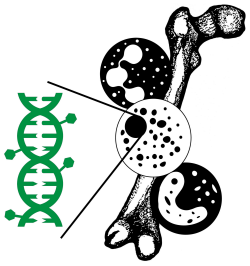
Nov 13, 2020

low input ChIP-sequencing of immune cells



In 1 collection

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Protocol status: Working
we developed this low input ChIPseq protocol to look at genome-wide epigenetic changes in histone modifications. we start with just 60,000 immune cells isolated directly ex vivo by flow sorting from mouse spleens and bone marrow. for examples of the quality of the generated data see Figure 6 and S8 of our publication: www.biorxiv.org/content/10.1101/2020.10.01.322180v1. all ChIPseq data is publicly available (GEO accession number GSE150478).

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Protocol Integer ID: 39995

Keywords: ChIPseq, histone modification, epigenetic reprogramming, low input,

Abstract

Cells can stably (and heritably) alter their gene expression profile through epigenetic modifications. Histones package DNA into chromatin and can be post-translationally modified - most prominently by methylation and acetylation. These histone modifications alter chromatin structure and DNA accessibility. We optimised a protocol for reliable high quality chromatin immunoprecipitation followed by DNA sequencing (ChIPseq) starting with just 60,000 monocytes isolated directly from mouse tissues by flow sorting. Our protocol can easily be adapted to other mouse or human cell types to interrogate the genome-wide distribution of histone modifications or transcription factor binding sites in immune cells directly *ex vivo*.

Image Attribution

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Materials

MATERIALS

- Chloroform-Isoamyl Alcohol **Sigma Aldrich Catalog #**25666
- 20X EvaGreen **Biotium Catalog #**31000
- Agencourt AMPure XP magnetic beads **Beckman Coulter Catalog #**A63880
- Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #**5067-4626
- cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack **Roche Catalog #**05 892 791 001
- Sodium Butyrate 500 mg **Stemcell Technologies Catalog #**72242
- Glycine **Sigma Catalog #**50046
- Qubit® Assay Tubes **Life Technologies Catalog #**Q32856
- DynaMag™-2 Magnet **Life Technologies Catalog #**12321D
- 4% Paraformaldehyde in PBS **Alfa Aesar Catalog #**J61899-AK
- DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #**0030108051
- UltraPure™ DEPC-treated Water **Thermo Fisher Catalog #**10813012
- IMDM **Thermo Fisher Catalog #**12440053
- UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher Catalog #**15593049
- PBS, pH 7.2 **Thermo Fisher Catalog #**20012019
- RNase Cocktail™ Enzyme Mix **Thermo Fisher Catalog #**AM2286
- DNAZap™ PCR DNA Degradation Solutions **Thermo Fisher Catalog #**AM9890
- Qubit™ dsDNA HS Assay Kit **Thermo Fisher Catalog #**Q32851
- Eppendorf Safe-Lock Tubes 1.5 mL PCR clean colorless 500 tubes **Eppendorf Catalog #**022363212
- True MicroChIP kit **Diagenode Catalog #**C01010130
- 1.5 ml Bioruptor Pico Microtubes with Caps **Diagenode Catalog #**C30010016
- Corning 15mL PP Centrifuge Tubes with CentriStar Cap Sterile **Corning Catalog #**430791
- Falcon® 5 mL Round Bottom High Clarity PP Test Tube with Snap Cap Sterile **Corning Catalog #**352063
- Drosophila spike-in chromatin **Active Motif Catalog #**61686
- spike-in antibody **Active Motif Catalog #**61686
- H3K27ac Antibody - ChIP-seq Grade **Diagenode Catalog #**C15410196
- H3K4me1 Antibody - ChIP-seq Grade **Diagenode Catalog #**C15410037-50
- H3K9me3 Antibody - ChIP-seq Grade **Diagenode Catalog #**C15410193
- DiaMag protein A-coated magnetic beads (ChIP-seq grade) **Diagenode Catalog #**C03010020

MicroPlex Library Preparation Kit v2 (12 indexes) **Diagenode Catalog #**C05010012
MicroChIP DiaPure columns **Diagenode Catalog #**C03040001
Lightcycler 480 multiwell plate 96 clear **Roche Catalog #**05102413001
MicroAmp Optical 8-Cap Strip lids **Thermo Fisher Catalog #**4323032
TE Buffer Tris-EDTA 1X Solution pH 8.0 **Fisher Scientific Catalog #**10224683
Premium Fetal Bovine Serum (FBS) **Thermo Fisher Catalog #**16000044
HBSS no calcium no magnesium no phenol red **Thermo Fisher Catalog #**14175053
Certified Molecular Biology Agarose **BIO-RAD Catalog #**1613100

FLOW BUFFER
PBS
2 % filtered (0.22 µm) heat-inactivated FBS
5 mM EDTA

> can be stored for 2 weeks at 4 °C

FIXATION BUFFER –1 ml per 1×10^7cells
PBS
10 % filtered (0.22 µm) heat-inactivated FBS
1 % Paraformaldehyde

> make fresh just before use, equilibrate to Room temperature before use

QUENCH BUFFER – use at final concentration of 125 mM Glycine
2.5 M Glycine in PBS (1.87 g in 10 ml PBS; gently heat to 37 °C in water bath, then rotate for 03:00:00 at Room temperature to dissolve completely)

> can be stored for 2 weeks at 4 °C, equilibrate to Room temperature before use

COLLECTION MEDIA
IMDM (specifically formulated for mouse cells - if working with human cells use RPMI instead)
5 % filtered (0.22 µm) heat-inactivated FBS

HBSS + PIC (protease inhibitor cocktail)
1 complete ULTRA Protease Inhibitor Cocktail tablet per 10 ml HBSS, rotate for 3 h at Room temperature to dissolve completely

> store at 4 °C for no longer than 1 week

100 mM sodium butyrate – use at final concentration of 5 mM sodium butyrate
dissolve in DEPC water

> store aliquots (single use – do not freeze/thaw) at -20 °C for no longer than 3 months

***Drosophila* spike in chromatin**
stock: 10 ng/ µl
dilute in DEPC water to 40 pg/ µl

> store aliquots (single use – do not freeze/thaw) at -80 °C for no longer than 6 months

Protocol materials

- Falcon® 5 mL Round Bottom High Clarity PP Test Tube with Snap Cap Sterile **Corning** **Catalog #**352063
- UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher** **Catalog #**15593049
- DNAZap™ PCR DNA Degradation Solutions **Thermo Fisher** **Catalog #**AM9890
- Agilent High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626
- Qubit® Assay Tubes **Life Technologies** **Catalog #**Q32856
- DNA LoBind Tubes, 1.5 mL **Eppendorf** **Catalog #**0030108051
- RNase Cocktail™ Enzyme Mix **Thermo Fisher** **Catalog #**AM2286
- True MicroChIP kit **Diagenode** **Catalog #**C01010130
- H3K4me1 Antibody - ChIP-seq Grade **Diagenode** **Catalog #**C15410037-50
- MicroChIP DiaPure columns **Diagenode** **Catalog #**C03040001
- HBSS no calcium no magnesium no phenol red **Thermo Fisher** **Catalog #**14175053
- cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack **Roche** **Catalog #**05 892 791 001
- Glycine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**50046
- 20X EvaGreen **Biotium** **Catalog #**31000
- Qubit™ dsDNA HS Assay Kit **Thermo Fisher** **Catalog #**Q32851
- 4% Paraformaldehyde in PBS **Alfa Aesar** **Catalog #**J61899-AK
- IMDM **Thermo Fisher** **Catalog #**12440053
- Lightcycler 480 multiwell plate 96 clear **Roche** **Catalog #**05102413001
- Sodium Butyrate 500 mg **STEMCELL Technologies Inc.** **Catalog #**72242
- MicroAmp Optical 8-Cap Strip lids **Thermo Fisher** **Catalog #**4323032
- TE Buffer Tris-EDTA 1X Solution pH 8.0 **Fisher Scientific** **Catalog #**10224683
- H3K27ac Antibody - ChIP-seq Grade **Diagenode** **Catalog #**C15410196
- Certified Molecular Biology Agarose **Bio-Rad Laboratories** **Catalog #**1613100
- Chloroform-Isoamyl Alcohol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**25666
- Eppendorf Safe-Lock Tubes 1.5 mL PCR clean colorless 500 tubes **Eppendorf** **Catalog #**022363212
- 1.5 ml Bioruptor Pico Microtubes with Caps **Diagenode** **Catalog #**C30010016
- Drosophila spike-in chromatin **Active Motif** **Catalog #**61686
- spike-in antibody **Active Motif** **Catalog #**61686
- DiaMag protein A-coated magnetic beads (ChIP-seq grade) **Diagenode** **Catalog #**C03010020
- DynaMag™-2 Magnet **Life Technologies** **Catalog #**12321D

- UltraPure™ DEPC-treated Water **Thermo Fisher** **Catalog #**10813012
- PBS, pH 7.2 **Thermo Fisher** **Catalog #**20012019
- H3K9me3 Antibody - ChIP-seq Grade **Diagenode** **Catalog #**C15410193
- MicroPlex Library Preparation Kit v2 (12 indexes) **Diagenode** **Catalog #**C05010012
- Premium Fetal Bovine Serum (FBS) **Thermo Fisher** **Catalog #**16000044
- Agencourt AMPure XP magnetic beads **Beckman Coulter** **Catalog #**A63880
- Corning 15mL PP Centrifuge Tubes with CentriStar Cap Sterile **Corning** **Catalog #**430791
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- Agilent High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626
- Agencourt AMPure XP magnetic beads **Beckman Coulter** **Catalog #**A63880

DNA-Protein crosslinking and cell sorting

1d

1 prepare single cell suspensions and lyse erythrocytes at Room temperature

RECOMMENDATION: work in 15 ml sterile, RNAse/ DNAse free, non pyrogenic polypropylene conical tubes until step 11.

NOTE: this protocol was optimised to isolate monocytes from mouse spleens and bone marrow - but it can be easily adapted to work with most other mouse and human tissues and cell types. adhere to best practice for your tissue when preparing single cells suspensions and lysing erythrocytes.

2 count cells, then Fc block and antibody stain in **FLOW BUFFER*** (scale appropriately: stain max 2×10^7 cells in 1 mL) for 00:20:00 at Room temperature

* Buffers and Solutions in bold capitals are described in detail in Materials section

NOTE: design, titrate and test your antibody panel carefully beforehand.

3 wash cells twice in PBS 350 x g, Room temperature, 00:05:00

4 gently resuspend cells in **FIXATION BUFFER*** (1 mL for every 1×10^7 cells)

CRITICAL: warm FIXATION BUFFER to Room temperature before use.

5 incubate for exactly 00:10:00 at Room temperature , gently flick to mix occasionally

6 add Room temperature **QUENCH BUFFER** (final Glycine concentration [M] 125 millimolar (mM) : for every 1 mL FIXATION BUFFER added in step 4 add 50 µL QUENCH BUFFER)

7 incubate for 00:05:00 at Room temperature , gently flick to mix occasionally

8 450 x g, 4°C, 00:10:00 , slow brake

NOTE: faster, longer centrifugations going forward, since cell velocity changes after fixation.

9 aspirate supernatant carefully (leave approx. 100 µL) and resuspend cells in 12 mL cold PBS

10 450 x g, 4°C, 00:10:00 , slow brake

11 aspirate supernatant carefully (leave approx. 100 µL) and resuspend cells in 3 mL cold **FLOW BUFFER**

12 SORT 60,000 desired cells on BD FACS Aria III or similar cell sorter (85 um nozzle, sort precision mode: purity, sample and collection chamber 4 °C) into 5 ml polypropylene FACS tubes with 2.5 mL **COLLECTION BUFFER.**

RECOMMENDATION: sort several technical replicates from one biological sample and chromatin-immunoprecipitate each replicate with an antibody against a different histone modifications. in this way you will get a more detailed picture of the epigenetic landscape within each biological sample.

NOTE: always perform a **test sort** beforehand, where you set up all parameters, gates and compensation ready for your big ChIPseq sort day. check cell recovery - some cell types are very fragile and may require alteration of sort or collection parameters for optimal viability. our recovery was 40 - 60 % of sorted cells i.e. we continue the protocol with approx 30,000 cells. always check the purity of your sort before and after your last sample (and in between if you encountered any problems): > 95% of sorted cells should fall in the gates for your population of interest and debris should be minimal.

13 450 x g, 4°C, 00:10:00 , slow acceleration, slow brake

14 aspirate supernatant carefully (leave approx 50 µL behind)

15 resuspend in 2 mL cold **HBSS + protease inhibitors (PIC)** + [M] 5 millimolar (mM) **sodium butyrate**

16 450 x g, 4°C, 00:10:00 , slow acceleration, slow brake

17 aspirate supernatant carefully (leave approx 50 µL behind)

18 flash freeze cell pellet in methanol bath on dry ice and store at -80 °C for up to 3 months

cell lysis and chromatin shearing

4h

19 *the following section uses reagents from the* **True MicroChIP kit** *(Diagenode, #C01010130) with a modified protocol*

NOTE: work in area designated for low input DNA work, use designated pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNAZap before starting.

True MicroChIP kit **Diagenode Catalog #**C01010130

TrueMicroChIP-kit-manual.pdf

20 equilibrate lysis buffer tL1 to Room temperature (all crystals should be dissolved) and add protease inhibitor cocktail (PIC, from True MicroChIP Kit)
for 1 sample: 25 µL tL1 + 0.125 µL PIC (1:200)

21 thaw samples slowly On ice and add 1 mL ice-cold **HBSS + PIC**

22 450 x g, 4°C, 00:10:00 , slow acceleration, slow brake

23 aspirate supernatant carefully (leave as little behind as possible), keep pellets
On ice

24 add 25 µL tL1 with PIC to the cell pellet – gently vortex to resuspend and flick until bubbles form

25 incubate for 00:05:00 On ice

26 add 75 µL HBSS with PIC, mix by pipetting and transfer to 1.5 ml Bioruptor Pico microtubes

27 using the Bioruptor Pico sonicate for 5 cycles 30 sec ON 30 sec OFF to shear the chromatin.

Equipment	
Bioruptor Pico sonication device	NAME
Sonicator	TYPE
Diagenode	BRAND
B01060010	SKU

NOTE: sonication time and intervals are unique for each cell type. for optimal ChIPseq chromatin should be sheared into 100 - 300 bp fragments. see [QC chromatin shearing] in step 30.1 for how to optimise shearing.

28 briefly vortex and place On ice

29 14000 x g, 4°C, 00:10:00

30 transfer supernatant (= 100 µL sheared chromatin) to 1.5 ml DNA LoBind tube

sheared chromatin can be stored at -80 °C for up to 8 weeks or immediately immunoprecipitated (see next section)

30.1 OPTIONAL: **optimise Chromatin shearing** for cell type of interest [uses one flow-sorted technical replicate] 8h

use reagents from the **True MicroChIP kit** *(Diagenode, #C01010130) with a modified protocol. during optimisation it may pay off to use the designated* **Chromatin shearing optimization kit – high SDS** *(Diagenode, #C01020012)*

1. start with 100 µL sheared chromatin in 1.5 ml DNA LoBind tube
2. dilute RNase cocktail (1 µL + 150 µL DEPC-treated water) and add 2 µL to the sheared chromatin

3. incubate 01:00:00 at 37 °C
4. add 100 µL elution buffer tE1 and 8 µL elution buffer tE2, mix thoroughly by pipetting
5. decrosslink proteins from DNA for at least 04:00:00 or Overnight in a ThermoMixer (1300 rpm) at 65 °C
6. spin tubes briefly
7. add 200 µL Room temperature Phenol/Chloroform/Isoamyl alcohol 25:24:1
8. vortex for 00:00:15, incubate for 00:10:00 on rotating wheel at Room temperature
9. 14000 x g, Room temperature, 00:02:00
10. transfer aqueous phase to new 1.5 ml DNA LoBind tube
11. add 200 µL Room temperature Chloroform/Isoamyl alcohol 24:1
12. vortex for 00:00:15, incubate for 00:10:00 on rotating wheel at Room temperature
13. 14000 x g, Room temperature, 00:02:00
14. transfer aqueous phase to new 1.5 ml DNA LoBind tube (approx 150 µL)
15. to precipitate the DNA add:

15 µL tP1

2 µL tCP1

2 µL tCP2

1 µL ice cold 100 % Ethanol

16. incubate at -80 °C for 00:30:00
17. 14000 x g, 4°C, 00:25:00
18. carefully discard supernatant and add 500 µL ice cold 70 % Ethanol
19. 14000 x g, 4°C, 00:10:00
20. carefully remove all supernatant and allow pellet to air-dry for max. 00:05:00
21. resuspend pellet in 12 µL TE: to assess DNA fragment size distribution and integrity use 1 µL for Bioanalyzer HS DNA Chip (see step 113 for details) and 10 µL to run on a 1.5 % Agarose gel at 100 V for 01:00:00 (use a 100 kb ladder)
22. stain and assess gel image

Expected result

sheared chromatin fragments should be between 100 and 300 bp

chromatin immunoprecipitation (ChIP)

1d

31 *the following section uses reagents from the* **True MicroChIP kit** *with a modified protocol*

NOTE: work in area designated for low input DNA work, use designated pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNAZap before starting.

32 add protease inhibitor cocktail (True MicroChIP Kit) to Chip buffer tC1
for 1 sample: 100 µL tC1 + 0.5 µL PIC

33 add 100 µL tC1 + PIC to 100 µL sheared chromatin

34 OPTIONAL: to normalise for technical variation between samples from this point onwards spike in a small amount of *Drosophila melanogaster* chromatin into each sample. an antibody against the *Drosophila*-specific histone variant H2Av then reliably pulls down a fraction of the *Drosophila* chromatin. this should happen consistently across all samples. after sequencing, the ratio of data mapping to the *Drosophila* genome vs your organisms genome creates a normalisation factor for each sample. you can then normalise your experimental tag counts by this factor. for more information: https://www.activemotif.com/catalog/1091/chip-normalization

add 140 pg ***Drosophila* spike-in chromatin**
(if you do this also add 0.3 µg **spike-in antibody** in step 36)

CITATION

Egan B, Yuan CC, Craske ML, Labhart P, Guler GD, Arnott D, Maile TM, Busby J, Henry C, Kelly TK, Tindell CA, Jhunjhunwala S, Zhao F, Hatton C, Bryant BM, Classon M, Trojer P (2016). An Alternative Approach to ChIP-Seq Normalization Enables Detection of Genome-Wide Changes in Histone H3 Lysine 27 Trimethylation upon EZH2 Inhibition.. PloS one.

LINK

https://doi.org/10.1371/journal.pone.0166438

35 CRITICAL: remove 10 µL (5 %) of sheared chromatin as ***input sample***, store at 4 °C in a 1.5 ml DNA LoBind tube.

NOTE: *input samples* (one for each biological replicate) are essential to analyse ChIPseq. it is the measurement of epigenetic landscape in your cells before immunoprecipitation - all enrichment is measured relative to it.

36 add your antibody of interest to the remaining 95% of sheared chromatin for immunoprecipitation:

we used antibodies against H3K27ac (2 µg), H3K4me1 (5 µg) and H3K9me3 (1 µg) to investigate activation and repression of transcription as well as the future potential to respond to stimuli.

H3K27ac marks transcription start sites to activate transcription
H3K4me1 marks enhancers and superenhancers to promote gene expression
H3K9me3 condenses DNA into heterochromatin to silence gene expression

NOTE: other ChIPseq-grade antibodies (for example against transcription factors) can be used. titrate all antibodies for optimal ChIPseq. some protocol recommend qPCR for validating titrations; however, we find that qPCR results do not predict ChIP sequencing outcome. we instead recommend a test sequencing run to validate antibodies and the concentrations they are used at.

OPTIONAL: add 0.3 µg **spike-in antibody** (see step 34)

37 incubate Overnight on a rotating wheel (40 rpm) in the cold room 4 °C

38 next morning: prepare DiaMag Protein A-coated magnetic beads for 1 sample mix 10 µL beads (pipette up and down > 20 times to get an even suspension) with 50 µL beads wash buffer tBW1 in a 1.5 ml tube

39 place in the DynaMag- 2 magnet and wait for 00:01:00
DynaMag™-2 Magnet **Life Technologies Catalog #**12321D

40 discard the supernatant (keep tube in magnet)

41 take tube out of magnet and gently resuspend the beads in 50 µL tBW1

42 place in the magnet and wait for 00:01:00

43 discard the supernatant (keep tube in magnet)

44 take tube out of magnet and gently resuspend the beads in 10 µL tBW1

45 remove samples from rotating wheel (keep On ice) and spin briefly to collect all liquid in the bottom of the tube

46 add 10 µL of washed beads

47 incubate for 06:00:00 on a rotating wheel (40 rpm) in the cold room 4 °C

washes 2h

48 *the following section uses reagents from the* **True MicroChIP kit** *with a modified protocol*

thorough, careful washing is key for high quality ChIPseq, since it removes non-antibody bound chromatin fragments and therefore reduces background.

NOTE: work in area designated for low input DNA work, use designated pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNAZap before starting.

49 place magnet On ice and keep samples and all buffers ice-cold throughout

50 remove samples from rotating wheel (keep On ice) and spin briefly to collect all liquid in the bottom of the tube

51 place your samples in the magnet and wait for 00:01:00 - the beads (and the immunoprecipitated chromatin bound to them) will bind to the side of the tube facing the magnet

52 discard the supernatant (keep tube in magnet)

53 take tube out of magnet and gently resuspend the beads in 100 µL ice cold wash buffer tW1

NOTE: do not create bubbles.

54 incubate for 00:04:00 on a rotating wheel (40 rpm) in the cold room 4 °C

55 remove samples from rotating wheel (keep On ice) and spin briefly to collect all liquid in the bottom of the tube

56 place your samples in the magnet and wait for 00:01:00

57 discard the supernatant (keep tube in magnet)

58 take tube out of magnet and gently resuspend the beads in 100 µL ice cold wash buffer tW2

NOTE: do not create bubbles.

59 incubate for 00:04:00 on a rotating wheel (40 rpm) in the cold room 4 °C

60 remove samples from rotating wheel (keep On ice) and spin briefly to collect all liquid in the bottom of the tube

61 place your samples in the magnet and wait for 00:01:00

62 discard the supernatant (keep tube in magnet)

63 take tube out of magnet and gently resuspend the beads in 100 µL ice cold wash buffer tW3

NOTE: do not create bubbles.

64 incubate for 00:04:00 on a rotating wheel (40 rpm) in the cold room 4 °C

65 remove samples from rotating wheel (keep On ice) and spin briefly to collect all liquid in the bottom of the tube

66 place your samples in the magnet and wait for 00:01:00

67 discard the supernatant (keep tube in magnet)

68 take tube out of magnet and gently resuspend the beads in 100 µL ice cold wash buffer tW4

NOTE: do not create bubbles.

69 incubate for 00:04:00 on a rotating wheel (40 rpm) in the cold room 4 °C

70 remove samples from rotating wheel (keep On ice) and spin briefly to collect all liquid in the bottom of the tube

71 place your samples in the magnet and wait for 00:01:00 1m

72 discard the supernatant (keep tube in magnet)

DNA decrosslinking 18h

73 *the following section uses reagents from the* **True MicroChIP kit** *with a modified protocol*

NOTE: work in area designated for low input DNA work, use designated pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNAZap before starting.

74 after removing wash buffer tW4 take tube out of magnet and gently resuspend the beads in 200 µL elution buffer tE1 (equilibrate to Room temperature before use - tE1 should be a clear solution)

75 take the *input samples* (10 µl) you saved in step 35 out of the fridge and add 190 µL elution buffer tE1

76 incubate *both ChIP and input samples* for 00:30:00 on a rotating wheel (40 rpm) at Room temperature

77 remove samples from rotating wheel and spin briefly to collect all liquid in the bottom of the tube

78 place *ChIP samples* in the Room temperature magnet and wait for 00:01:00

79 *ChIP samples*: keep the tube in the magnet and transfer the supernatant (= your immunoprecipitated chromatin) to a new 1.5 ml DNA LoBind tube

80 add 8 µL elution buffer tE2 to *both ChIP and input samples*

81 decrosslink proteins from DNA (for both *ChIP and input samples*) Overnight in a ThermoMixer (1300 rpm) at 65 °C

DNA purification using Micro ChIP DiaPure columns 1h

82 *the following section uses* **Micro ChIP DiaPure columns** *(Diagenode, #C03040001) according to manufacturers instructions*

NOTE: work in area designated for low input DNA work, use designated pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNAZap before starting.

MicroChIP DiaPure columns **Diagenode Catalog #**C03040001

MicroChIP_DiaPure_manual.pdf

83 spin decrosslinked samples (*ChIP and input samples*: 200 µl Volume) briefly to collect all liquid in the bottom of the tube

84 add 1000 µL (5 Vol) Room temperature ChIP DNA binding buffer and mix gently by pipetting

85 transfer 600 µL to the spin column in its collection tube

86 10000 x g, Room temperature, 00:00:30

87 discard the flow-through

88 transfer the remaining 600 µL to the spin column in its collection tube

89 10000 x g, Room temperature, 00:00:30

90 discard the flow-through

91 add 200 µL Room temperature DNA wash buffer

CRITICAL: make sure Ethanol was added to the buffer.

92 10000 x g, Room temperature, 00:00:30

93 add 200 µL Room temperature DNA wash buffer

94 10000 x g, Room temperature, 00:00:30

95 transfer the column to a new 1.5 ml DNA LoBind tube

96 to elute the DNA add 15.2 µL Room temperature DNA elution buffer directly onto the column matrix and incubate for 00:03:00 at Room temperature

97 10000 x g, Room temperature, 00:00:30 then discard the column and transfer tube with DNA On ice

98 DNA can be stored at -20 °C before library preparation for up to 2 weeks

Library preparation

1d

99 *the following section uses the* **Diagenode MicroPlex Library preparation kit v2** *(Diagenode, #C05010012) according to manufacturers instructions*

1d

NOTE: work in area designated for library preperation (distinct from low input DNA area), use designated library preparation pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNA-ZAP before starting.

MicroPlex Library Preparation Kit v2 (12 indexes) **Diagenode Catalog** #C05010012

MicroPlex-Libary-Prep-Kit-v2-man...

NOTE: keep samples and reagents On ice throughout.

100 in clear 96 well Lightcycler 480 plate mix 10 µL ChIP-ed, purified DNA with 2 µL template preparation buffer and 1 µL template preparation enzyme

101 gently mix by pipetting, cap using strip lids and spin briefly to collect all liquid in the bottom of the wells

102 run on a standard PCR machine (settings: plate, 13 µl Volume, heated lid)

	temperature	time
	22 °C	25 min
	55 °C	20 min
	4 °C	∞

103 transfer plate back On ice as soon as PCR machine has cooled to 4 °C, spin briefly to collect all liquid in the bottom of the wells

104 carefully open lids and add 1 µL library synthesis buffer and 1 µL library synthesis enzyme

105 gently mix by pipetting, cap using strip-lids and spin briefly to collect all liquid in the bottom of the tube

106 incubate once more using the same standard PCR machine (settings: plate, 15 µl Volume, heated lid)

	temperature	time
	22 °C	40 min
	4 °C	∞

107 transfer plate back On ice as soon as samples PCR machine has cooled to 4 °C, spin briefly to collect all liquid in the bottom of the wells

108 carefully open lids and add 30 µL library amplification master mix:

	reagent	volume/reaction
	Library amplification buffer	25 µl
	Library amplification enzyme	1 µl
	EvaGreen	2.5 µl
	Nuclease free water	1.5 µl

109 add 5 µL indexing reagent (total volume 50 µl) - to avoid cross-contamination spray index lid with DNAZap and wipe dry, change gloves after each index

NOTE: carefully consider your sequencing requirements and plan which/how many libraries you are going to pool in each lane and index samples accordingly. see below for details of the standard Illumina indices supplied with the MicroPlex Library preparation kit v2 (12 indices, Diagenode #C05010012,). a kit with 48 indices is also available: MicroPlex Library Preparation Kit v2 (48 indexes, Diagenode #C05010014).

	index number	index ID	index sequence
	1	iPCRtagT1	ATCACGTT
	2	iPCRtagT2	CGATGTTT
	3	iPCRtagT3	TTAGGCAT
	4	iPCRtagT4	TGACCACT
	5	iPCRtagT5	ACAGTGGT
	6	iPCRtagT6	GCCAATGT
	7	iPCRtagT7	CAGATCTG
	8	iPCRtagT8	ACTTGATG

	9	iPCRtagT9	GATCAGCG
	10	iPCRtagT10	TAGCTTGT
	11	iPCRtagT11	GGCTACAG
	12	iPCRtagT12	CTTGTACT

110 gently mix by pipetting, use sealing foil and 1300 x g, 4°C, 00:02:00

111 run on real time quantitative PCR machine (Roche Lightcycler 480) to monitor library amplification

Equipment

LightCycler® 480 Instrument II — NAME

real-time quantitative PCR machine — TYPE

Roche — BRAND

05015278001 — SKU

		temperature	time	ramp rate
	extension	72 °C	3 min	3° C/sec
	cleavage	85 °C	2 min	3° C/sec
	denaturation	95 °C	2 min	3° C/sec
	addition of indices	98 °C	20 sec	3° C/sec
		67 °C	20 sec	2.2 °C/sec

		72 °C	40 sec	3 °C/ sec
	repeat steps 6 to 8 four times			
	library amplification	98 °C	20 sec	3 °C/ sec
		72 °C	50 sec: record fluorescence using "single acquisition"	2.2 ° C/sec
	repeat steps 11 & 12 for x number of cycles*			
	cool - hold	37 °C **	1 h	2.2 ° C/sec

* monitor fluorescence after each cycle: the optimal phase is reached when Fluorescence (465-510) linearly increases to 3.5 – 4.5. at this point stop library amplification and move to step 15 - cooling. we find it takes approx 8 - 13 cycles to amplify libraries sufficiently. the exact number of cycles will depend on how much chromatin your antibody pulls down.
** 37°C is the lowest temperature the Roche Lightcycler 480 will cool to: transfer plate containing amplified library to ice 1 min after 37°C is reached

112 use 1 µL of amplified library to quantify the amount of DNA with **Qubit dsDNA HS assay Kit** according to manufacturers instruction

Qubit™ dsDNA HS Assay Kit **Thermo Fisher Catalog #**Q32851

Equipment

Qubit Fluorometer	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238	LINK

Expected result

5 - 15 ng/ µl
if concentration is significantly lower return sample to real time PCR machine for extra amplification

113 dilute 1 µL of amplified library in 4 µL TE to asses DNA intergrity and size distribution using Bioanalyzer **High Sensitivity DNA Kit** according to manufacturers instructions

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #**5067-4626

Equipment

2100 Bioanalyzer Instrument — NAME

Sizing, quantification, and sample quality control of DNA, RNA, and proteins on a single platform — TYPE

Agilent Technologies — BRAND

G2939BA — SKU

Expected result

bell curve 200 - 2000 bp, average size approx. 400 bp
amplified_library.pdf

114 pool libraries with different indices at equal molarities in 1.5 ml DNA LoBind tube (aim to reach a volume just over 100 µL)

Note

molecular mass of dsDNA = 660 [g/mol/bp]

calculation:
concentration [ng/ul] * 10^6 * 1/660 * 1/average size [bp] = molarity [nM]

115 transfer library from plate to 1.5 ml DNA LoBind tube (keep On ice)

NOTE: libraries (individual or pooled) can be stored at -20 °C for up to 2 weeks

Library purification using AMPure beads

4h

116 NOTE: work in area designated for library preperation (distinct from low input DNA area), use designated library preparation pipettes with sterile RNAse and DNAse free tips, clean area and pipettes as well as all other equipment with 1 % Distel, 70 % Ethanol and DNA-ZAP before starting.

117 bring pooled libraries, **AMPure beads** and freshly prepared 80 % Ethanol to Room temperature

Agencourt AMPure XP magnetic beads **Beckman Coulter Catalog #**A63880

118 resuspend AMPure beads until homogenous solution and add 100 µL to 100 µL of pooled library (1:1 ratio); mix until homogenous

119 incubate at Room temperature for 00:05:00

120 spin briefly (00:00:03) to collect all liquid in the bottom of the tube

121 place tube in DynaMag- 2 magnet, wait for 00:02:00 until all beads are bound (solution clear)

122 discard supernatant

123 add 300 µL 80 % Ethanol

124 rotate the tube clockwise by 90 °, wait for 00:00:10 and repeat 3 more times 10s

125 discard supernatant

126 add 300 µL 80 % Ethanol

127 rotate the tube clockwise by 90 °, wait for 00:00:10 and repeat 3 more times 10s

128 discard supernatant

129 spin briefly (00:00:03) to collect all liquid in the bottom of the tube

130 place tube in DynaMag- 2 magnet, wait for 00:02:00

131 remove all residual Ethanol

132 remove tube from magnet and dry the beads with lid open for max 00:02:00 in ThermoMixer (37 °C)

133 resuspend the beads in 50 µL TE, spin briefly (00:00:03) to collect all liquid in the bottom of the tube

134 place tube in magnet, wait for 00:02:00

135 carefully transfer the eluted DNA to a new 1.5 ml DNA LoBind tube

136 use 1 µL of pooled purified library to quantify the amount of DNA with **Qubit dsDNA HS assay Kit** according to manufacturers instruction (see step 112 for details)

Expected result

expect 10 ng/ µl (i.e. a total of 500 ng in 50 µl)

137 dilute 1 µL of pooled purified library in 4 µL TE to asses integrity and size distribution of libraries using Bioanalyzer **High Sensitivity DNA Kit** according to manufacturers instructions (see step 113 for details)

Expected result

bell curve 200 - 2000 bp (all small fragments removed by AMPure XP bead purification), average size approx. 400 bp

pooled_purified_library.pdf

138 pooled, purified libraries can be stored at -20 °C before sequencing for up to 2 months

Note

our samples were sequenced by Edinburgh Genomics https://genomics.ed.ac.uk on the Illumina NovaSeq S1 yielding approx 750 x 10^6 100 bp paired end reads per lane. we aimed for a depth of 70 x 10^6 paired end reads for each sample. ChIPed samples (for all different histone modifications) and matched input sample should be sequenced on the same lane. we used the motif discovery software HOMER for data anlaysis (**http://homer.ucsd.edu/homer/**). our ChIPseq data is publicly available: GEO accession number **GSE150478**.

NOTE: we subscribe to the notion that ChIPseq is *qualitative* (it can reveal the presence or absence of a histone modification at a particular genomic location) - not *quantitative* (it does not reveal biologically meaningful differences in peak height, which are often influenced by the efficiency of immunoprecipitation). please consider this when analysing your results.

CITATION

Ma Z, Wang H, Cai Y, Wang H, Niu K, Wu X, Ma H, Yang Y, Tong W, Liu F, Liu Z, Zhang Y, Liu R, Zhu ZJ, Liu N (2018). Epigenetic drift of H3K27me3 in aging links glycolysis to healthy longevity in Drosophila.. eLife.

LINK

https://doi.org/pii:e35368.10.7554/eLife.35368

CITATION

Orlando DA, Chen MW, Brown VE, Solanki S, Choi YJ, Olson ER, Fritz CC, Bradner JE, Guenther MG (2014). Quantitative ChIP-Seq normalization reveals global modulation of the epigenome.. Cell reports.

LINK

https://doi.org/10.1016/j.celrep.2014.10.018

Citations

Step 34

Egan B, Yuan CC, Craske ML, Labhart P, Guler GD, Arnott D, Maile TM, Busby J, Henry C, Kelly TK, Tindell CA, Jhunjhunwala S, Zhao F, Hatton C, Bryant BM, Classon M, Trojer P. An Alternative Approach to ChIP-Seq Normalization Enables Detection of Genome-Wide Changes in Histone H3 Lysine 27 Trimethylation upon EZH2 Inhibition.
https://doi.org/10.1371/journal.pone.0166438