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Optimized ChIP-exo for mammalian cells and patterned sequencing flow cells

 G3: Genes, Genomes, Genetics

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We use this protocol and it's working

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Abstract

ChIP-exo is a genome-wide protein-DNA binding assay with unrivalled resolution, but its widespread adoption has been hindered by technical challenges, particularly when applied to mammalian cells or when used with recent sequencing platforms. We introduce a Mammalian-Optimized ChIP-exo (MO-ChIP-exo) protocol with key modifications that overcome previous technical hurdles. We demonstrate that our optimized protocol produces high-quality data comparable to previously published protocols and is adaptable for use with both suspension (K562) and adherent (HepG2, mESC) cell lines.

Materials

Buffers:

- Cell Lysis Buffer / Modified Farnham Lysis Buffer (CLB): 20mM Tris pH 8.0, 85mM KCl, 0.5% NP-40 (Igepal), 0.5% Triton X-100
- RIPA Variant Nuclear Lysis Buffer (NLB): 1x PBS, 1% NP40 (Igepal), 0.5% NaDeoxycholate, 0.1% SDS
- ChIP Elution Buffer: 100 mM NaHCO₃, 1% SDS
- 5M NaCl
- IP Dilution Buffer (IPDB): 20 mM Tris, pH 8.0, 2 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton x-100)
- Wash Buffer 1 (FA Lysis Buffer): 50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, 0.1% Sodium Deoxycholate and 1% Triton-X 100. *Add 1X CPI before use*
- Wash Buffer 2 (NaCl 250): 50 mM HEPES-KOH pH 7.5, 250 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton-X 100 and 0.1% Sodium Deoxycholate. *Add 1X CPI before use*
- Wash Buffer 3 (LiCl 250): 100 mM Tris-HCl pH 8.0, 250 mM LiCl, 1% NP-40 and 1% Sodium Deoxycholate. *Add 1X CPI before use*
- Wash Buffer 4 (10 M Tris-HCl pH 8). *Add 0.4 µL of 25% Triton-X per mL of buffer*
- 1X TE: 10 mM Tris-HCl, pH 8 and 1 mM EDTA
- ChIP Elution Buffer: 25 mM Tris-HCl pH 7.5, 2 mM EDTA pH 8.0, 200 mM NaCl and 0.5% SDS

Reagents/consumables:

- PBS (VWR Cat# VWRL0119-0500)
- Protease Inhibitor Cocktail Set V, EDTA-Free, Calbiochem® (CPI) (Millipore Sigma Cat# 539139-10VL)
- 32% Formaldehyde (VWR Cat# 100504-858)
- Tris (Neta Scientific Cat# RPI-T60040-1000.0)
- Glycine (Fisher Scientific Cat# AC120070010)
- 1.5 mL sonication tubes (Diagenode Cat# No. C30010016)
- RNase A 10 mg/mL (Thermo Fisher Scientific Cat# EN0531)
- Proteinase K 20 mg/mL (Life Technologies Cat# EO0491)
- QIAquick PCR purification kit (Qiagen Cat# 28104)
- D5000 ScreenTapes (Agilent Cat# 5067-5588)
- D5000 reagents (Agilent Cat# 5067-5589)
- Protein A Dynabeads (Invitrogen Cat# 10002D)
- Protein G Dynabeads (Invitrogen Cat# 10004D)
- tRNA (Millipore Sigma Cat# R8508-1ML)
- NEBNext Ultra II DNA Library Prep Kit for Illumina (Cat# E7645)
- ExB2_i##/ExA2B 15 uM (Rossi et al. 2018)
- 10X BSA 1 mg/mL (Thermo Fisher Scientific Cat# J64100-18)
- Phi 29 Polymerase 10 U/µL and 10X Phi29 DNA Polymerase Reaction Buffer (NEB Cat# M0269L)
- dNTP's (Promega Cat# U1330)
- Lambda Exonuclease 5 U/µL and 10 X Lambda Exonuclease Reaction Buffer (NEB Cat# M0262S)
- 10% Triton X (Sigma-Aldrich Cat# X100-1L)
- DMSO (Sigma-Aldrich Cat# D2650-100ML)



- Mag-Bind® TotalPure NGS (Omega Bio-Tek M1378-01)
- T4 DNA Ligase MBG 500 U and 10x T4 Ligation Buffer (Qiagen Cat no. / ID. EN11-050)
- ExB1-iX/ExB1B-5N 3 uM (Rossi et al. 2018)
- Phusion™ High-Fidelity DNA Polymerase 2 U/μL and 5X HF Buffer (Thermo Fisher Scientific Cat# F549L)
- PCR primers 1.3 and 2.1 (Rossi et al. 2018)
- SPRIselect DNA Size Selection reagent (Beckman Coulter B23317)
- HS D5000 ScreenTapes (Agilent Cat# 5067-5592)
- HS D5000 reagents (Agilent Cat# 5067-5593)
- NEB Next® Library Quantification Kit, For Illumina® (NEB Cat# E7645L)

Equipment:

- Rotator at 4°C
- Minifuge
- Vortex
- Benchtop centrifuge
- Refrigerated benchtop centrifuge
- Magnet for collection of dynabeads
- Heatblock and thermomixer
- Thermocycler
- Agilent 2100 Bioanalyzer Instrument or equivalent fragment analyzer
- ABI StepOne Plus Real Time PCR System (or similar)

Antibodies:

The following antibodies were used successfully for MO-ChIP-exo:

	A	B
	Antibody	Catalog Number
	Anti-CTCF	EMD Milipore 07-729
	IgG	Sigma i5006
	Anti-USF1	DSHB USF1-1B8

Day 1 - CROSSLINKING AND HARVEST. *Cells are crosslinked to preserve DNA-protein interactions and harvested, taking precautions to preserve cell and chromatin integrity.*

1 Crosslinking and harvest

- 1.1 Count cells (detach first if using adherent cells).
- 1.2 Adjust cell density to 1M cells/mL with fresh media.
- 1.3 Choose the size (allow ample space for gentle shaking - wide bottom is preferred) and number of flasks to be used based on the volume. Example: 1 Lt Erlenmeyer for 200 M cells in 200 mL media.
- 1.4 Add formaldehyde (FA) to a final concentration of 1%. To prevent oxidation and contamination, the use of ampules is recommended. Example: Use 1.5 mL of 32% FA for 50 M cells in 50 mL of media (or vehicle).
- 1.5 Incubate with gentle shaking at RT for 5-10 min.
- 1.6 Quench the crosslinking reaction by adding 3 M Tris-HCl pH 7.8 in a 2-fold molar excess. Alternatively, use 2.5 M Glycine to a final concentration of 125 mM. Example: Use 12.5 mL of 3 M Tris-HCl pH 7.8 (or 2.5 mL of 2.5 M Glycine) for 50 mL of crosslinking reaction.
- 1.7 Continue to incubate with gentle shaking at RT for 5 min.
- 1.8 Divide the crosslinked cell suspension into 50 mL tubes and centrifuge at 1,500 xg for 5 min at 4°C in pre-chilled centrifuge.
- 1.9 Keep tubes on ice from this point.
- 1.10 Discard the supernatant and resuspend the cells in ice-cold PBS + 1X CPI.

- 1.11 Transfer to microfuge tubes in volumes corresponding to 10-50M cells per tube (or adjust to desired cell number) for freezing.
- 1.12 Pellet cells by centrifuging for 3min at 10k rpm at 4°C.
- 1.13 Remove and discard supernatant and immediately flash freeze pellets in liquid nitrogen.
- 1.14 Store at -80°C until ready to use.

Day 2 - LYSIS AND SONICATION. *Cell and nuclear lysis are performed. The released chromatin is sheared through sonication and checked using a fragment analyzer.*

2 Lysis

- 2.1 Turn on water cooler ~30min before sonicating.
- 2.2 Thaw crosslinked cell pellets on ice for 10-30min.
- 2.3 Resuspend 10-30 M cells in 1 mL of CLB + 1X CPI.
- 2.4 Incubate on a rotator for 10min at 4°C.
- 2.5 Needle lyse (25 G needle) for 10 syringe pumps to assist in cell lysis.
- 2.6 Incubate on a rotator for 10min at 4°C.
- 2.7 Spin nuclei at 4°C, 10K rpm, 3 min.

- 2.8 Remove and discard the supernatant.
- 2.9 Resuspend the nuclei in 1 mL of NLB + 1X CPI.
- 2.10 Incubate on a rotator for 20 min at 4°C.
- 2.11 Spin at 4°C, 10K rpm, 3 min.
- 2.12 Remove and discard the supernatant.
- 2.13 Resuspend chromatin in 300 µL of 1X cold PBS + 1X CPI per 10 M cells.
- 2.14 Needle lyse (25 G needle) for 2 syringe pumps to assist resuspension.

3 **Sonication**

Note: The first time using a cell line, optimization of shearing conditions is recommended (for the Diagenode Bioruptor® Pico, starting with 1 cycle – with 1 cycle increments – is suggested).

- 3.1 Transfer 300 µL of chromatin (from 10M cells) to 1.5 mL Diagenode tubes for sonication.
- 3.2 Balance the carousels using dH₂O for blanks to complete the spaces if needed.
- 3.3 Sonicate in a Diagenode Bioruptor® Pico using the 'Go & Shear' setting with the validated parameters on the 'Easy Mode' for 30" ON/OFF for **4 cycles** (or as determined).
- 3.4 Remove a 15 µL aliquot (~0.5 M cells) to reverse crosslink and purify the DNA for a sonication assessment using the Agilent Tape Station D5000 kit (an alternative fragment analyzer or gel electrophoresis can be used).



- 3.5 Keep chromatin on ice/cold at all times for short term storage (24-48 hrs) or freeze (-80°C) for long term.

4 **Crosslinking Reversal and sonication assessment**

- 4.1 To the 15 µL aliquot removed for sonication assessment, add 185 µL of ChIP Elution Buffer to bring the volume to 200 µL.
- 4.2 Add 16 µL of 5M NaCl and 1 µL of RNase A (10 mg/mL). Mix well.
- 4.3 Incubate for 1 hr at 65°C.
- 4.4 Add 3 µL of proteinase K (20 mg/mL). Mix well.
- 4.5 Incubate for 1 hr at 45°C.
- 4.6 Purify the DNA using the Qiagen QIAquick PCR purification kit (or alternative column-based PCR purification kit).
- 4.7 Assess DNA size distribution using an Agilent TapeStation and D5000 kit. If >30% of DNA is between 100 – 500 bp, proceed to ChIP using the stored chromatin.

Day 3 - LIBRARY CONSTRUCTION (Part I) - ChIP. *Crosslinked sheared chromatin is captured using an antibody of interest to target DNA associated with a specific protein. Note: Day 2 and 3 can be combined.*

5 **Preparing and Blocking Dynabeads**

Note: Determine antibody isotype compatibility with Invitrogen Protein A or G Dynabeads according to the manufacturer's recommendations.

- 5.1 Keep all buffers, reagents and tubes on ice.
- 5.2 Mix stock of dynabeads by vortexing and transfer the total volume of beads (50 µL per ChIP) to be used to a 1.7 mL microfuge tube pipetting slowly.



- 5.3 Place the tube on the magnet for 1 min to collect the beads.
- 5.4 Remove and discard the supernatant.
- 5.5 Remove the tube from the magnet and add 1 mL of IPDB + 1X CPI.
- 5.6 Place the tube on the magnet for 1 min to collect the beads and remove the supernatant.
- 5.7 Remove the tube from the magnet and add 50 μ L of blocking solution (IPDB + 1X CPI + 0.2 mg/mL tRNA).
- 5.8 Mix and place on a rotator at 4°C for 15 min.
- 5.9 Proceed to antibody coating or store at 4°C until ready to use.

6 **Coating Dynabeads with specific antibodies and chromatin pre-clearing**

- 6.1 Add 3-10 μ g of antibody to the tube containing 50 μ L of blocked Protein A/G Dynabead slurry.
- 6.2 Mix by flicking and incubate for 1-2 hrs on a rotator at 4°C.
- 6.3 ~30min before antibody coating is complete, pre-clear the chromatin to lower the amount of nonspecific binders in the cell lysate: If frozen, thaw sonicated crosslinked chromatin for 5 min at 37°C. Otherwise remove from fridge and directly centrifuge at max speed (~14 K) for 15 min at 4°C.
- 6.4 Once antibody coating is complete, spin tubes in a minifuge to remove any condensation.
- 6.5 Place tubes against the magnet for 1 min to collect beads.

6.6 Remove and discard the supernatant.

6.7 Immediately add 200 μ L of IPDB + 1X CPI.

6.8 Repeat the spin and magnet placement to remove the supernatant.

7 **Chromatin Immunoprecipitation (ChIP)**

7.1 Immediately add the pre-cleared chromatin to each tube containing antibody/bead complexes.

Optional: save 5 μ L from the pre-cleared chromatin to be used as INPUT.

7.2 Incubate on a rotator at 4°C **overnight**.



Day 4 - LIBRARY CONSTRUCTION (Part II) - First adapter ligation, fill-in, exonuclease digestion, and reverse crosslinking. *Antibody-bound chromatin is subjected to end-repair and A-tailing before the first adapter is ligated (using TA ligation). The first adapter is filled in before exonuclease digestion takes place. Chromatin fragments are then reverse crosslinked.*

8 **Washes**

Before starting the library construction, bead-bound complexes are washed with:

Wash Buffer 1: FA Lysis + 1X CPI

Wash Buffer 2: NaCl 250 + 1X CPI

Wash Buffer 3: LiCl 250 + 1X CPI

Wash Buffer 4: 10 M Tris-HCl pH 8 + 0.4 μ L of 25% Triton-X at per mL of buffer

8.1 Spin the tubes on a minifuge and place the tubes on a magnet for 1 min.

8.2 Remove and discard the supernatant (unbound portion).

8.3 Add 150 μ L of wash buffer and incubate for 1 min.



8.4 Spin the tubes on a minifuge and place the tubes on a magnet for 1 min.

8.5 Remove wash buffer.

8.6 Repeat for each Wash Buffer (1-4) in the order above.

8.7 After the final wash, directly add the A-tailing master mix.

9 A-tailing

9.1 Prepare the A-tailing master mix (60 μ L per sample) according to the table below:

A	B
Reagent	Vol. per sample (μ L)
1X TE	50
NEBNext Ultra II End Prep Enzyme Mix	3
NEBNext Ultra II End Prep Reaction Buffer	7
Total	60

9.2 Add 60 μ L of the A-tailing master mix to the washed beads and gently pipette up/down 10 times.

9.3 Incubate for 30 min at 20°C followed by 30 min at 65°C.

9.4 Proceed directly to ligation.

10 First Adapter Ligation (i# adapters)

10.1 Prepare the First Adapter (i#) Ligation master mix according to the table below:

A	B
Reagent	Vol. per sample (μL)
NEBNext Ultra II Ligation Master Mix	30
NEBNext Ultra II Ligation Enhancer	1
PCR grade H ₂ O	1.5
Total	32.5

10.2 Quick spin tubes to collect the 60μL at the bottom and directly add 32.5 μL of the First Adapter (i#) Ligation master mix to all tubes.

10.3 Immediately add 1 μL of the first adapter ExB2_i##/ExA2B (15 uM) to each library.

10.4 Gently pipette up/down 10 times to mix and incubate for 15 min at 20°C.

10.5 Wash bead-bound chromatin with Wash Buffers 2-4 as described under 'Washes'.

11 Fill-in Reaction

11.1 Prepare the master mix for the Fill-in reaction according to the table below:



A	B	C
Reagent	Vol. per sample (μL)	Final Concentration
Water	24.6	
10X BSA (1 mg/mL)	8	200 ug/mL
10X Phi29 DNA Polymerase Reaction Buffer	4	1X
3 mM dNTP's	2.4	180 uM
Phi29 DNA Polymerase (10 U/μL)	1	10 U
Total	40	

- 11.2 Quick spin tubes on a microfuge and place them on a magnet for 1 min.
- 11.3 Remove and discard the supernatant.
- 11.4 Add 40 μL of the Fill-in master mix and gently pipette up/down 10 times to mix.
- 11.5 Incubate for 20 min at 30°C with shaking at 1k rpm.
- 11.6 Wash dynabeads with Wash Buffer 4 as described under 'Washes'.

12 Lambda exonuclease digest

- 12.1 Prepare the Lambda exonuclease master mix according to the table below:



A	B	C
Reagent	Vol. per sample (μL)	Final Concentration
Water	31.6	
10X Lambda Exonuclease Reaction Buffer	4	1X
10% Triton X	0.4	0.10%
DMSO	2	5%
Lambda Exonuclease (5 U/μL)	2	10 U
Total	40	

- 12.2 Quick spin tubes on a microfuge and place them on a magnet for 1 min.
- 12.3 Remove and discard the supernatant.
- 12.4 Add 40 μL of the Lambda exonuclease master mix and gently pipette up/down 10 times to mix.
- 12.5 Incubate for 30 min at 37°C with shaking at 1k rpm.
- 12.6 Wash dynabeads with Wash Buffer 4 as described under 'Washes'.

13 Elution and Reverse Crosslink

- 13.1 Prepare the ChIP elution Buffer with Proteinase K according to the table below:



	A	B	C
	Reagent	Vol. per sample (μL)	Final Concentration
	ChIP Elution Buffer	40	
	Proteinase K (20 mg/mL)	1.5	0.7 mg/mL

- 13.2 Quick spin tubes on a microfuge and place them on a magnet for 1 min.
- 13.3 Remove and discard the supernatant.
- 13.4 Add 40 μL of ChIP Elution Buffer with Proteinase K and incubate overnight at 65°C.

Day 5 - LIBRARY CONSTRUCTION (Part III) - Second adapter ligation, enrichment and quantification. *DNA fragments undergo 2nd adapter ligation. Libraries are then enriched and size selected before final quantification.*

14 DNA Purification

- 14.1 Quick spin tubes on a microfuge and place them on a magnet for 1 min.
- 14.2 Transfer and KEEP the supernatant (eluate) in a new tube.
- 14.3 Add 1.8 Volumes of TotalPure Beads (72 μL for 40 μL eluted samples).
- 14.4 Mix by pipetting up and down 10 times and incubate at RT for 5 min.
- 14.5 Place tubes on a magnet for 1 min and remove the supernatant.



- 14.6 Keep tubes on magnet and add 180 μL of freshly made 70% ethanol without disturbing the pellet.
- 14.7 Incubate for 30 sec without disturbing the beads.
- 14.8 Remove the supernatant and repeat the ethanol wash.
- 14.9 Dry the beads at RT for 5 min.
- 14.10 Add 21 μL of PCR-grade H_2O and mix by pipetting up and down 10 times.
- 14.11 Place tubes on a magnet and collect 20 μL of the eluted sample in a fresh tube.

15 Second Adapter Ligation (iX adapters)

- 15.1 Prepare the Second Adapter (iX) Ligation master mix according to the table below:

	A	B	C
	Reagent	Vol. per sample (μL)	Final Concentration
	Water	9	
	10X T4 Ligase Buffer	4	1X
	T4 Ligase (600 U/ μL)	2	1200 U
	Total	15	

- 15.2 Add 15 μL of the Second Adapter (iX) Ligation master mix to each tube containing 20 μL of eluted DNA.

15.3 Immediately add 5 μ L of the second adapter ExB1-iX/ExB1B-5N (3 μ M) to each library.

15.4 Gently pipette up/down 10 times to mix and incubate for 1 hr at 25°C.

16 Repeat DNA Purification

16.1 Repeat step 14 to clean up DNA fragments containing both adapters.

17 PCR

17.1 Prepare the PCR Master Mix (plan to include negative and positive controls) according to the table below:

A	B	C
Reagent	Vol. per sample (μ L)	Final Concentration
Water	6.8	
5X Phusion HF Buffer	8	1X
3 mM dNTP's	2.67	200 μ M each
P1.3 primer (20 μ M)	0.8	500 nM
P2.1 primer (20 μ M)	0.8	500 nM
Phusion Hot Start DNA Polymerase (2 U/ μ L)	1	2 U
Total	20	



17.2 Add the 20 μ L of master mix to each library (20 μ L resuspended DNA).

17.3 Vortex tubes and spin down before placing them in the thermocycler using the following settings:

End Extension		72°C 2 min
Amplification (12 cycles)	Denature	98°C 10 sec
	Anneal	52°C 30 sec
	Extension	72°C 30 sec
End Extension		72°C 2 min
		4°C Hold

18 **Double-sided Size selection:**

This step uses bead to sample ratios of 0.7X and 1X to select for ~150 - 500 bp fragments. An alternative to this step is gel excision and purification as in ChIP-exo 5.0

18.1 Spin tubes to collect libraries at the bottom of the tube.

18.2 Add 10 μ L 10 mM pH 7.5 to each 40 μ L library for a total starting volume of 50 μ L.

18.3 Gently resuspend SPRI beads.

18.4 Add 0.7 volumes (35 μ L) of beads (50 μ L * 0.7x vol) to each library.

18.5 Mix by pipetting 10 times and incubate at RT for 5 min.

18.6 Place samples on magnet and allow the beads to settle for 1-3 min.

18.7 Collect the supernatant (contains the Right Side Size Selected sample) and transfer to fresh tubes.



- 18.8 Gently resuspend fresh SPRI beads.
- 18.9 Add 0.3 volumes (15 μ L) of beads. Calculated as: Sample Vol. * (1X – initial ratio (0.7X)).
[50 μ L * (1-0.7) = 15 μ L of SPRIselect beads]
- 18.10 Mix by pipetting 10 times and incubate at RT for 5 min.
- 18.11 Place samples on magnet and allow the beads to settle for 1-3 min.
- 18.12 Remove and discard the supernatant.
- 18.13 With the tubes still on the magnet, add 180 μ L of freshly made 85% ethanol and incubate at RT for 30 sec.
- 18.14 Remove and discard the supernatant. Be careful not to remove any beads at this step as it may result in reduced yield.
- 18.15 Repeat the ethanol wash.
- 18.16 Air dry pellet for 5 min.
- 18.17 Remove the samples from the magnet and resuspend the pellet in 21 μ L of 10 mM Tris pH 8.
- 18.18 Mix by pipetting 10 times and incubate for 5 min.
- 18.19 Place samples back on the magnet and allow the beads to settle for 1-3 min.
- 18.20 Transfer 20 μ L of the supernatant to fresh tubes.



19 Repeat DNA Purification

- 19.1 Repeat step 14 to clean up DNA fragments containing both adapters.

20 Library assessment

- 20.1 Assess DNA size distribution using an Agilent Tape Station and High Sensitivity D5000 kit. >90% of each library is expected to be between 200-500 bp with an average fragment size of 300-350 bp. It's important to also confirm the absence of free adapters (~75 bp) or adapter dimers (~150 bp).
- 20.2 Finally, library quantification is performed using NEB Next® Library Quantification Kit, For Illumina® in an ABI StepOne Plus Real Time PCR System.

ADDITIONAL DETAILS

21 Adapter pre-annealing

ChIP-exo oligos are single stranded and need to be pre-annealed for each adapter as below. For MO-ChIP-exo oligo's are HPLC purified and pre-annealed adapters are used as UDI's.

21.1 ExB2-iX (Number Adapter i01- i16) – 15 uM

- Mix ExA2B with each ExB2-i# (i01- i16) (15 uM each) in 1X SSC.
- Incubate 2 min at 95°C followed by 1 hour at room temp.
- Store at -20C until ready to use.

21.2 ExC1-iX (Letter Adapters iA-iP) – 3 uM

- Mix ExA1-SSL_N5 with each ExC1-iX (iA-iP) (15 uM each) in 1X SSC.
- Incubate 2 min at 95°C followed by 1 hour at room temp.
- Dilute 1:5 in 10 mM pH 7.5 for 3 uM working stocks.
- Store at -20C until ready to use.

Protocol references

Protocol

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