Chromatin Immunoprecipitation (ChIP)

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1 Introduction

This protocol aims to describe the standard chromatin immunoprecipitation protocol using **Agilent sonication buffer** with sonication in the Covaris sonicator and using **magnetic bead** pull-down. This protocol is standardized for histone and histone modification ChIP, additional considerations may be necessary for application of this protocol to transcription factor or TF co-factor ChIP (*i.e.* consider micrococcal nuclease digestion in place of sonication.

A note on condition optimization:
The two most critical parameters to optimize are the sonication and the fixation conditions, with the former predominating. These conditions should be fixed for a given sample type or cell line but may vary quite significantly across cell/tissue types and should be set up carefully.

2 Required Reagents and Equipment

2.1 Reagents

- Lysis buffer (Covaris)
- Wash buffer (Covaris)
- Wash buffer A
- Wash buffer B
- Sonication buffer (Agilent)
- Blocking buffer
- ChIP IP buffer
- Elution buffer
- Methanol-free formaldehyde
- Glycine (1.25 M)
- PBS
- Dyna Beads (coated with protein A, G or A/G)

2.2 Equipment

- Magnetic bead separation rack (pre-cooled at 4℃)
- Rotating rack stored in 4℃ closet
- Covaris sonication system
- Low-retention pipette tips
- Low-retention 1.5 ml conical tubes

3 Protocol

DAY 1

[*n.b.*] Before beginning, check that all buffers (Covaris lysis, wash, and Agilent sonication) contain fresh protease inhibitor cocktail.

3.1 Crosslinking

1. Harvest cells in log phase growth. Collect a maximum of $50 * 10^6$ cells in 30 ml.
2. Add 2 ml of 16% methanol-free formaldehyde (final 1% formaldehyde). Invert several times and incubate 10' (RT) on a shaking platform.
3. Stop the crosslinking reaction by adding glycine at a final concentration of 0.125 M (3.5 ml/tube from 1.25 M glycine stock). Incubate for 5' (RT) on a shking platform.
4. Centrifuge 5' @ 500 g (4℃), aspirate supernatent and place cells on ice.
5. Wash cells twice in ice-cold PBS with 5'/500 g (4℃) centrifugation.
6. **Optionally freeze fixed cells** **Warning:** it is preferable to continue through the sonication and instead freeze sonicated chromatin.
   (a) Completely aspirate supernatent from fixed cell pellet and snap-freeze in lN_2 or dry-ice ethanol bath.
   (b) Store fixed cell pellet at -80℃.

3.2 Chromatin Sonication

1. Resuspend fixed cell pellet (max $100 * 10^6$ cells) in 10 ml of ice-cold Covaris lysis buffer in a 15 ml conical tube. Incubate rotating 10' (4℃).
2. Centrifuge 5'/4000 rpm (4℃) to pellet nuclei.
3. Wash nuclear pellet (resuspend) in 10 ml ice-cold Covaris wash buffer, incubate rotating for 10' (4℃), then centrifuge 5'/4000 rpm (4℃) to pellet nuclei.
4. Resuspend the nuclear pellet in the appropriate amount of ice-cold Agilent sonication buffer to achieve $10 - 20 * 10^6$ cells/ml.
5. Sonication should be performed uniformly (constant volume, cell concentration, cell number, *etc.*) in up to 1 ml total volume per sonication tube using the Covaris sonicator. Sonication time should be carefully optimized to achieve a fragment length distribution between 200-500 bp.
6. ***Optionally freeze chromatin***
   (a) Split sonicated chromatin into eppendorf tubes and centrifuge 15'/13,000 rpm (4℃)
   (b) remove supernatent and place in a new eppendorf tube for snap-freezing (in lN_2 or dry ice ethanol bath) and store at -80℃.

3.3 Immunoprecipitation

1. Pool sonicated chromatin samples (by biological condition) in a 15 ml conical tube. Add TritonX-100 to 1% final concentration. Briefly vortex and divide equally among eppendorf tubes according to the total number of IP reactions.
2. Set aside 50 µl of sonicated chromatin as input and store at 4℃ until IP is complete.
3. Add the appropriate antibody (Ig control or specific Ab). *n.b.* Antibody amount needs to be empirically determined, normally in range 1-6 µg per IP.
4. Incubate overnight at 4℃ with rotation.

DAY 2

3.4 Pull-down

1. Wash 100 µl of protein A (or G) magnetic beads three times in 1 ml of ice-cold blocking buffer by inverting several times in a 1.5 ml tube. After each wash, collect beads using a pre-cooled magnetic rack.

   [*n.b.*] If downstream analysis is qPCR, 50 µl of beads is sufficient, however for ChIP-seq 100 µl is required.
2. Transfer the washed beads to the tube containing chromatin+Ab.
3. Incubate on a rotating platform at 4℃ for 4-5 hours.
4. Remove supernatent from beads using the pre-cooled magnetic stand. Add 1 ml of ice-cold ChIP IP buffer (sonication buffer + 1% TritonX-100) and incubate with rotation 5' (4℃). Repeate once.
5. Remove supernatent from beads using the pre-cooled magnetic stand. Add 1 ml of ice-cold Wash buffer A and incubate with rotation 5' (4℃). Repeate once.
6. Remove supernatent from beads using the pre-cooled magnetic stand. Add 1 ml of ice-cold Wash buffer B and incubate with rotation 5' (4℃). Repeate once.
7. Remove supernatent from beads using the pre-cooled magnetic stand. Add 1 ml of ice-cold 1X TE buffer (pH 8.0) with 50 mM NaCl. Incubate 10' (4℃) with rotation.
8. Remove supernatent from beads using the pre-cooled magnetic stand. Add 210 µl of Elution buffer to the beads. Mix by *delicate* vortexing.
9. Incubate beads in Elution buffer 16' (65℃) resuspending by *delicate* vortexing every 2'.
10. Centrifuge 2'/10,000 rpm (RT) and recover the supernatent in new, low-binding tubes.

    [!] From this point forward use only **low binding** tips and tubes.
11. Add 150 µl of Elution buffer to the input chromatin (from previous day).

3.5 Reverse crosslinking

1. Reverse crosslinking in both the IPs and input by incubating in 65℃ water bath over-night (12-14 hours).

DAY 3

3.6 ChIP DNA Clean-up

1. Add 200 µl of 1X TE buffer (pH 8.0, 50 mM NaCl) to each tube to dilute the SDS.
2. Add 40 µl of RNAse A (molecular grade) and incubate at at least 2 hours (37℃).
3. Add 80 µg of proteinase K (4 µl of our stock) and incubate 30' (55℃).
4. Perform DNA clean-up using Qiagen PCR Purification kit.
5. Recover ChIP DNA in low-binding tubes. Recovered ChIP-DNA and Input DNA can be stored at -80℃ or immediately used for qPCR or HTS library prep.

4 Recipes

** should be added immediately prior to use.*
‡ use only freshly prepared stock.

4.1 Covaris Lysis Buffer

	Stock	Final	Amount
Hepes-KOH pH 7.5		50 mM	
NaCl		140 mM	
EDTA pH 8.0		1 mM	
Glycerol		10%	
Triton X-100		0.25%	
NP40*		0.5%	
Protease Inhibitors*		1X	

4.2 Covaris Wash Buffer

For 50 mL:

	Stock	Final	Amount
Tris-HCl pH 8.0		10 mM	
NaCl		200 mM	
EDTA pH 8.0		1 mM	
EGTA pH 8.0		0.5 mM	
Protease Inhibitors*		1X	

4.3 Agilent Sonication Buffer

For 50 mL:

	Stock	Final	Amount
Tris-HCl pH 8.0		10 mM	
NaCl		100 mM	
EDTA pH 8.0		1 mM	
EGTA pH 8.0		0.5 mM	
N-lauroylsarcosine Sodium Salt		0.5%	
Na-deoxycholate‡*		0.1%	
Protease Inhibitors*		1X	

4.4 Blocking Buffer

Filter and store at 4 ℃. For 50 mL:

	Stock	Final	Amount
PBS		1X	
BSA		0.5%	

4.5 Wash Buffer A

For 50 mL:

	Stock	Final	Amount
Hepes pH 7.8		50 mM	
NaCl		500 mM	
EDTA		1 mM	
Triton X-100		1%	
Na-deoxycholate‡*		0.1%	
SDS*		0.1%	
Protease Inhibitors*		1X	

4.6 Wash Buffer B

For 50 mL:

	Stock	Final	Amount
Tris-HCl pH 8.0		20 mM	
LiCl		250 mM	
EDTA		1 mM	
NP40		0.5%	
Na-deoxycholate‡*		0.1%	
Protease Inhibitors*		1X	

4.7 Elution Buffer

For 50 mL:

	Stock	Final	Amount
Tris-HCl pH 8.0		50 mM	
EDTA		10 mM	
SDS*		1%	