000 µL N2P Buffer and 200 µL sample).
    2. Mix well.
2. Bind DNA sample to column
    1. Place PrepEase™ Clean-Up Column into a 2 ml PrepEase™ Collecting Tube.
    2. Pipet 700 µL of the sample directly into the center of the column.
    3. Centrifuge 1 min at 11,000 x g.
    4. Discard flow-through.
    5. Repeat Step B to Step D for the remaining sample.
3. Wash column
    1. Add 600 µL NT3 Buffer to column.
    2. Centrifuge 1 min at 11,000 x g.
    3. Discard flow-through. Place column back into collecting tube.
4. Dry column

    Centrifuge 2 min at 11,000 x g.
5. Elute DNA
    1. Place the column into a clean 1.5 ml microcentrifuge tube.
    2. Add 30-40 µL NE Buffer to column.
    3. Incubate at room temperature for 1 min.
    4. Centrifuge 1 min at 11,000 x g.
6. Take 2 µL from each sample to determine the yield by spectrophotometric UV measurement at 260 nm, 280 nm and 320 nm:

    Concentration of Double-Stranded cDNA (µg/µL) = $[A_{260} - A_{320}]$ x 0.05 x dilution factor

    µg DNA = eluate in µL x DNA in µg/µL

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