Ultra-low-input native ChIP-seq for rare cell populations

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Abstract

Combined chromatin immunoprecipitation and next generation sequencing \(ChIP-seq) has become an extremely popular method to generate genome-wide epigenetic profiles from numerous cell lines and tissue types. Typical ChIP-seq experiments require large number of cells, making them ill-adapted to the study of rare cell populations. This procedure describes an ultra-low-input \(ULI) micrococcal nuclease-based native ChIP \(NChIP) and sequencing library construction method to generate genome-wide chromatin profiles from as few as 10^3 cells \(Brind'Amour et al., 10.1038/ncomms7033). In addition, ULI-NChIP-seq has been validated in vivo, by generation of H3K9me3 and H3K27me3 profiles from E13.5 primordial germ cells isolated from single embryos \(Liu, Brind'Amour et al., 10.1101/gad.244848.114). ULI-NChIP-seq should be useful to generate high quality and complexity libraries from rare cell populations, allowing to decrease colony breeding size or to analyze rare clinical samples. Due to often variable cell numbers obtained during isolation of in vivo cell population, the procedure described here allows for flexibility, with some suggestions on adaptation of buffer or volume conditions at various points during the procedure.

Introduction

Overview of the ULI-NChIP procedure See figure in Figures section.

Reagents

EQUIPMENT Vortex Rotator Cold Room Magnetic rack for 200 µl PCR strip tubes Magnetic rack for 1.5 ml sample tubes Agilent TapeStation or Agilent Bioanalyser **REAGENTS** 100 mM dATP solution \(New England Biolabs _N0440S_) 10× MNase digestion buffer \(New England Biolabs _M0247_) 25 mM dNTP solution \(New England Biolabs _N0447L_) 3M Sodium acetate solution \(Sigma-Aldrich _71196_) 50% Polyethylene glycol 6000 \(PEG 6000) solution \(Sigma-Aldrich _81304_) Agencourt Ampure XP DNA purification kit \(Beckman-Coulter _A63880_) Buffer EB \(Qiagen _19086_) DL-Dithiothreitol \(DTT, Sigma-Aldrich _D9779_) Ethanol Ethylenediaminetetraacetic acid \(EDTA, Sigma-Aldrich _EDS_) Glycerol \(Sigma-Aldrich _G5516_) Klenow fragment \(3' to 5' exo-, New England Biolabs _M0212L_) Linear polyacrylamide \(LPA, Sigma-Aldrich _56575_) MaxTract High Density tubes \(Qiagen _129046_) Micrococcal nuclease \(MNase, New England Biolabs _M0247_) Nuclear isolation buffer \(Sigma-Aldrich _NUC-101_) Phosphate Buffered Saline \(Life Technologies _10010-023_) Phusion HF Buffer Master Mix \(New England Biolabs _F531S_) Polymerase II large fragment \(Klenow, New England Biolabs _M0210L_) Protein A Dynabeads \(Life Technologies _1006D_) Protein G Dynabeads \(Life Technologies _1007D_) Quick DNA ligation kit \(New England Biolabs _M2200L_) Sodium chloride \(Sigma-Aldrich _746398_) Sodium deoxycholate \(Sigma-Aldrich _D6750_) T4 DNA ligase reaction buffer \(New England Biolabs _B0202S_) T4 DNA polymerase \(New England Biolabs _M0203L_) T4 polynucleotide kinase \(T4 PNK, New England Biolabs _M0201L_) Trizma base \(Sigma-Aldrich _T1503_) Triton X-100 \(Sigma-Aldrich _T8787_) Ultrapure H_2O \(Life Technologies _10977-023_) UltraPure Phenol:Chloroform:Isoamyl Alcohol \(25:24:1, v/v) \(Life Technologies _15593-031_) **SOLUTIONS** **Complete

immunoprecipitation buffer** 20 mM Tris-HCl pH 8.0 2 mM EDTA 150 mM NaCl 0.1% Triton X-100 1x Protease inhibitor cocktail 1 mM PMSF **Elution buffer** 100 mM NaHCO3 1% SDS **Triton-Deoxycholate solution** \(in H_20) 1% \(w/v) Triton X-100 1% \(w/v) sodium deoxycholate **Low salt wash solution** 20 mM Tris-HCl pH 8.0 2 mM EDTA 150 mM NaCl 1% Triton X-100 0.1% SDS **High salt wash solution** 20 mM Tris-HCl pH 8.0 2 mM EDTA 500 mM NaCl 1% Triton X-100 0.1% SDS

Equipment

Vortex Rotator Cold Room Magnetic rack for 200 µl PCR strip tubes Magnetic rack for 1.5 ml sample tubes Agilent TapeStation or Agilent Bioanalyser

Procedure

1. ULI-NChIP PROCEDURE **A. SAMPLE ISOLATION BY FLOW CYTOMETRY** _As ULI-NChIP is dilution-based, the conditions of cell isolation and storage are important for the subsequent steps in the procedure. When the expected isolated cell number is sufficient for the formation of a visible cell pellet, we suggest sorting the cells directly in PBS containing protease inhibitor cocktail and pelleted prior to flash freezing and storage. However, sample loss is much higher when the isolated cell number is insufficient for the formation of a visible pellet and thus, rarer cell types can be sorted and stored directly in the nuclear isolation buffer that will be used later during micrococcal nuclease \(MNase) digestion of chromatin \(**B**). There is a certain degree of flexibility in the isolation condition, and cell numbers higher than 2×10^4 cells can also be sorted directly in nuclear isolation buffer, as long as the volume of nuclear isolation buffer is appropriately adjusted._ **When expecting > 2×10^4 cells** 1. Label clean 1.5 ml sample tubes. 2. Add 100 µl of PBS containing 1x protease inhibitor cocktail. 3. Sort desired cell population directly in the 1.5 ml sample tubes. 4. Pellet cells by centrifugation \(500 g for 5 minutes at 4°C). 5. Remove supernatant. 6. Flash freeze cell pellets by immersing sample tubes in liquid nitrogen for 0.5 minute. 7. Store samples at -80°C. Cell pellets can be stored from a few weeks to a few years. **When expecting < 2×10^4 cells** 1. Label clean 1.5 ml sample tubes. 2. Add 10-20 µl of complete nuclear isolation buffer to each tube. Suggested volumes of buffer per expected sample size are presented in **Table 1**. 3. Sort desired cell population directly in complete nuclear isolation buffer. 4. Estimate the total volume of sample, cells and sheath buffer. The contribution of sheath buffer to the final volume should remain below 1/3 of the final volume. 5. \[optional] If sheath buffer contribution is above 1/3 of the final volume: i) Pellet cells by centrifugation \(500 g for 5 minutes at 4°C). ii) Remove all but 10 µl of nuclear isolation buffer + sheath buffer. iii) Add 10 µl of complete nuclear isolation buffer. 6. Flash freeze samples in complete nuclear isolation buffer by immersing sample tubes in liquid nitrogen for 0.5 minute. 7. Store samples at -80°C. Samples can be stored from a few weeks to a few years, and be pooled together in subsequent steps. **Table 1: Suggested conditions for cell isolation and storage** See figure in Figures section. **B. CHROMATIN PREPARATION** _Preparation of MNase fragmented chromatin is one of the most critical steps of native ChIP. Insufficiently digested chromatin will lead to input bias and higher background levels, while the yield of overdigested chromatin will be reduced._ _A small amount of

detergents is added to the nuclear isolation buffer to increase accessibility of MNase to the chromatin, and PEG 6000 is included as a crowding agent to allow for more uniform digestion of smaller inputs. Please note that MNase is very sensitive to temperature changes, and will lose efficiency with time. Small aliquots of MNase should be prepared and transported to the bench no more than 2-3 times for consistent results. It is recommended to test each new batch of MNase with low numbers of cultured cells prior to use for in vivo samples._ 8. Thaw frozen cell pellet or, for lower cell numbers, cells in nuclear isolation buffer. _At this stage, samples isolated on different days can be pooled together._ 9. \[optional] If cells are frozen as a pellet, re-suspend in appropriate volume of complete nuclear isolation buffer \(**Table 2**). 10. Pipette samples up and down 15-20 times while swirling and place back on ice. 11. Prepare dilute MNAse stock enzyme in MNAse dilution buffer to 200 U/µl \(0.5 µl stock in 4.5 µl MNase dilution buffer). **\!CAUTION\!** Add MNase enzyme only when you are ready to proceed to digestion. 12. Prepare MNAse digestion buffer as described in **Table 2** \(always prepare just before digestion). 13. Place samples on rack at 21-25°C. 14. Add MNase master mix \(+MNase enzyme) to each sample. Mix very well \(15-20 times) with pipettor. **\!CAUTION\!** When digesting multiple samples at the same time, it is important to leave the MNase master mix on ice. 15. Allow reaction to proceed according to the conditions suggested in **table 2** . 16. Stop reaction by adding 10% of the reaction volume of 100 uM EDTA and mix very well with pipette \(20-30 times). Place samples immediately on ice. 17. Add 1% Triton/1% deoxycholate solution as per indicated in **Table 2** . 18. Rest samples on ice for 15 minutes. 19. Vortex samples \(medium setting) for approximately 30 seconds. 20. Add complete immunoprecipitation buffer to the digested chromatin. Digested chromatin should take < 25% of the immunoprecipitation volume. Depending on the amount of cells available and digestion volume, immunoprecipitation will be performed in a 100-200 µl final volume. 21. Rotate chromatin at 4°C for 1 hour. 22. Vortex \(medium setting) for 30 seconds 23. Take out an input control aliquot \(for small samples, inputs tend to be larger than for normal ChIP, to reduce errors due to low cell numbers. For < 20,000 cells input per immunoprecipitation, the input should be approximately 10% of the sample). 24. This input can be readily extracted if you want to control for fragmentation size. Add 10% volume of 10% SDS, mix well and add EB to obtain a final volume of 100 µl. Proceed to DNA extraction as described in **Section D**. **Table 2: Suggested conditions for MNase digestion of native chromatin** See figure in Figures section. **C. CHROMATIN IMMUNOPRECIPITATION** _A challenge of performing ChIP on very small samples is finding the balance between obtaining sufficient material for sequencing and using conditions that are stringent enough to reduce the unavoidable background associated to such samples. A good knowledge of the antigen being pulled down and of the antibody used should guide the experimental design. Antigen that can be pulled down at a higher frequency allow for the use of smaller samples, while it may be difficult to generate high resolution enrichment profiles of a mark which is present in only a small percentage of a cell population. This protocol has been successfully used down to 103 cells using the following antibodies; H3K9me3 \(Active Motif #39161), H3K27me3 \(Diagenode pAb-069-050), H3K36me3 \(Abcam #9050) and H3K4me1 \(Upstate #07-436). This protocol has been successfully used down to 5 × 103 cells using the following antibody; H3K4me3 \(Abcam #1012), but remains to be tested for smaller inputs._ 25. Pre-wash Protein A/G magnetic beads \(Dynabeads, Life Technologies #1006D and 1007D) 3 times in complete immunoprecipitation buffer. Each

immunoprecipitation will require 5 µl \(<10,000 cells input) or 10 µl \(10-100,000 cells input) of beads for pre-clearing the chromatin and 5-10µl of beads for immunoprecipitation. 26. Prepare antibody beads complexes \(as described in **Table 3 \(A)**) in 200 µl PCR strip tubes. Rotate at 4°C for 3-8 hours. 27. Pre-clear chromatin \(**Table 3 \(B)**) prepared in **section B**: add 5 or 10µl of pre-washed magnetic beads per immunoprecipitation, depending on input. 28. Rotate for 2-6 hours at 4°C. 29. After incubation, place antibody-beads complexes \(**Table 3 \(A)**) on magnetic rack and take out supernatant. 30. Place pre-cleared chromatin \(**Table 3 \(B)**) on a magnetic rack. Transfer chromatin \(supernatant) to antibody-beads complexes. 31. Rotate overnight at 4°C. 32. Place PCR strip tubes on a magnetic rack. 33. Discard unbound chromatin. 34. Re-suspend the beads in complete immunoprecipitation buffer and transfer to labeled 1.5 ml sample tubes. 35. Place on a magnetic rack. Remove supernatant. 36. Wash the antibody-beads complexes as follows: i) Re-suspend beads in 200 µl low salt wash buffer. Take out the wash buffer and repeat. ii) Re-suspend beads in 200 µl high salt wash buffer. Take out the wash buffer. iii) Re-suspend beads in 200 µl high salt wash buffer. Transfer beads to a clean 1.5 ml sample tube. iv) Take out wash buffer. Close the tubes and pulse-spin the beads. Use a gel loading pipette tip to remove the last few drops of wash buffer. 37. Re-suspend the antibody-beads complexes in 30 µl freshly prepared ChIP elution buffer. 38. Elute DNA for 1-1.5 hours in a 65°C water bath. Vortex tubes regularly. 39. Pulse-spin the sample tubes and place on a magnetic rack. 40. Transfer the eluted chromatin to a clean 1.5 ml sample tube. 41. Wash the beads with 70 µl of EB and combine with the eluted chromatin. **Table 3: Suggested conditions for native ChIP** See figure in Figures section. **D. DNA EXTRACTION** _Various methods can be used for extraction and purification of DNA from chromatin samples. As a general rule, it is preferable to avoid any DNA purification procedure that is column-based, as a significant proportion of smaller samples will not be recovered._ 41. Transfer eluted chromatin to a pre-spun phase lock tube. 42. Add 100 µl of phenol: chloroform: isoamyl alcohol 25:24:1 to each sample. Vortex vigorously for 20-30 seconds. 43. Spin at 13,000g for 5 minutes at 21-25°C. 44. Transfer the upper phase to a new 1.5 ml tube. **\!CAUTION\!** Make sure to take all the liquid and to not touch the new tube with the tip of the pipette \(which could transfer some of the grease in the following reaction). 45. Add 10 µl of 3M sodium acetate and 1 µl of LPA \(linear polyacrylamide). Mix well. \(LPA can be replaced by glycogen as a co-precipitating agent). 46. Add 275 µl of cold Ultrapure Ethanol \(keep at -20°C). 47. Mix very well and allow DNA to precipitate for at least 30 minutes \(can be overnight) at -20°C. 48. Spin down DNA at 13,000g for 30 minutes at 4 degrees. 49. Take out supernatant and wash the pellet with 200 µl of freshly prepared 70% ethanol. 50. Allow pellet to dry. 51. Re-suspend in 20 \(input < 20,000 cells) or 30 \(input > 20,000 cells) µl of buffer EB. **NOTE** At this point, if the DNA is re-suspended in a low volume, the concentration of salts/SDS present will interfere with qPCR or the first step of library construction. We recommend re-purifying the DNA with 1.8 volumes of Agencourt Ampure XP beads. **NOTE** RNAse A treatment is recommended prior to qPCR, depending on amplicon. For 1,000 cells ChIP, we typically take out 10-15% of the eluate and dilute it for qPCR. We can run 2-3 multi-copy amplicons with such an aliquot, but there is typically not enough material for single copy genes. **E. DNA PURIFICATION USING AMPURE XP BEADS** 52. Make sure the beads are well re-suspended. Add 1.8x volume \(36 µl if sample was re-suspended in 20 µl EB) of bead slurry per sample. 53. Mix very well with a pipette \(10-12 times). Once the beads have been added to all samples pulse-vortex 3-4 times. 54. Incubate at room temperature

for 10 minutes. 55. Place the samples on a magnetic rack for 2 minutes or until the sample is clear of beads. 56. Remove the liquid from the beads. 57. Wash the beads \(leave on the magnetic rack) twice with 100 µl of freshly prepared 70% ethanol. 58. Take out the ethanol, pulse-spin and remove the last bit of ethanol using a gel loading tip. 59. Allow the beads to air dry. 60. Add 20 \(< 20,000 cells input) or 30 \(>20,000 cells input) µl of buffer EB to the beads, making sure they are well covered. 61. Allow beads to rehydrate at room temperature for approximately 5 minutes \(they are rehydrated when you can re-suspend them by flicking the tube). Pipette up and down 15-20 times to re-suspend completely. 62. Place the tubes back on the magnetic rack. 63. Transfer the eluted DNA buffer to a new tube, taking care not to transfer any magnetic beads. **2. LIBRARY CONSTRUCTION** _Raw ChIP material generated by ULI-NChIP-seq will range from a few pg to a few ng of DNA, depending on the input size and antigen. There are various methods and kits that can be used for construction of libraries from low inputs of material. Described here is an adapter ligation-based followed by PCR amplification library construction procedure that works from picograms of DNA material. Indexed libraries can be pooled prior to size selection, thus reducing sequencing costs and the number of PCR cycles required to generate sufficient amounts of material for sequencing._ **F. END REPAIR OF FRAGMENTED DNA** 64. Thaw ChIP samples \(in 20 or 30 µl of EB buffer). Keep on ice until End Repair Master Mix is prepared. 65. Prepare the End Repair Master Mix in a clean 1.5 ml tube as follows: See figure in Figures section. **NOTE** 10X Phosphorylation buffer is T4 DNA ligase buffer \(NEB B0202S) 66. Mix thoroughly and pulse spin. Keep master mix on ice while distributing to samples. 67. Add 5 \(if <10,000 cells per ChIP) or 7.5 µl \(if >10,000 cells per ChIP) of End Repair Master Mix to each sample tube. Mix very well \(10-15 times) using pipettor. 68. Incubate at room temperature for 30 minutes. 69. Proceed to DNA purification using Ampure XP beads \(**steps 52-63**). In **step 60**, elute DNA in 17 \(< 20,000 cells input) or 25.5 \(> 20,000 cells input) µl of EB. **G. A-TAILING** 70. Set the heating block or waterbath at 37°C. 71. Thaw and pulse-spin end-repaired \(or digested with blunt cutter) samples \(in 17 or 25.5 µl buffer EB). 72. Prepare A-tailing Master Mix in a clean tube as follows: See figure in Figures section. 73. Vortex and pulse-spin A-tailing Master Mix and add 3 or 4.5 µl to each sample. Mix thoroughly by pipetting/stirring. 74. Incubate at 37°C for 30 minutes. 75. Proceed to DNA purification using Ampure XP beads \(**steps 52-63**). In **step 60**, elute DNA in 6.67 \(< 20,000 cells input) or 10 \(> 20,000 cells input) µl of EB. **H. ADAPTER LIGATION** 76. Prepare Adaptor Ligation Master Mix in a clean tube as follows: See figure in Figures section. 77. Add 13.34 or 20 µl of Adaptor Ligation Master Mix to each sample, mixing very well. 78. Incubate at 21-25°C for at least 30 minutes. Can be extended to O/N ligation. 79. Proceed to DNA purification using Ampure XP beads \(**steps 52-63**). In **step 52**, add 0.8x volume \(16 or 24 µl of Ampure XP beads). In **step 60**, elute DNA in 11 µl of EB. **I. LIBRARY AMPLIFICATION** 80. Prepare PCR Master Mix as follows \(keep on ice until use): See figure in Figures section. 81. Distribute 1 µl of the appropriate indexed primers PE 2.x into a PCR plate or 8-tubes strips. 82. Distribute 13.5 µl of PCR Master Mix into sample tubes. 83. Add 10.5 µl of adaptor-ligated samples to PCR tubes and mix very well \(10-20 times) with pipette. 84. Pulse-spin tubes or plate 85. Run following PCR program \(select appropriate, depending on the material to amplify, suggested in **Table 8**): Hot start 98°C 4:00 × 10-18 amplification cycles \(see **Table 8**) 98°C 0:30 65°C 0:30 72°C 0:30 1 cycle: 72°C 5:00 12°C \(hold) **Table 8: Suggested number amplification cycles of constructed libraries depending on input size and immunoprecipitated chromatin mark** See figure in

Figures section. 86. Proceed to DNA purification using Ampure XP beads \(**steps 52-63**). In **step 52**, add 0.8x volume \(16 or 24 µl of Ampure XP beads). In **step 60**, elute DNA in 15 µl of EB. 87. Evaluate library yield and quality using an Agilent Bioalalyzer \(High Sensitivity DNA detection kit) or Agilent TapeStation \(D1000 High Sensitivity screentape). **NOTE** Due to the small amount of template in the library construction procedure, it is normal at this stage to observe a large amount of adapter dimers. These will be removed prior to sequencing. **3. LIBRARY POOLING AND SIZE SELECTION** _Libraries should be pooled according to the number of reads that are required. As low input libraries will have a tendency to have a higher proportion of unmapped and duplicate reads, it is a good idea to sequence deeper than the number of distinct reads required. As low-input samples are often from precious samples, we recommend using paired-end sequencing, which will enable inferring exact fragments size as reducing the number of reads that will be flagged as duplicates. For heterochromatic marks such as H3K9me3 and H3K27me3, we recommend sequencing 30-40 million read pairs \(60-80 million reads). For H3K4me3, sequencing 5-15 million read pairs \(10-30 million reads) should yield sufficient distinct reads to generate high resolution peaks._ 88. Pool libraries to be sequenced together according to their molarity and the desired number of reads. 89. Run the pooled libraries on an agarose gel. _In order to prevent having constructed library fragments floating around and potentially contaminating experiments, we recommend using a buffer-free system such as E-gel \(Life Technologies)._ 90. Cut out the 250-650 bp fragments from the gel and transfer to a new sample tube. 91. Proceed to gel extraction of DNA and elute in 15 µl of EB. We recommend the Zymoclean Gel DNA Recovery kit from Zymo Research. 92. Confirm the absence of adapter dimers \(~ 130 bp) in the purified library pool using an Agilent Bioalalyzer \(High Sensitivity DNA detection kit) or Agilent TapeStation \(D1000 High Sensitivity screentape).

Troubleshooting

Table 9: Troubleshooting guide See figure in Figures section. **Figure 2: Illustration of ideal MNase fragmentation** See figure in Figures section. **Figure 3: Example illustrating the effect of antibody concentration on H3K9me3 ULI-NChIP profiles** See figure in Figures section.

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Figures

	1,000-5,000 cells (µl)	5,000-20,000 (µl)	>20,000 (µl)
Nuclear isolation buffer (Sigma NUC-101)	10	20	-
PBS	-	-	20-50
	Flash freeze. Keep at -80°C.	Spin down and reduce volume to 10 µl. Flash freeze. Keep at -80°C.	Spin down, remove PBS and flash freeze pellet. Keep at -80°C.

Figure 1

Table 1 Suggested conditions for cell isolation and storage

	1,000,000 cells (µl)	100,000 cells (µl)	5,000-10,000 cells (µl)	1,000 cells (µl)
Nuclear isolation buffer (+ sheath)	50	20	~20	~10
1% Triton, 1% DOC sol.	-	-	2	1
MNase Master Mix				
10 X MNase Buffer (NEB)	6	4	10	5
200 mM DTT	0.44	0.37	0.75	0.5
50% PEG 6000 (Sigma 81304)	-	-	10	5
1:10 MNase enzyme (NEB M0247)	0.6	0.4	1	0.5
Ultrapure H_20	2.96	15.23	58.76	29.38
Final MNase Concentration	$2U*µl^{-1}$	$2U*µl^{-1}$	$2U*µl^{-1}$	$2U*µl^{-1}$
Digestion	**7.5 minutes @ 37°C**	**5 minutes @ 37°C**	**7.5 minutes @ 21°C**	**7.5 minutes @ 21°C**
100 mM EDTA	6.6	4.4	11	5.5
1% Triton, 1% DOC sol.	6.6	4.4	8	4
Vortex, 30 seconds				

Figure 2

Table 2 Suggested conditions for MNase digestion of native chromatin

	1,000-10,000 cells	100,000 cells
A- Antibody-beads complex		
Complete IP buffer	100 µl	100 µl
Protein A:protein G Dynabeads 1:1	5 µl	10 µl
Antibody	0.25 µg	1 µg
Incubate > 3hrs on a rotator at 4°C		
B- Chromatin		
Chromatin lysate	10-50 µl	10-50 µl
Complete IP buffer	to 100-200 µl*	to 100-200 µl*
Protein A:protein G Dynabeads 1:1 (Life Technologies 1006D and 1007D)	5 µl	10 µl
Incubate > 2 hrs on a rotator at 4°C		
Immunoprecipitation		
Take out NChIP buffer from tube A.		
Transfer chromatin lysate from tube B to beads from tube A		
Incubate overnight (8-12 hours) on a rotator at 4°C		

Figure 3

Table 3 Suggested conditions for native ChIP

	100-10,000 cells per ChIP (µl)	>10,000 cells per ChIP (µl)
Raw ChIP material in EB	20	30
End Repair Master Mix		
***10X Phosphorylation buffer**	2.5	3.75
10 mM dNTP mix (NEB N0447L)	0.5	1.5
T4 DNA polymerase (NEB M0203L)	0.5	0.75
Klenow DNA polymerase (NEB M0210L)	0.1	0.15
T4 PNK (NEB M0201L)	0.5	0.75
Ultrapure H_2O	0.4	0.6
Total master mix volume	*5*	*7.5*

Figure 4

Table 4 Conditions for end-repair of ChIP material

	100-10,000 cells per ChIP (µl)	>10,000 cells per ChIP (µl)
End-repaired/blunt-ended material in EB	17	25.5
A-tailing Master Mix		
10X NEB buffer 2	2	3
10 mM dATP (NEB N0440S)	0.5	0.75
Klenow (3'-5' exo-) (NEB M0212L)	0.5	0.75
Total master mix volume	*3*	*4.5*

Figure 5

Table 5 Conditions for A-tailing of end-repaired material

	100-10,000 cells per ChIP (µl)	>10,000 cells per ChIP (µl)
Adapter ligated material in EB	10.5	10.5
10 µM Illumina paired-end indexed primer 2.x (different for each sample)	1	1
PCR amplification master mix		
2X Phusion HF Master Mix (NEB M0531L)	12.5	12.5
10 µM Illumina paired-end primer 1.0	1	1
Total master mix volume	*13.5*	*13.5*

Figure 6

Table 6 Conditions for Illumina adapter ligation on A-tailed material

	100-10,000 cells per ChIP (µl)	>10,000 cells per ChIP (µl)
A-tailed material in EB	6.67	10
Adaptor ligation master mix		
2x Quick DNA ligation buffer (NEB)	10	15
1 µM annealed Illumina adapters	0.67	1
Quick DNA ligase (NEB M2200L)	2.67	4
Total master mix volume	*13.34*	*20*

Figure 7

Table 7 Conditions for PCR amplification of constructed Illumina libraries

Cells in ChIP:	**1,000-10,000 cells**	**100,000 cells**
H3K9me3	10-12	8-10
H3K27me3	10-12	8-10
H3K4me3	14-15	12

Figure 8

Table 8 Suggested number amplification cycles of constructed libraries depending on input size and immunoprecipitated chromatin mark

Problem	Possible Cause	Solution
1. Improper MNase fragmentation	**1.1 Underdigestion**	
	1.1.1 MNase has gone through too many freeze-thaw cycles	**1.1.1** Use a fresh batch of MNase (test each new batch), aliquot each batch of enzyme, use benchtop cooler (not ice)to carry enzyme to bench.
	1.1.2 MNase added too early to MNase Master Mix (all Ca++ used up)	**1.1.2** Add MNase to Master Mix immediately before proceeding to digestion. Keep on ice while distributing. Do not digest more than 6-10 samples at a time (per batch of Master Mix).
	1.1.3 MNase has no access to the nucleus	**1.1.3** Make sure complete nuclear isolation buffer represents > 2/3 of the final volume cells are sorted in. Double the concentration of Triton-Deoxycholate solution in nuclear preparation prior to adding MNase Master Mix.
	1.1.4 Final EDTA concentration too high	**1.1.4** Reduce EDTA concentration in digestion buffer (i.e increase digestion volume).
	1.1.5 Insufficient digestion time	**1.1.5** Increase digestion time.
	1.1.6 Digestion temperature too low	**1.1.6** Proceed to digestion in a waterbath adjusted at 25 or 37°C, depending on input size.
	1.2 Overdigestion	
	1.2.1 MNase concentration is too high for cell type	**1.2.1** Reduce MNase concentration in MNase Master Mix
	1.2.2 Digestion time is too long for cell type	**1.2.2** Reduce digestion time or temperature.
	1.3 Combination of under- and over digestion	
	1.3.1 Sample is not mixed properly	**1.3.1** Swirl the sample while pipetting up and down 15-20 times when adding MNase Master Mix. Vortex lightly after adding MNase Master Mix.
	1.3.2 MNase does not have access to all nuclei in suspension	**1.3.2** Mix properly cells in complete nuclear isolation buffer + Triton/Deoxycholate by pipetting up and down 10-15 times and incubate the sample for ~15 minutes on ice prior to adding the MNase Master Mix.
	1.3.3 MNase has gone through too many freeze/thaw cycles	**1.3.3** Use a fresh batch of MNase (test each new batch), aliquot each batch of enzyme, use benchtop cooler (not ice)to carry enzyme to bench.
2. Very low yield of constructed library	*If adapter dimers are present in the constructed library:*	
	2.1 Loss of raw ChIP material.	
	2.1.1 Use of a column-based DNA purification method.	**2.1.1** Not well adapted for very small DNA samples. Purify DNA with suggested phenol:chloroform and Ampure XP beads procedures.
	2.1.2 Excess salt in eluted ChIP material.	**2.1.2** Excess salt interferes with End-repair, leading to very low complexity libraries. Add a second 70% ethanol wash after ethanol DNA precipitation. As suggested, follow phenol:chloroform DNA extraction/ethanol precipitation by an Ampure XP beads DNA purification.
	2.1.3 No co-precipitating agent during ethanol precipitation or co-precipitating agent not mixed in with the sample.	**2.1.3** Add LPA to phenol:chloroform extracted DNA prior to add NaOAc and ethanol. Make sure to mix well the LPA with the extracted DNA.
	2.1.4 Mark covers a small portion of the genome	**2.1.4** If a mark covers <2-3% of the genome, additional PCR cycles could be required during library construction.
	If no adapter dimers are present in the constructed library:	
	2.2 Inefficient library construction	
	2.2.1 Low quality Illumina adapters	**2.2.1** Use new batch of adapters. Test new batch of adapters against an aliquot that is known to work (always keep one aliquot to test new adapter batches).
	2.2.2 Inefficient adapter ligation step	**2.2.2** Make sure all components of the adapter ligation Master Mix are present. Use fresh batch of ligase enzyme.
	2.2.3 Amplification of constructed libraries failed	**2.2.3** Repeat PCR amplification step. Use fresh aliquots of PE primers and enzyme.
3. High background signal	**3.1** Antibody concentration is too high	**3.1** Titrate down antibody concentration. Signal-to-noise ratio can be estimated by qPCR at known positive and negative loci. We suggest performing qPCR on constructed and amplified material for ChIP on very low inputs.
	3.2 Non-specific signal is insufficiently washed	**3.2** Increase the volumes of the washes. Transfer antibody beads complexes into a new 1.5 ml sample tube
	3.3 Nature of the histone mark	**3.3** Some histone marks (i.e H3K9me2) have a broader distribution and thus have a lower signal to noise ratio. These should be sequenced more deeply.
4. Low ChIP signal	**4.1** Antibody concentration is too low	**4.1** Titrate up the antibody concentration.
	4.2 Poor antibody quality	**4.2** Only use ChIP grade antibodies
	4.3 Antigen is very rare	**4.3** Increase the input size for ChIP on this antigen.
	4.4 Post immunoprecipitation washes are too harsh	**4.4** Replace the two high salt washes by two additional low salt washes.

Figure 9

Table 9 Troubleshooting guide

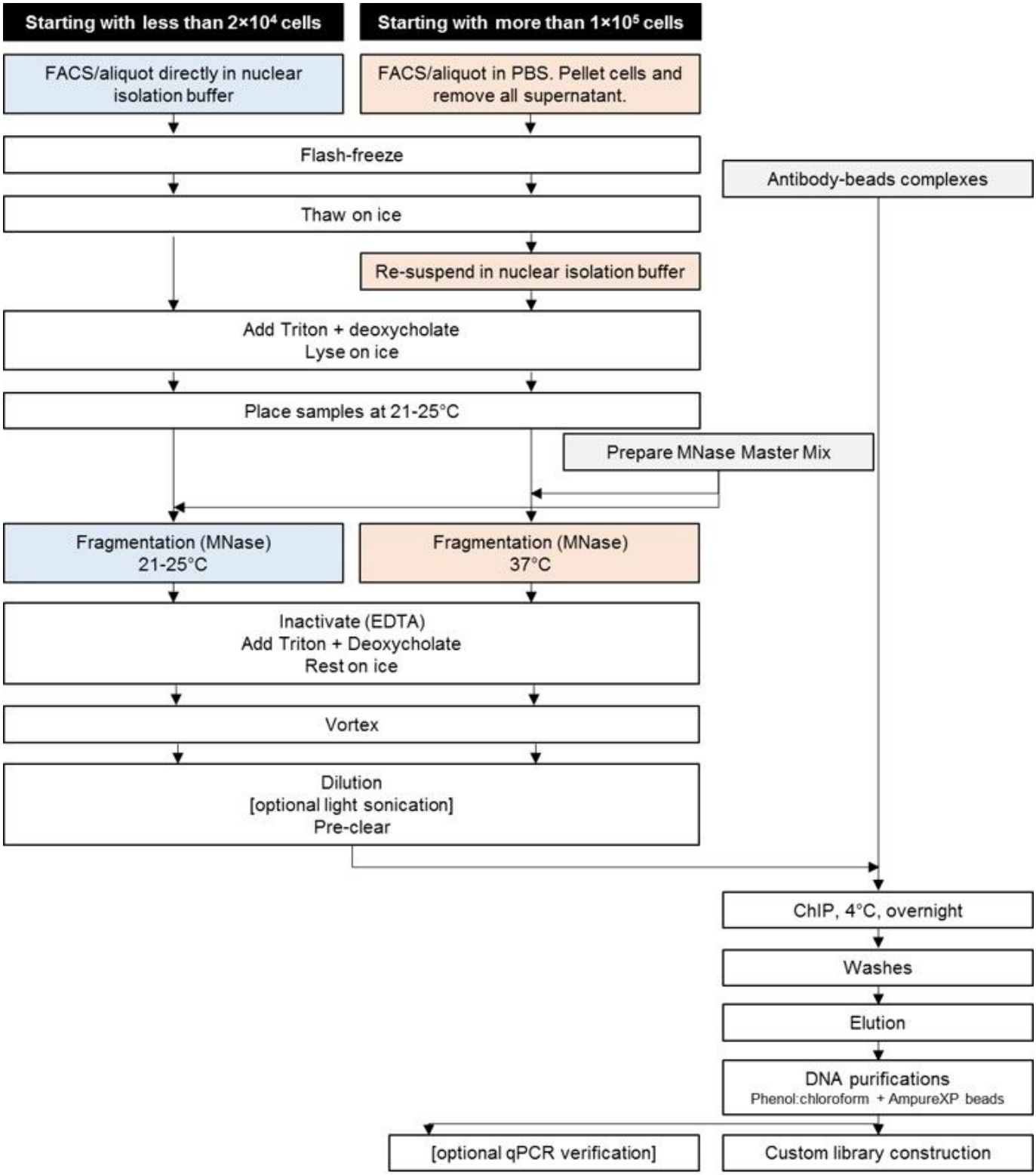

Figure 10

Figure 1 Overview of the ULI-NChIP procedure

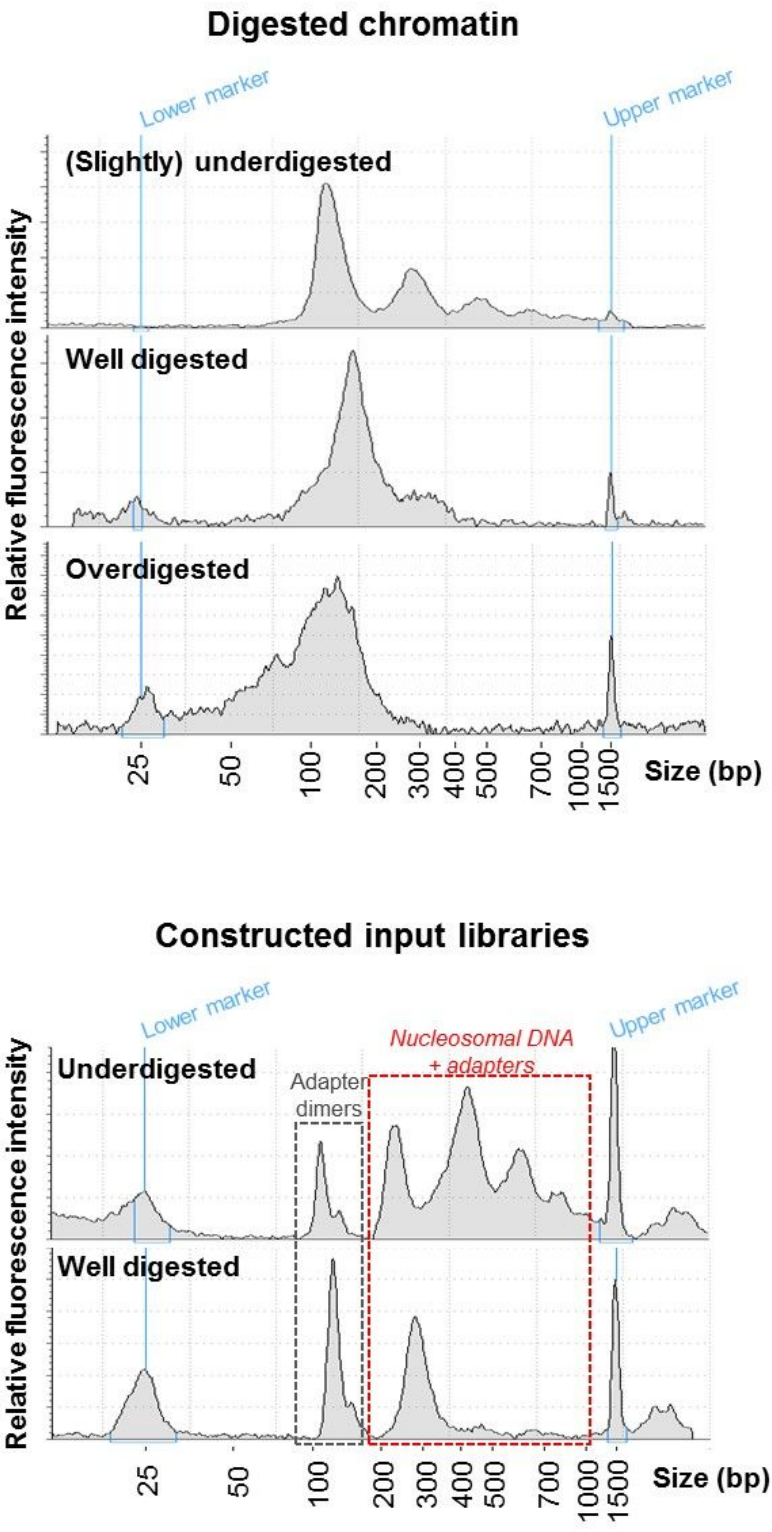

Figure 11

Figure 2 MNase fragmentation

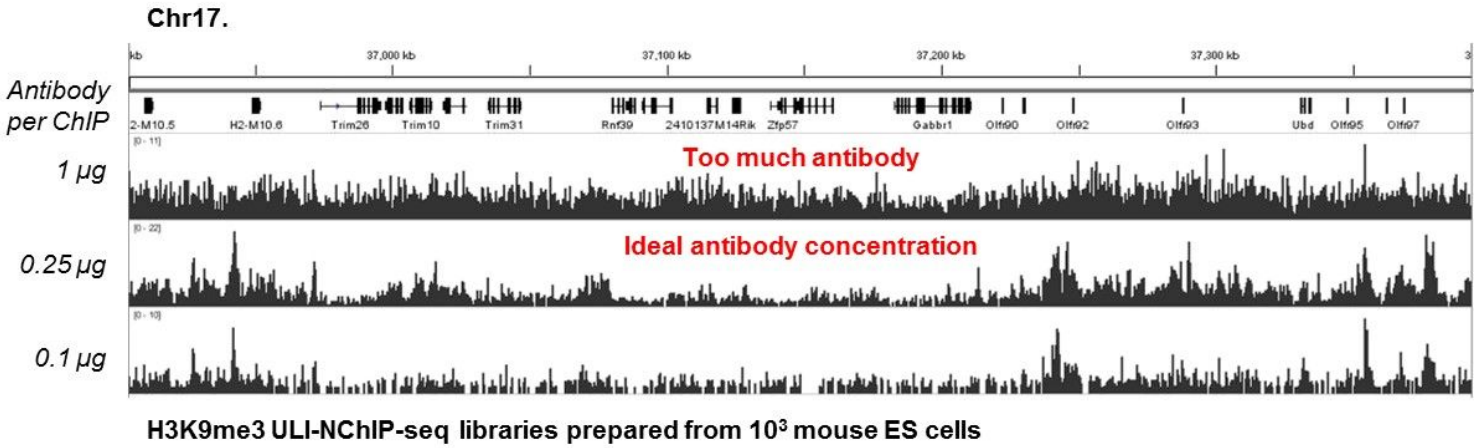

Figure 12

Figure 3 Effect of antibody titration on H3K9me3 ULI-NChIP profiles