Native ChIP Using 100,000 Cells

I. Purpose

To provide details for immunoprecipitation reactions using 100,000 cells from cells or tissues

II. Scope

All procedures are applicable to the BCGSC Library Core and Library TechD groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QS associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Production Coordinator to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
ndChIP-seq	EGL017.07
Preparation and QC of SeraMag Bead Solution	EGL008.2

VI. Related Documents

Document Title	Document Number
N/A	

VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material safety data sheet (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number	Model or Catalogue #	
Small size safetouch nitrile gloves	Ultident	296359683		✓
Wet ice	In house	N/A	N/A	N/A
Ice bucket	Fisher	11-675-58		✓
1.5 mL Microtubes	Diamed	PRE150-B		✓
15 mL Conical Tubes	BD Falcon	352097		✓
50 mL Conical Tubes	BD Falcon	352070		
Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		✓
Gilson P200 pipetman	Mandel	GF-23601		✓
Gilson P1000 pipetman	Mandel	GF-23602		✓
Diamond Filter tips DFL10 (10 Tipacks of 96 racked filter tips)	Mandel Scientific	GF-F171203		✓
Diamond Filter tips DFL30 (10 Tipacks of 96 racked filter tips)	Mandel Scientific	GF-F171303		✓
Diamond Filter tips DFL200 (10 Tipacks of 96 racked filter tips)	Mandel Scientific	GF-F171503		✓
Diamond Filter tips DFL1000 (10 Tipacks of 96 racked filter tips)	Mandel Scientific	GF-F171703		✓
Galaxy mini-centrifuge	VWR	37000-700		✓
Large Kimwipes (Kimberly Clark/Kimtech)	Fisher Scientific	06-666-1A		✓
Black ink permanent marker pen	VWR	52877-310		✓
Small Autoclave waste bags 10”X15”	Fisher Scientific	01-826-4		✓
Ultra Pure Water (RNase/DNase free)	Invitrogen	10977-023		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	Commercial Alcohols	People Soft ID: 23878		✓
DNA Away	Molecular Bioproducts	7010		✓
1M Tris Solution, pH 8.0	Ambion	AM9856		✓
Triton® X-100, laboratory grade	Sigma	X100-100ML		✓
20% SDS Solution	Ambion	AM9820		✓
Centrifuge, Eppendorf 5417R, refrigerated high-speed, 115V	Fisher Scientific	5417 R	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
Eppendorf Thermomixer 1.5 mL	Eppendorf	21516-166	✓	
Parafilm	Fisher Scientific	13-374-12		✓
Water Bath	Fisher Scientific	Isotempn 220	✓	
5M NaCl	Ambion	AM9760G		✓
1M DTT	Invitrogen	P2325		✓
1M Tris HCl pH 7.5	Invitrogen	15567-027		✓
0.5M EDTA	Ambion	AM9260G		✓
Deoxycholic Acid, Sodium Salt	Fisher Scientific	AC218590250		✓

Sodium Bicarbonate	Sigma Aldrich	S5761-500G		✓
PCR Clean ChIPSeq Beads with 30% PEG	Aline Biosciences	C-1007		✓
0.2 mL Ultra Rigid Skirted 96 well PCR plate	Thermoscientific	FSSP9741450		✓
Micrococcal Nuclease	NEB	M0247S		✓
Nonstick 1.5 mL tubes	Ambion	AM12450		✓
1M DTT	Invitrogen	P2325		✓
RNase Away	Invitrogen	10328-011		✓
Dynabeads Protein A,5 mL size	Invitrogen	100-02D		✓
Protease Inhibitor Cocktail	Calbiochem	539134		✓
LabQuake Shaker/Rotator with clips	Barnstead	415110	✓	
Buffer G2	Qiagen	1014636		✓
Qiagen Protease	Qiagen	19155		✓
DynaMag 2 Magnet	Invitrogen	12321D		✓
Disposable Trough	VWR	21007-972		✓
Multi 12-channel Pipette P20	Rainin	17013803		✓
Multi 12-channel Pipette P200	Rainin	17013805		✓
EB Buffer	Qiagen	19086		✓
96-well Plate Magnet	Alpaqua	002523		✓
Tape Pads: Adhesive Plate Sealer	Qiagen	19570		✓
Aluminum Foils	VWR	60941-126		✓
Rainin 200µl barrier tips	Rainin	RT-L200F		✓
Rainin 20µl barrier Tips	Rainin	RT-L20F		✓
Sodium Butyrate	Millipore	19-137		✓
Sodium Azide	Sigma-Aldrich	S2002-100G		✓
Liquid Nitrogen	Praxair	NI M-FILTER		✓
Dry Ice	In house	N/A		✓
Steel plate	In house	N/A		✓
Liquid Nitrogen Cooled Mini Mortar and Pestle Set, Bel-Art	VWR	89233-994		✓
Single Edge Industrial Razor Blades	VWR	55411-050		✓
Sterile Disposable Forceps	VWR	12576-934		✓
Sterile Cell Culture Dishes (150mmx25mm)	Fisher Scientific	08-772-25		✓
Sterile Cell Culture Dishes (60mmx16mm)	Fisher Scientific	08-772-31		✓
H3K4me1 Antibody	Consult Supervisor	N/A		
H3K4me3 Antibody	Consult Supervisor	N/A		
H3K9me3 Antibody	Consult Supervisor	N/A		
H3K27me3 Antibody	Consult Supervisor	N/A		
H3K36me3 Antibody	Consult Supervisor	N/A		
H3K27ac Antibody	Consult Supervisor	N/A		
PCR tube strip, Domed 12 cap strip	Biorad	TCS 1201		✓
Eppendorf Thermomixer C	Fisher Scientific	05-412-503		✓
96 Well PCR plate Smartblock for Thermomixer C	Fisher Scientific	05-412-512		✓
T36 Disinfex	VWR	CA11007-034		✓
PEG-8000 (molecular biology grade)	Sigma-Aldrich	P5413-500G		✓
Seramag speed beads	Fisher Scientific	09-981-123		✓

Portable Pipet Aid,Multispeed XP, rechargeable	Fisher Scientific	13-681-15E		✓
50 mL conical tube	VWR	CA21008-940		✓
15 mL conical tube	VWR	CA21008-918		✓

IX. Procedure

1. Retrieval of reagents and equipment preparation

1.1. Put on a lab coat and clean pair of gloves.

1.2. Wipe down the assigned specific workstation, pipettes, and small equipment.

1.3. Lay down new bench coat.

1.4. Change gloves.

1.5. Confirm with supervisor as to sample type (tissue or cells).

1.5.1. If sample source is cells, verify the number of cells/vial.

1.6. Prepare worksheets keeping in mind:

- What is the sample source (i.e. tissue or cells)
- Number of IPs and what are the targets/antibodies to IP
- Include positive control IPs (100K HL60 cells: whole histone modified panel of antibodies)

1.7. Note that this protocol is optimized for **100,000 cells (100K)/IP**.

1.8. A minimum of 2L of liquid nitrogen is required for tissue samples.

1.9. A small amount of dry ice is required for tissue samples.

1.10. Dilute antibodies if needed. See Appendix B if dilutions are required. Can be prepared ahead of time and stored at -20°C as working aliquots are for one time use.

1.11. Prepare ahead of time 30% PEG SeraMag beads as detailed in Appendix C.

1.12. Ensure all buffers, reagents and enzymes have been validated using the Native ChIP production pipeline up to library qPCR QC.

1.13. Prepare buffers and reagents and keep on ice.

DAY 1

2. Preparation of Ab-Bead Complex

Prior to starting protocol ensure the following:

Prepare worksheets and plate layout before starting protocol

Ensure there is enough diluted stock of antibodies. See Appendix B if not

2.1. Add 20µL of Calbiochem protease inhibitor cocktail (500x) into 10mL of IP Buffer. (diluting PIC to 1x). Mix well and keep on ice.

2.2. Add 100µL of 1M sodium butyrate to give a final concentration of 10mM to IP buffer + 1xPIC. Use this buffer for the entire IP panel as sodium butyrate is a histone deacetylase inhibitor. Mix well and keep on ice.

2.3. Retrieve Dynabeads Protein A from the 4°C and mix the bottle very well to achieve homogenous bead slurry. The beads can be vortexed gently.

2.4. Label a 1.5mL non-stick tube with 'Dyna A beads' and the date.

2.5. Transfer appropriate amount of Dyna A protein beads to the labeled 1.5mL non-stick tube by using the following formula:

(46µL Protein A + dead volume*) x # of IPs = amount of beads
*dead volume is 3 reactions worth of beads

2.6. Place bead tube on magnet stand and let separate. Remove supernatant.

2.7. Remove bead tube from magnet stand and place on ice.

2.8. Add equal volume of IP Buffer + 1xPIC to beads. (1:1 v/v bead : IP buffer)

2.9. Mix by pipetting up and down. Ensure the beads are thoroughly mixed.

2.10. Place bead tube back on magnet stand and let separate. Remove supernatant.

2.11. Repeat wash 2 more times with IP + 1xPIC buffer.

2.12. Resuspend beads in equal volume of IP Buffer + PIC. Keep on ice.

2.13. On ice, aliquot 46µL of washed Dynabeads per well of sample into a new plate.

2.14. On another new plate, aliquot 130µL per well of IP buffer + 1xPIC. A reagent trough can be used for this.

2.15. Add 20µL of washed Dynabeads to each well containing the 130µL of IP buffer + 1xPIC. Mix by pipetting up and down.

2.16. Seal the remaining Dynabeads plate and keep on ice. This will be used for pre-clearing chromatin later.

2.17. Addition of Antibodies:
If necessary dilute the stock antibody to a concentration that is reasonable to pipette. See Appendix B, if dilution of stock antibody is necessary. Do not pipette less than 1µL. Add 0.5-1.0µg of antibody to the wells containing Dynabeads and IP buffer + 1xPIC (to plate from Step 2.15) to generate the Ab-bead complex. See Table 1 below for the amount of antibody to add. Ensure that a positive control IP has been set up as well using H3K4me3 antibody.

Name	Amount (µg)
H3K4me3	0.75
H3K4me1	0.5
H3K27me3	0.5
H3K36me3	1.0
H3K27ac	0.5
H3K9me3	0.5

Table 1: Amount of Antibody

2.18. Set multi-channel P200 pipette to 100µL and mix each row up and down 10 times. Change tips between each row.

2.19. Cover the Ab-bead complex plate with BioRad domed cap strip lids securely. Ensure the beads are mixed thoroughly and no air pockets are visible in the wells. If there are air pockets, gently flick the lid(s) of the wells(s) to disrupt the air pocket.

2.20. Incubate the plate at 4°C on a rotating platform for a minimum of 2.5 hours or until pre-clearing is done.

2.21. Proceed to Step 5 after incubation.

3. Cell Lysis and MNase I Digestion of Chromatin

3.1. Retrieve lysis buffer from 4°C and place on ice.

3.2. Aliquot 10mL into 15mL tube.

3.3. Add 20µL of Calbiochem Protease Inhibitor cocktail (500x) to the buffer. Add 100µLof 1M sodium butyrate, which is an inhibitor of histone deacetylase, to the Lysis Buffer to give a final concentration 10mM. Mix and keep on ice.

3.4. Prepare sample for lysis and MNase I digestion.

For fresh or frozen 100K cells follow Steps 3.5.1. For tissue samples follow Step 3.6.

3.5. **For Fresh/Frozen Cells:**

3.5.1. Verify the number of cells/vial prior to proceeding as mentioned in Step 1.5.1. Consult with supervisor if unsure.

3.5.2. Depending on the number of cells/vial and the number of IPs to set up: Thaw 100K cells/1 IP + 1 extra IP (to account for loss) at 37°C for a few seconds in a water bath.

****For a complete histone panel, use 700,000 cells for 100K IPs. Equally divide the extra 100K cells amongst the 6 IPs of a whole panel; 117K cells / well****

*****Any reference to a '100K' IP using a cells sample includes the extra 17K cells*****

Note: If the sample has more than the # of cells required for the Native ChIP process, supervisor will advise to split the frozen cell stock. In that case, please refer to the following instruction in step 3.5.2.1-3.5.2.13:

3.5.2.1. In a 15mL falcon tube, pipette 5mL of PBS and add 10uL of 500x PIC. Keep on ice.

3.5.2.2. Pre-chill Eppendorf centrifuge to 4˚C.

3.5.2.3. Retrieve the source cell tube in a small styrofoam box containing dry ice.

3.5.2.4. Label a pre-chilled 1.5mL Eppendorf tubes for each cell aliquot.

3.5.2.5. Thaw the source tube quickly either by hand or by dipping in a 37˚C water bath.

3.5.2.6. Gently flick the source tube to ensure all the cells are thawed and evenly suspended.

3.5.2.7. Add an appropriate amount of PBS containing 1xPIC based on the number of cells reported by the collaborator.

3.5.2.8. Mix the cells by pipetting up and down gently to ensure there are no cell clumps.

3.5.2.9. Aliquot out, the volume of cells needed into a previously pre-chilled 1.5mL Eppendorf tube.

3.5.2.10. Spin down both the source and cell aliquot tubes at 2,655g (5,000rpm in Eppendorf Centrifuge 5417R), for 5 minutes, at 4˚C.

3.5.2.11. Pipette off and discard the supernatant from both the source and aliquot cell tubes.

3.5.2.12. Place the source tube back on dry ice

3.5.2.13. With the cell aliquot, proceed to Step 3.5.3.

3.5.3. Top up each 100K worth of cells with 30µL of cold Lysis buffer + 1xPIC per IP. Scale up if necessary. Pipette up and down at least 10 times to mix. Make sure there are no clumps.

3.5.4. Also thaw out a pellet of HL60 cells as a positive control. Follow Step 3.6.20.

3.5.5. Transfer each 100K cell lysate to each well of a 96 well plate. Refer to the plate layout worksheet to ensure that the correct lysate is transferred to the correct well in the Ab-bead complex plate.

3.5.6. Cover the plate with a plastic seal and incubate on ice for 20 minutes. During this incubation prepare MNase I dilution and master mix as described in Step 3.7.

3.6. **For Tissue Samples:**

3.6.1. Ensure 2L of liquid nitrogen and a small quantity of dry ice is present in the lab before starting.

3.6.2. To cut a piece of tissue from the original source, collect the following items per 1 **sample** and put them in the -80°C for at least 1 hour prior to cutting the tissue. It is also acceptable to put the apparatus and supplies in the -80°C overnight. **It is crucial that all reagents and supplies used in cutting and grinding of the tissue remain extremely cold.**

- Steel plate
- 2 razor blades
- 1 disposable forcep
- 1 small petri dish

- large lid of a styrofoam box
- 1 non-stick 1.5mL tube
- 15mL tube (to hold disinfected pestle)
- Mini Mortar and Metal Pestle Set (ensure it is thoroughly disinfected with DNA Away, rinsed with sterile ultrapure water, and then wiped with 70% ethanol.

***If working with multiple tissue samples, gather all the supplies needed. See Steps 3.6.23-3.6.30 for additional set up of processing multiple tissue samples.**

3.6.3. Prepare the BSC by turning it on for 15-20 minutes before use. After purging the BSC, wipe it clean with T36 disinfectant. Wipe with 70% ethanol to remove traces of T36 disinfectant. Tape a small biohazard waste bag to the inside wall to dispose off any biohazard waste.

3.6.4. Wipe down the bottom of the non-consumable supplies with 70% ethanol and transfer all items from Step 3.6.2 into the BSC carefully. It is not necessary to wipe down the steel plate as it was previously decontaminated.

3.6.5. Transfer dry ice into the large styrofoam lid, in a single layer. Lay the steel plate onto the dish. Onto the lid containing dry ice, place the remaining supplies except the mini mortar. Ensure the supplies are fully covered by dry ice.

3.6.6. Carefully transfer liquid nitrogen into the mortar bowl. Fill the bowl about half way to avoid spilling once the mortar is placed back onto it. Tightly secure the mortar onto the bowl to prevent rapid evaporation of the liquid nitrogen. Place the 1.5mL non-stick tube in the tube holder of the mortar bowl. See Figure 1 for set up.

Figure 1: Adding liquid nitrogen to mortar bowl

3.6.7. Wait until the mortar and the bowl become 'frosty' to start. This will take around 5 minutes. The apparatus will stay extremely cold for about 10 minutes before the liquid nitrogen needs to be topped up. It is important to work fast but safely.

3.6.8. Retrieve the source tissue on dry ice. Leave on dry ice until ready to cut.

3.6.9. Place the small petri dish onto the steel plate, and leave open. Carefully remove paper protection wrap from 1 or 2 razor blades and place in the lid of the petri dish. Try to minimize handling to prevent warming up of supplies.

3.6.10. Change gloves and double gloving is optional. Transfer the source tissue onto the small petri dish. Cut a small piece using the chilled razor blade(s), approximately $2mm^3$. Be extremely careful when cutting the tissue. If necessary, use the lid of the petri dish as a shield to deflect any tissue(s) piece(s) that become airborne. $2mm^3$ of tissue is required only if setting up a whole histone modification panel (6 IPs). Adjust the amount of tissue to be cut if setting up fewer or more IPs. Quickly transfer the source tissue back into source tube and put on dry ice. Consult with supervisor if unsure of size amount.

3.6.11. Transfer the cut tissue into the chilled 1.5mL non-stick tube sitting in the mortar bowl using the razor blade or disposable forceps. Leave the lid open.

3.6.12. Using the chilled metal pestle, apply controlled yet substantial pressure against the frozen tissue piece while turning the pestle within tube. The tissue should pulverize into a powder within a few turns.

3.6.13. **DO NOT** remove the tube from the tube holder of the mortar as this action will thaw the tissue. If it is absolutely necessary to check the grinding efficiency, place the tube immediately on dry ice. Also keeping the metal plunger in the air will warm it up as well. Be aware of all supplies and apparatus as this process is only successful if everything is very cold, at the same time ensure your safety.

3.6.14. After the grinding, place the tube on dry ice.

3.6.15. Remove the sample tube from dry ice and carefully add 30µL of Lysis Buffer + PIC + 10mM sodium butyrate /IP. Scale up if required as noted below. Once the ground up tissue is resuspended, it is safe to put the tube on wet ice. With the tube in one hand, ensure the ground up tissue thaws as it is being lysed at the same time.

For an entire histone modification panel: (6 IPs + 1 extra for loss) 30µL=210µL Lysis Buffer + PIC +** 10mM Sodium butyrate **

3.6.16. Pulse spin very gently if necessary to bring down any lysis buffer from the sides of the tube. Leave sample tube on wet ice for 10 minutes once the ground up tissue has thawed. During the 10 minutes incubation proceed to Step 3.6.20 at the 6 minutes mark.

3.6.17. Pulse spin the tube on a microfuge to pull down cellular debris. Gently put back on ice to avoid disturbing the pellet.

3.6.18. Carefully and thoroughly transfer the supernatant, which contains the chromatin, to a fresh 1.5mL non-stick tube. Avoid disturbing the pellet.

3.6.19. Put sample tube back on wet ice and equally distribute the supernatant amongst wells assigned for an IP of a 96-well plate. See plate layout worksheet. For example, if the entire histone modification panel of antibodies is going to be used then equally divide the supernatant amongst 6 assigned wells.

3.6.20. Pull out HL60 control cells from the -80°C freezer on dry ice. Each cell pellet is at 1 million cells/100µL of lysis buffer 1xPIC +10mM Sodium butyrate. Add 150µL of lysis buffer + PIC+ 10mM sodium butyrate, thaw and resuspend cells simultaneously. Submerge the tube in a 37°C water bath for a quick thaw, is an option as well. Aliquot 30µL of cell suspension to appropriate assigned wells

according the plate layout worksheet. There will be approximately 117K cells/well for a 100K IP.

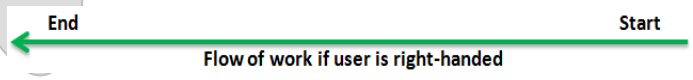
3.6.21. Cover the plate with a seal and let incubate on ice for 20 minutes. It is safe to go over by 5-10 minutes since there is PIC present in the lysis buffer.

3.6.22. During the 20 minutes incubation, proceed to Step 3.8-3.16 BUT DO NOT ADD the micrococcal nuclease until ready to proceed to the digestion step. After the 20 minutes incubation is complete, proceed to **Step 3.17**.

3.6.23. If processing multiple tissue samples it is necessary to keep the pulverized tissue frozen as subsequent samples are being cut and crushed. Until all samples are processed, they can be thawed all at once for lysis and subsequent steps. Along with the supplies listed in Step 3.6.2, a few additional items are required and are listed below:

- Small container of liquid nitrogen close by in case a top up is required
- 15mL falcon tube(s) containing 9mL DNA Away
- 1 falcon tube to hold disinfected pestle
- squirt bottle with ultrapure water
- P200 pipette and filtered tips
- lid of a large styrofoam box filled with 1 layer of dry ice pellets
- small styrofoam box filled with wet ice to contain lysis buffer
- 1 non-stick tube/sample pre-labeled with sample ID on lid and side of tube with a permanent marker

3.6.24. See Figure 2 below as an example of processing multiple tissue samples. As visualized a substantial amount of supplies are required. Ensure the set up follows proper aseptic techniques as best as possible and the flow of work starts at one end of the BSC and ends at the other, as directed in Figure 2 as well. This also provides guidelines to ensure safety of the user as well. This allows minimum disruption of the airflow as possible.



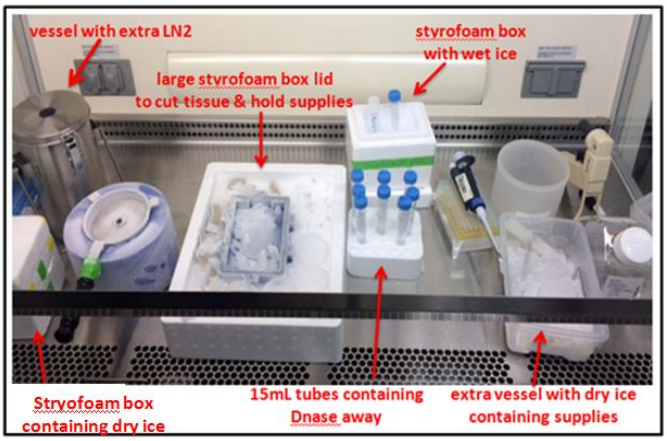


Figure 2: Additional Set Up For Processing Multiple Tissue Samples

3.6.25. Retrieve the tissue source tubes on dry ice. Process 1 sample at a time. After cutting the tissue sample, place any remaining piece(s) back into source tube. Immediately put back on dry ice to avoid thawing. Once the cut tissue piece has been pulverized, snap the lid shut while the tube is still in the mortar.

3.6.26. By holding the tube firmly on the top (closest to the lid), transfer it to box containing dry ice. This is to prevent the tissue from warming up.

3.6.27. Immerse the metal portion of the pestle used to grind the tissue piece into a tube containing DNA Away. Leave for about 1 minute to disinfect. Pull it out, rinse the pestle with ultrapure water and wipe dry with a kimwipe. Remove traces of water by spraying with 70% ethanol and wipe dry. Put the pestle in a clean 15mL tube that has been sitting in dry ice to keep thoroughly cold.

3.6.28. Change gloves and proceed to the next sample.

3.6.29. Once all samples have been processed, safely remove all tubes from liquid nitrogen and add 30µL of lysis buffer + PIC + 10mM sodium butyrate /IP. Scale up as required. Resuspend slowly by pipetting up and down and place on wet ice to let completely thaw. Continue protocol with Step 3.6.16.

3.6.30. When ready, carefully and thoroughly clean and tidy the BSC, discarding all disposable supplies that came into contact with tissue into the biohazard waste bag. Dispose of razors into the sharps container. Soak the mortar bowl, pestle and steel plate in DNA Away and then in ultra pure water to disinfect. Remove traces of water by spraying with 70% ethanol.

3.7. Prepare the MNase I dilution buffer in a 1.5mL tube, as according to Table 2. Make sure to mix it very well and keep it on ice. Make the buffer **fresh** for every use.

Reagent	Amount (µL)
1M Tris, pH 7.5	10
5M NaCl	10
0.5M EDTA	2
Glycerol	500
Ultrapure Water	478

Table 2: MNase I dilution buffer

3.8. Dilute Stock MNase enzyme (2000U/µL):

Amount of Cells	Dilution Factor	Amt Dilution Buffer (µL)	Amt Stock Enzyme (µL)
100K	1/10 (200U/µL)	18	2

Table 3: Dilution of Stock MNase Enzyme

3.9. Mix well by pipetting gently up and down. Scale up if needed. Avoid pipetting less than 2µL of enzyme. Keep diluted enzyme on ice.

3.10. Prepare the MNase I digestion Master Mix in a 1.5mL tube, based on the worksheet.

Reagent	Volume (µL)/Rxn

20mM DTT*	1
MNase (200U/µL)	0.45
10X MNase Buffer	4
Ultrapure Water	4.5
Total/Reaction	10

Table 4: MNase I Digestion Master Mix

*Note: 1M DTT stock is diluted to 200mM which is further diluted 1:10 to get 20mM.

3.11. Mix the brew very well by pipetting up and down slowly. Keep on ice.

3.12. Turn on thermoblock and set temperature to 25°C.

3.13. In a new 96 plate aliquot 10µL of the MNase I Master Mix per each row of samples plus 5µL of dead volume. Keep on ice.

Example: For 2 rows the volume should be (10µL x 2) + 5µL = 25µL/well x # of IPs.

3.14. Aliquot 1% Triton-X100 containing 1% DOC into a new 96-well plate. Label the plate. Aliquot 5.5µL per each row of samples plus 5µL of dead volume. Keep on ice.

Example: For 2 rows the volume should be (5.5µL x 2) + 5µL = 16µL/well x # of IPs.

3.15. In a new 96 well plate, Aliquot 5.5µL of 250µM EDTA per each row of samples plus 5µL of dead volume. Keep at room temperature.

Example: For 2 rows the volume should be (5.5µL x 2) + 5µL = 16µL/well x # of IPs.

3.16. After the lysates finish incubating for 20 minutes on ice, remove the plate from ice and put on the bench for a few minutes.

3.17. It is important to ensure the plate is at room temperature, before proceeding to the next step.

3.18. If necessary, pulse spin the plate at room temp, at 1500rpm.

3.19. Place the plate on the thermoblock preheated to 25°C. Using a P20 multichannel pipette, add 10µL of MNase I Master Mix to the first row of samples.

3.20. Mix by pipetting up and down 15times. Start the timer for 6 minutes. Gently place a cover seal on the plate and let incubate at 25°C on a thermoblock with lid on.

3.21. Proceed to the second row, if required. Make note of the mixing time.

Note: Do not process more than 2 rows at a time to make sure that each digestion is exactly for 6 minutes.

3.22. At the halfway mark of the incubation time, remove seal and mix by pipetting up and down 10 times. Cover with a plastic seal. Change tips between rows.

3.23. Add 5.5µL of 250µM EDTA, with the plate on the thermoblock, to stop the digestion using a P20 multichannel pipette. Set the pipette to 20µL and mix 15 times by pipetting up and down at room temp. Repeat with the second row if necessary. Ensure timing is equal between rows.

Note: This step should be performed so that each row of samples is digested with MNase I for exactly 6 minutes.

3.24. Repeat the process of MNase I digestion + EDTA quenching 2 rows at a time if processing more than 2 rows of samples.

3.25. When completed place the plate on ice.

3.26. Using a P20 multichannel, add 5.5µL of 1% Triton-x100 containing 1% DOC into each row of samples. Adjust the pipette volume to setting to 20µL and mix 15 times by pipetting up and down.

3.27. Seal the plate with a plastic cover, and spin at 200g, for 1 minute, at 4°C.

3.28. Incubate on ice for 20 minutes. Proceed to Step 4.1 during incubation time.

4. Input Separation and Pre-clearing

4.1. While the MNase I digestion plate is incubating, aliquot 130µL of IP Buffer + 1xPIC + 10mM sodium butyrate into 1 row of a new 96-well plate, if working with just one row. Seal plate with plastic cover and keep on ice. If processing multiple rows of samples pour the IP Buffer + 1xPIC + 10mM Sodium butyrate into a disposable plastic reservoir.

4.2. After 20 minutes incubation, using a P200 pipette pool all samples (of the same cell type) into a clean non-stick 1.5mL tube. Mix well by gently inverting tube and pulse spin. Transfer 12µL of the pool into a new non-stick 1.5µL tube. This will be the INPUT DNA for that sample. Store at 4°C until the following day.

4.3. Using a P200 pipette measure the volume of each pool. Note the volume. Distribute the sample equally (amongst number of IPs to set up) into a new 96 well plate. Put plate on ice. (See worksheet for plate layout).

4.4. Add 100-110µL of IP Buffer + 1xPIC + 10mM sodium butyrate (from Step 4.1) to each sample well and mix 10x by pipetting up and down. If the volume of the chromatin/IP is high (>50 µL), add 100 µL. If the volume of the chromatin/IP is <50 µL then, add 110 µL of the IP Buffer + 1xPIC + 10mM Sodium Butyrate per well for the entire IP panel. Make sure that each well is well mixed before proceeding to the next step. Keep plate on ice.

4.5. Retrieve the pre-washed and aliquoted Dynabeads that were kept on ice from Step 2.16.

4.6. Using a P20 multichannel pipette, mix the beads by pipetting up and down a few times. Try not to introduce bubbles.

4.7. Using a P20 multichannel pipette, transfer 20µL of beads to each well of sample. After each addition, mix up and down a few times. Use fresh tips for each row.

4.8. To make sure that the samples are well mixed, use a P200 multichannel pipette set to 100µL and mix each row 10 times by pipetting up and down. Change tips between rows.

4.9. Seal the plate very well with Biorad domed cap strip. Ensure there are no air bubbles present in the wells. If there are, gently flick the wells to pop them.

4.10. Incubate the plate on a rotating platform at 4°C for 1.5 hour.

5. Immunprecipitation Reaction

5.1. Place the Ab-Bead complex (from Step 2.20) on a magnet and wait for the beads to separate.

5.2. Carefully remove and discard the supernatant.

5.3. Remove the plate from the magnet and place it on ice.

5.4. Place the pre-clearing reaction plate (from Step 4.10) on a magnet and wait for the beads to separate.

5.5. Set the P200 multichannel pipette to 160µL and transfer the supernatant from the pre-clearing reaction plate to the Ab-bead complex plate kept on ice. Ensure that no beads are transferred.

5.6. Repeat the transfer using a low volume multichannel pipette to ensure all of the supernatant from the pre-clearing reaction plate has been transferred to the Ab-bead complex plate.

5.7. Gently mix 15 times by pipetting up and down. Ensure no air bubbles are present in the wells. Gently flick the plate if there are to pop them.

5.8. Seal the plate well with domed cap strip and incubate at 4°C on a rotating platform over night.

DAY 2

6. Washes and Elution

6.1. Put on a clean lab coat, fresh gloves, wipe down bench and pipettes with DNA Away. Turn on and set thermomixer temperature setting to 65°C.

6.2. Change gloves.

6.3. Retrieve ice and place Low Salt Wash Buffer and High Salt Buffer on it.

6.4. Prepare Elution Buffer as described in Appendix A.

6.5. Aliquot 40µL of Elution Buffer per row of samples into a new 96-well plate. Cover and store at room temperature.

6.6. Spin down the IP reaction plate (from Step 5.8) at 200g, for 1 minute, at 4°C.

6.7. Place the IP reaction plate on a magnet and wait for the beads to separate.

6.8. Carefully remove and discard the supernatant.

6.9. Remove the IP reaction plate from the magnet and place on ice.

6.10. Pour out 3mL of Low Salt Wash buffer per row of samples into a trough and keep on ice.

6.11. Add 120µL of Low Salt Wash buffer per well to the IP reaction plate.

6.12. Slowly mix 10 times to resuspend the beads.

6.13. Place the IP reaction plate on a magnet.

6.14. Set the multichannel pipette at 130µL and discard the supernatant.

6.15. Repeat the Low Salt buffer wash for a total of 2 washes.

6.16. Place the IP reaction plate on ice.

6.17. Pour out 3mL of High Salt Wash buffer per row of samples into a trough and keep on ice.

6.18. Add 120µL of High Salt Wash buffer per well to the IP reaction plate.

6.19. Slowly mix 10 times to resuspend the beads.

6.20. Place the IP reaction plate on a magnet.

6.21. Set the multichannel pipette at 130µL and discard the supernatant.

6.22. Remove the IP reaction plate from the magnet and place it on ice. Place a new plate next to it.

6.23. Add 120µL of High Salt Wash buffer to each well of the IP reaction plate.

6.24. Slowly mix 10 times to resuspend the beads.

6.25. Transfer the resuspended beads into the new plate. Place the plate on magnet. Do not discard the source plate yet. Keep it on ice.

6.26. Once the bead suspension has started to clear, aspirate 20µL of supernatant from the new plate in Step 6.25 and rinse out the wells of the source plate to make sure all the beads are transferred. Ensure the aspirant is going into the corresponding wells from new plate to source plate and vice versa. Discard source plate.

6.27. Aspirate supernatant from new plate and discard the supernatant.

6.28. Take the plate off the magnet. Keep it at room temperature.

6.29. Add 30µL of Elution buffer to each well and mix slowly 10 times to resuspend the beads.

6.30. Seal the plate with domed cap strip and incubate at 65°C for 1.5 hours with a mixing speed of 1350rpm.

6.31. Check the elution reactions every 15 minutes for the first 30 minute to ensure the beads do not settle. In this step we want to agitate the beads enough to prevent them from settling while avoiding splashing up of the liquid. If the beads are on the bottom, increase the mixing step to 1800rpm for a few seconds.

6.32. Spin down the plate at 200g, for 1 minute, at room temperature.

6.33. Place the elution reaction plate on a magnet.

6.34. Using P200 multichannel set to 50µL in volume. Transfer all of the supernatant to a new 96 well plate. Make sure not to transfer any beads.

6.35. Cover the IP plate and keep at room temperature and proceed to the next step.

7. DNA Purification

7.1. Retrieve INPUT sample tube from 4°C storage.

7.2. Spin down at 200g, for 1 minute, at **ROOM TEMPERATURE** and place at room temperature.

7.3. Add 18µL of Qiagen EB buffer to each Input sample and mix slowly by pipetting up and down 10 times. The final volume should be 30µL, to match the volume of the IPs.

7.4. Using a single channel P200 pipette, transfer the INPUT(s) to the IP plate as according to the plate layout worksheet. Cover the plate.

7.5. On ice, prepare the DNA Purification Master Mix. Scale up as calculated on worksheet.

Reagent	Volume (µL)/Rxn
Qiagen EB	31
Qiagen Buffer G2	8
Qiagen Protease	1.75
Total	**40.75**

Table 5: DNA Purification Master Mix

7.6. On ice, aliquot 40µL of DNA Purification Master Mix per row of samples plus 5µL dead volume into a new 96 well plate.

7.7. Using a P200 multichannel, add 40µL of DNA Purification Master Mix to IP plate (including INPUT wells). Mix slowing up and down 15 times. Change tips after each row.

7.8. Seal the plate with Bio-Rad domed cap strips and incubate in a thermomixer at 50°C for 30 minutes with a speed of 600 rpm (volume should be 70µL).

7.9. In preparation for Step 8, retrieve SeraMag beads and leave at room temperature for at least 30 minute in the dark. In addition, retrieve a new aliquot of 70% EtOH and keep at room temperature.

7.10. After incubation, spin plate at **room temperature,** at 200g, for 1 minute.

8. Manual SeraMag Bead Clean Up of IPs

8.1. Retrieve SeraMag beads (30% PEG) from 4°C fridge and incubate at room temperature for at least 30 minutes before use. It is important that the beads are at room temperature as a cooler solution will yield lower product. Mix the beads well immediately before use. Due to the high percentage of PEG, the solution is quite viscous. Vortexing the tube is required to reach a homogenous suspension.

8.2. Retrieve 5mL of 70% EtOH and place at room temperature. Pour in a plate reservoir and cover for later use.

8.3. In a new plate, add 35µL of Qiagen EB buffer plus 5µL of dead volume per sample well. Cover and leave at room temperature for later use.

8.4. To the IP plate samples, add an equal volume of beads (70µL). As long as the bead volume is equal or just a little more, it is sufficient.

8.5. Mix by pipetting up and down gently 10 times.

8.6. Cover the plate and incubate at room temperature for 10-12 minutes.

8.7. Put the plate on the magnet and let the solution become clear (approximately 10 minutes).

8.8. Remove and discard the supernatant. Leave the plate on magnet.

8.9. Add 150µL of 70% EtOH to each sample well.

8.10. Do not mix the beads and 70% EtOH as this is only a wash. Remove the supernatant.

8.11. Repeat 70% EtOH wash.

8.12. Take the plate off of the magnet.

8.13. Allow the beads to dry which takes a few minutes. (If the beads pellet appears cracked, then they are too dry).

8.14. Add 35µL of Qiagen EB buffer to the sample wells. Thoroughly mix by pipetting up and down at least 10 times or until the mixture appears homogenous. Cover.

8.15. Incubate the plate at room temperature for 3 minutes. Label a new 96 well plate with plate ID, date and initials and cover.

8.16. Place plate back on magnet and let sit for 2-3 minutes.

8.17. Carefully transfer the supernatant to the new 96 well plate pre-labeled in Step 8.15.

8.18. Seal the plate with a foil cover and store at 4°C overnight or at -20°C for long term storage.

Appendix A - Buffer Preparation

NOTE: All buffers have a 2 month expiry unless otherwise noted

Reagent	Stock Concentration	Volume	Final Concentration
IP Buffer			
Tris-HCl (pH 7.5)	1M	200µL	20mM
EDTA	0.5M	40µL	2mM
NaCl	5M	300µL	150mM
Triton X-100**	1% Solution	1mL	0.1%
Deoxycholate*			0.1%
Ultrapure Water	N/A	Up to 10mL	
Sodium Butyrate	1M	100µL	10mM
Protease Inhibitor	Refer to protocol-add at the time of use		

Lysis Buffer			
Triton X-100**	1% Solution	1mL	0.1%
Deoxycholate*			0.1%
Ultrapure Water	N/A	Up to 10mL	
Sodium Butyrate	1M	100µL	10mM
Protease Inhibitor	Refer to protocol-add at the time of use		

Low Salt Wash Buffer			
Tris-HCl (pH 8.0)	1M	200µL	20mM
EDTA	0.5M	40µL	2mM
NaCl	5M	300µL	150mM
Triton-X 100**	10%	1mL	1%
SDS***	10%	100µL	0.1%
Ultrapure Water	N/A	Up to 10mL	

High Salt Wash Buffer			
Tris-HCl (pH 8.0)	1M	200µL	20mM
EDTA	0.5M	40µL	2mM
NaCl	5M	1mL	500mM
Triton-X 100**	10%	1mL	1%
SDS***	10%	100µL	0.1%
Ultrapure Water	N/A	Up to 10mL	
Elution Buffer			
$NaHCO_3$****	1M****	100µL	100mM
SDS	10%	100µL	1%
Ultrapure Water	N/A	800µL	
Make a fresh solution for every use			

Stock Reagents:

*10% Triton X-100:

1. Add 9mL of ultrapure water to a 15mL falcon tube.
2. Cut the end of a P1000 tip and add 1mL of Triton X-100.
3. Vortex/shake the tube until Triton X-100 is completely dissolved.
4. Label and date. Store at 4°C.
5. Expiry date is 2 months.

**1% Triton X-100+1% Deoxycholate:

1. Using analytical scale, weigh out 100mg of sodium deoxycholate into a 15mL falcon tube.
2. Add 1mL of 10% Triton X-100*.
3. Top up to 10mL with ultrapure water.
4. Vortex/shake vigorously until all components are dissolved.
5. Label and date. Store at 4°C.
6. Expiry date is 2 months.

***10% SDS:

1. Add 5mL of 20% SDS solution to a 15mL falcon tube.
2. Top up to 10mL with ultrapure water.
3. Mix gently to resuspend.
4. Label and date. Store at room temperature.
5. Expiry is 6 months.

****1M $NaHCO_3$:

1. Using an analytical scale, weigh out 840mg of $NaHCO_3$ into a 15mL falcon tube.
2. Top up to a final volume of 10mL with ultrapure water.
3. Vortex the solution vigorously until $NaHCO_3$ is completely dissolved.
4. Label and date. Store at room temperature.
5. Expiry date is 1 month.

Note: All buffers, enzymes will need to be validated using the Native ChIP production pipeline up to library qPCR QC prior to being used in production pipelines.

Appendix B - Dilution of Stock Antibodies

- If the concentration of the stock is too high to pipette a minimum of 2µL/IP then it requires a dilution.
- The buffer to use to dilute the stock is the same buffer used to make up the stock, which is provided on the information sheet provided by the supplier with the shipment of the antibody.
- For example, Figure 4 details the components of the storage buffer of the H3K4me1 antibody

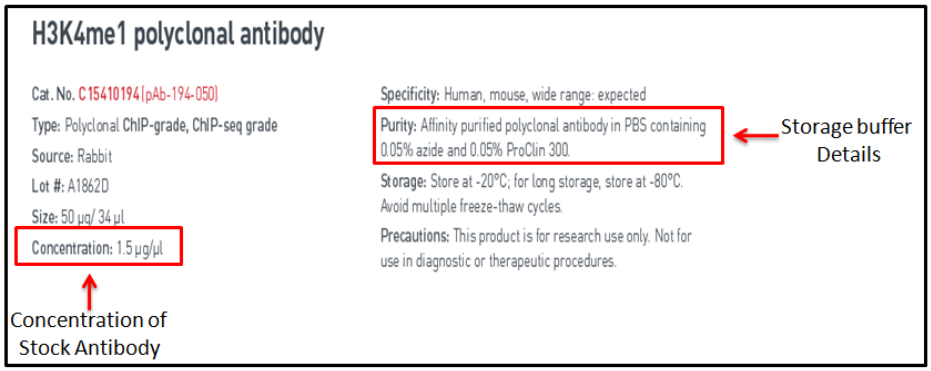


Figure 4: the components of the storage buffer of the H3K4me1 antibody

- For this particular antibody the concentration is 1.5µg/uL.
- The amount of antibody needed per IP is 0.5µg, which is 0.33µL. This is too low to pipette.
- It is recommended to take an aliquot of the antibody and dilute to a concentration that is easy to pipette.
- Consult with supervisor if unsure.

Appendix C - Preparation of 30% PEG SeraMAG Beads

1. 30% PEG in 1M NaCL buffer preparation

1.1. Place a new, sterile 50mL falcon tube on the analytical scale plate (use a styrofoam rack) and zero it. Accurately weigh 15.0g of PEG-8000 directly into the 50 mL tube. Repeat when preparing multiple tubes.

1.2. Add 20mL of ultrapure water (DNase/RNase free distilled water). Close the tube and mix by inverting.

1.3. Add 10mL of 5M NaCl.

1.4. Add 500µL of 1M Tris-HCl (pH8.0).

1.5. Add 100µL of 0.5M EDTA.

1.6. Mix by inverting until PEG goes into the solution and all of the components are uniformly dispersed. The solution should become completely clear. Use a nutating mixer if preparing more than one tube at a time (recommended).

1.7. Label the tube with a permanent marker and keep the mixture at room temperature while washing the beads.

2. Bead Washes

2.1. Remove SeraMag beads from its 4^{o}C storage, mix very well to make sure that the beads are evenly dispersed, aliquot 1mL into a new 1.5mL non-stick tube.

2.2. Place the tube in the DynaMag magnet stand and incubate for 2 minutes. Make sure all the beads are drawn to the sides and the solution is completely clear.

2.3. Remove the supernatant and discard. Be very careful not to remove any beads. Take the tube off the magnet stand.

2.4. Add 1mL of TE buffer to the beads, close the tube and mix by gentle, repeated pulse-vortexing.

2.5. Place the tube back on the magnet and incubate for 2 minutes. Make sure that all of the beads separate to the side and the solution becomes completely clear.

2.6. Remove and discard the supernatant. Be careful not to remove any beads.

2.7. Take the tube off the magnet and repeat TE buffer wash two more times (for a total of 3 washes).

2.8. After the last wash resuspend the beads in 1mL of TE buffer and keep it at room temperature.

3. SeraMag Bead Stock Preparation

3.1. Aliquot 1mL of the washed SeraMag beads to the 30% PEG, 1M NaCl solution. Resuspend the beads by inversion.

3.2. Top up the solution to 50mL with ultrapure water, close the lid and mix by inverting and *gentle*, repeated, pulse vortex until uniformly brown.

3.3. When preparing multiple 50mL conical tubes of beads at a time, mix all vials together in a pre-sterilized glass bottle to create one batch solution. This reduces the number of QC tests.

3.4. Label the tube (or the glass bottle) as “Do not use. QC in progress.”

3.5. Remove an aliquot of the freshly prepared SeraMag bead solution for QC testing. Store the rest at 4°C, **protected from light**.

4. Quality Control Testing

4.1. Test every new batch of beads prepared by performing Native ChIP using 100,000 HL60 cells. IP whole histone mod panel.

4.2. Perform library construction including Quant-iT QC for product amount and Caliper for size distribution.

4.3. Perform qPCR using histone modification target specific primers and calculate fold enrichment.

4.4. Compile all results and confer with supervisor.

4.5. If the bead batch passes all QC gates proceed to Step 5 below to aliquot beads. If batch production was unsuccessful prepare a fresh batch.

5. Aliquot Beads

5.1. After the new batch passes QC, retrieve the newly prepared SeraMag bead working stock from its designated 4°C storage. Make sure the stock fully reaches room temperature (for larger volumes this can take 1h or more).

5.2. Mix the SeraMag bead stock very well by inverting and gentle, repeated, pulse-vortex. Make sure that the beads are uniformly dispersed in the supernatant and that the solution is uniform in color.

5.3. Aliquot 1mL of beads to 1.5mL non-stick pre-labeled tubes.

5.4. Store aliquots at 4°C, **protected from light**. Make sure to label and date each new batch on the tubes and on the storage box.

5.5. Bead solution stored at 4°C and protected from light should be stable for at least 6 months. After 6 months, re-test monthly for performance.

Appendix D:

LIMS:

1. Native ChIP

2. Native ChIP qPCR (to be done after library construction and qPCR QC by supervisor)