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Mint-ChIP3: A low-input ChIP-seq protocol using multiplexed chromatin and T7 amplification

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ABSTRACT

This is a ChIP-seq protocol that uses native chromatin plus MNase digestion, and employs ligation of a barcoded adapter with a T7 promoter after MNase digestion and prior to the immunoprecipitation step. Due to the barcode on the ligated adapter, we have the option to mix multiple (typically 2 to 4) chromatin preps together, prior to the the immunopreciptation step. This adapter barcode becomes an "in-line" barcode, which is read during the first 8 cycles of Read 2 during the paired-end Illumina sequencing. Subsequent to the recovery of ChIP DNA, we will use the T7 promoter contained within the ligated adapter to *in vitro* transcribe the ChIP DNA, followed by reverse transcription and PCR amplification, to complete library construction. The PCR amplification incorporates a conventional Illumina barcode, typically one per antibody in use; primer designs are supplied for both single index and dual index Illumina sequencing. We find this protocol gives excellent results with about 50,000 cells per ChIP using antibodies to histone modifications. This protocol extends the method previously published (van Galen et al, 2016). While conceptually similar to the prior method, it differs in several ways from the prior publication, and 100% of the oligonucleotides have been redesigned to achieve higher efficiency.

GUIDELINES

Many methods exist for mapping histone modifications, almost all based on some form of chromatin immunoprecipitation and sequencing of associated DNA, known as ChIP-seq (Mikkelsen TS et al, 2007. Nature 448,553-60). Mint-ChIP3 has the following advantages over other ChIP-seq methods in common use: 1) High complexity libraries can be obtained from relatively low cell numbers (i.e. 10K to 100K per antibody; we seldom exceed 100K cells per antibody, but in this range, better results are obtained at the higher end); 2) Mint-ChIP3 is validated for both repressive and activating histone modifications; 3) Mint-ChIP3 is amenable to high-throughput processing, and at low cost. Mint-ChIP3 includes several optimizations over the original protocol (van Galen et al, 2016. Mol Cell 61,170-180) and an improved barcode positioning that results in 50-100% more sequencing reads of higher quality. The protocol has been extensively reconfigured, optimized and tested using numerous cell types. Here we describe our current workflow, which results in robust and reproducible ChIP-seq profiles.

Types of Cells: The current protocol has the following properties and constraints: It has been used extensively with cultured cells, primary lymphocytes, and **easily dissociated cells** such as those derived from organoids. Like the prior protocol, it should be applicable to dissociated cells derived using 0.05% trypsin, mechanical disruption, and flow sorting from mouse embryonic tissues (Gonen N et al, 2018. Science 360,1469-1473).

Physical condition of cells: This protocol can work starting either with (a) viable cells, (b) cells isolated and recovered using a flow cytometer, (c) viably frozen cells (i.e. in DMSO), or (d) snap-frozen cells, stored as a dry pellet. In the case of cells stored in DMSO, the cells must first be washed and resuspended in PBS. Regardless of how cells are obtained and stored, it will be necessary to recover the cells in 20 ul of ice cold PBS, at a density not to exceed 250,000 cells per 20 ul.

Limitations: To our knowledge, Mint-CHIP3 has not been used effectively with crude extracts of adult tissues. The current protocol employs **MNase digestion of unfixed chromatin**. To our knowledge, it has not been used with formaldehyde treated, cross-linked chromatin. The current protocol has been used with **antibodies to histone modifications**. To our knowledge, it has not been used effectively with antibodies to transcription factors or other peripheral factors.

Unless noted otherwise, perform all steps in a 96 well plate compatible with a thermal cycler.

Experimental design

Libraries created using Mint-ChIP3 will ultimately be sequenced and demultiplexed using two different types of barcodes -- the adapter barcode, that is ligated to the chromatin, and the Illumina barcode (whether single index or dual index), that is added during library PCR. We offer 8 different adapter barcodes at present, 36 different single index Illumina barcodes, and 8 different dual index Illumina barcodes (please contact the senior author if more dual index barcode designs are required). In our hands, the most typical experimental designs

have involved either (1) a large number of cell samples and only one antibody per cell type, or (2) a small number of cell samples and several antibodies per cell type. The challenge for Mint-ChIP experimental design is to accommodate the needs of each of these kinds of experiments, as well as hybrids of these two extremes. We have obtained excellent results using dual index PCR oligos on the Illumina NovaSeq, where it may be prudent to use dual indices.

The first situation (many samples, one antibody) is relatively straightforward: Let's assume you have about 50K cells from each sample, and a reasonably uniform number of cells across samples. You will set up a different lysis reaction with each sample. Each lysis reaction will take place in 40 ul (20 ul of cells in PBS plus 20 ul of Lysis/MNase mastermix). Following lysis, you will ligate one of the 8 available adapters to each lysate. Following ligation, you can make mixtures of the ligated reactions, taking care to ensure that each chromatin adapter is only represented one time in each mixture. We find that mixtures of 2 to 4 components work best, but up to 8 components are feasible. Remember that each component of the mixture is competing for the antibody, and that any imbalances in cell number will be propagated forward, resulting ultimately in imbalances in the yield of reads following the DNA sequencing. Each mixture should be PCR amplified using a different Illumina barcode PCR primer, to enable multiplex sequencing of distinct libraries derived from distinct cell preps that were ligated to the same chromatin adapter barcode.

The second situation (few samples, but multiple antibodies) poses a further challenge: you will likely need more than 50,000 cells (since we suggest about 50,000 cells per sample per antibody). As your cell number goes up, your lysis volume must eventually go up as well. We find that we can efficiently lyse up to about 250,000 cells in 40 ul (20 ul of cells in PBS plus 20 ul Lysis/MNase mastermix). Above that, efficiency falls and results deteriorate. Thus, if you either wish to use more than 50,000 cells per antibody, or more than 5 antibodies, at some point you have to adjust your lysis volume. As a practical matter, we suggest you divide your lysis into multiple replicate 40 ul aliquots. You will subsequently perform multiple replicate adapter ligation reactions. Since we use PCR plates and thermal cyclers for all steps, we achieve the most uniform and predictable temperature regulation by keeping volumes invariant, regardless of the experimental design. Moreover, given the solutions added at each step (including lysis, MNase digestion, adapter ligation and quenching), the initial lysis in 40 ul grows to 160 ul, approaching the 200 ul limit of most 96 well PCR plates. The replicate ligation reactions (from one or more cell types, each with its own chromatin adaptor barcode) are ultimately pooled in microfuge tubes, and then split to accommodate the diverse antibodies in use during the immunoprecipitation. In these sorts of experimental designs, we typically use a different Illumina barcode PCR primer for each antibody, once again enabling multiplex sequencing of all libraries.

Overview of steps in the protocol

Day 1: The starting material, usually from around 10,000 to 100,000 cells per antibody, either viably frozen or snap-frozen, is lysed and digested with MNase to achieve fragments that are 1-4 nucleosomes in length. After digestion, MNase activity is stopped by addition of EGTA. After EGTA addition, we use a DS DNA Qubit assay on the crude chromatin prep to achieve a non-calibrated, comparative reference value for the quantity of chromatin available. We find this is extremely useful for deciding which chromatin preps may be multiplexed after adapter ligation, and it is predictive of the relative yield of reads per library from the eventual DNA sequencing. We then ligate a custom DS DNA adapter to the digested chromatin. This adapter contains (5' to 3') a three carbon spacer forcing orientation of ligation, a T7 promoter, an Illumina PCR priming sequence, and a unique 8 base pair chromatin demultiplexing sequence. By employing adapters with distinct barcodes, chromatin can be mixed for multiplex processing after adapter ligation. This mixture of chromatin preparations can be divided as needed to afford a distinct aliquot for each intended ChIP antibody. A small aliquot of the chromatin mixture is reserved to enable an antibody free, input control. The immunoprecipitation is set up and typically run over night.

Day 2: After antibody adsorption, the immune complexes are recovered using pre-washed Protein A/G Dynabeads, which are then extensively washed to remove non-specifically adsorbed fragments. Samples are resuspended in ChIP Elution Buffer supplemented with Proteinase K (CEBPK). The reserved mixture(s) of chromatin preps (input controls) are added back to the work flow at this point. The volume of each sample is brought up to 100 ul with the CEBPK, and incubated at 62^{o}C in a thermocycler, to release the DNA from the immune complexes. After 1 hour, a 1x SPRI cleanup is done to retain fragments over 100 bp in length, and exclude unligated adapters. After the SPRI cleanup, the concentration of the recovered ChIP DNA is measured using a DS DNA Qubit assay. ChIP DNA is used to template *in vitro* transcription, which is allowed to run overnight.

Day 3: After the IVT, RNA is isolated using silane beads. The isolated RNA is reverse transcribed, primed using a custom oligo that includes a random hexamer. The resulting single stranded cDNA is subject to 1X SPRI cleanup to select for cDNA fragments greater than 100 bp in length. After the SPRI cleanup, we measure the concentration of the cDNA with an ssDNA Qubit. We then complete library construction by PCR, incorporating an Illumina demultiplexing barcode. After library PCR, a Dual SPRI Cleanup is done to isolate DNA fragments between 200 bp and 700 bp in length. Libraries are assessed by Qubit and Agilent 2100, and combined for multiplex DNA sequencing.

Concluding steps: DNA sequencing is performed using Illumina (single index or dual index) paired end sequencing. The Illumina index (or dual indices) are 8 cycles in length, and the chromatin demultiplexing index, which occurs at the start of Read 2, is a further 8 cycles in

length. The 8 bases of chromatin demultiplexing index must be spliced from the genomic DNA sequence prior to attempting to align sequences to a genome scaffold.

If you have previously used Mint-ChIP (as published, van Galen *et al*, 2016), please note the following crucial differences between Mint-ChIP and Mint-ChIP3 (the current protocol). All of the adapters have been redesigned, with the objective of moving the chromatin index to the start of Read 2, rather than the start of Read 1. Illumina technology (in our hands, on the HiSeq 2500) is relatively intolerant of synthetic DNA sequences in the first five cycles of Read 1. In addition, the chromatin adapters have two additional new features: phosphorothioate linkages in the terminal 3 positions (for nuclease resistance), and a 5' phosphate on the "lower" strand of the adapter (to support ligation). From an operational perspective, there is a key difference in how we perform the protocol. In van Galen *et al*, it was suggested that one end the MNase digestion step and start the adapter ligation step by simultaneous combination of chromatin, EGTA, adapters, and DNA Ligase master mix. In contrast, we first end the MNase digestion by simple addition of EGTA. This affords the opportunity to measure the chromatin concentration using a DS DNA Qubit, which provides a quality check for subsequent steps. We find this is essential, to avoid inadvertently combining chromatin preps with highly dissimilar concentrations (after completing the adapter ligation step). After the Qubit readings are taken, we then sequentially (a) combine the adapters with the chromatin, (b) mix, and (c) add the DNA Ligase master mix. In this way, we minimize the risk of active DNA Ligase encountering undiluted adapters and generating unproductive tail – to – tail adapter dimers.

MATERIALS

NAME	CATALOG #	VENDOR
Micrococcal Nuclease - 320,000 gel units	M0247S	New England Biolabs
Ethanol		
RLT Buffer		Qiagen
MagneSphere(R) Mag. Sep. Stand 12-hole, 1.5ml Vial	Z5342	Promega
HiScribe T7 Quick High Yield RNA Synthesis Kit - 50 rxns	E2050S	New England Biolabs
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific
Agencourt AMPure XP SPRI beads	A63881	Beckman Coulter
Dynabeads Protein A	10002D	Thermo Fisher Scientific
Proteinase K Solution (20 mg/ml) RNA grade	25530-049	Thermo Fisher Scientific
End-It DNA End Repair Kit	ER81050	Lucigen
Fast-Link DNA Ligation Kit	LK6201H	Lucigen
SuperScript™ III First-Strand Synthesis SuperMix	18080-400	Invitrogen - Thermo Fisher
RNase DNase-free	11119915001	Sigma-aldrich
PfuUltra II Hotstart	600850-51	Agilent Technologies
Dynabeads™ MyOne™ Silane	37002D	Thermo Fisher Scientific
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
Qubit™ ssDNA Assay Kit	Q10212	Thermo Fisher Scientific
cOmplete™ EDTA-free Protease Inhibitor Cocktail	4693132001	Roche

MATERIALS TEXT

Pre-mixed Buffer Cocktails: Make in large volumes, sufficient for a series of experiments.

Stored at room temprature, protected from light (since these contain deoxycholate):
2x MINT Lysis Buffer (100mM Tris-HCL- pH 8.0, 300mM NaCl, 2% Triton,0.2% NaDOC)
MINT Lysis Dilution Buffer (50mM Tris-HCl – pH8.0, 150mM NaCl, 1% Triton X-100, 0.1% NaDOC, 50mM EGTA, 50mM EDTA)

Stored at 4C:
Low Salt RIPA (0.1% SDS, 1% Triton X-100, 1mM EDTA, 20mM Tris-HCl pH8.1, 140mM NaCl, 0.1% DOC)
High Salt RIPA (0.1% SDS, 1% Triton X-100, 1mM EDTA, 20mM Tris-HCl pH8.1, 500mM NaCl, 0.1% DOC)
LiCl Wash Buffer (250mM LiCl, 0.5% NP40, 0.5% NaDOC, 1mM EDTA, 10mM Tris-HCl pH 8.1)

Other solutions and standard laboratory reagents:

stored at room temperature:
ChIP Elution Buffer (10mM Tris-HCl- pH 8.0, 5mM EDTA, 300mM NaCl, 0.1% SDS)
Tris EDTA (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0)
PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4)
EGTA (0.5M)

Sodium butyrate (1M) (small aliquots, stored at -20C)

MNase (see source above) should be aliquotted in very small aliquots when freshly purchased, and stored at -80, to ensure that your MNase activity does not drift over a series of experiments.

DNA Sequences of Adapters and Primers:
Sequences of Adapters, random priming RT primer, and PCR primers are available in the following Excel Workbook. For each tab, the column on the far right may be copied and pasted into the bulk order interface of Integrated DNA Technologies (IDT) to effortlessly place your order for oligos.

Mint-ChIP3 all Adapters and Primers v3 clean.xlsx

Apparatus:
To wash Protein A/G beads, and to wash ChIPs in 1.5 ml tubes, you must have a suitable way to subject the tubes to a magnetic field, such as a Promega MagneSphere Magnetic Separation Stand, catalog # Z5342.

SAFETY WARNINGS

Standard precautions must be followed for handling human cell lines and primary human cells and tissues.

BEFORE STARTING

Oligonucleotides. Before you perform this protocol, you will need to purchase, solubilize, and otherwise prepare the following three types of oligos. An Excel spreadsheet attached to this "Materials" section of this protocol supplies all oligo sequences in a format ready for copy / paste into the bulk-entry interface at Integrated DNA Technologies (IDT).

[1] Barcoded DS DNA Adapters to Ligate to the Chromatin. These are the CBE1XX series oligos in the attached Excel spread sheet. You must purchase both "top" and "bottom" strands of each adapter, and then anneal the strands as follows to achieve a DS DNA Adapter. We routinely purchase these oligos at a 100 nMoles synthesis scale, and solubilize them to a concentration of 500 uM (i.e. 200 ul per 100 nMoles; please use the IDT certificate of authenticity (COA) and do not assume you have received 100 nMoles of each oligo). Then, to anneal the oligos to make DS DNA, proceed as follows:

Mix together equal volumes of "top" and "bottom" strands (typically, 125 µl of each to fit in a 250 ul well of a PCR plate).
Cover the plate with a foil seal. Place in a thermal cycler programmed as follows (these are typical temperatures for these oligos and are optimized for a DS DNA molecule with a melting temperature of about 74 degrees C):

1. 95°C for 3 minutes
2. 94–79°C go down 1 degree per 10 seconds
3. 79–69°C go down 1 degree per 30 seconds
4. This represents the annealing step and is specified for the range Tm+5 to Tm-5.
5. 69°C for 5 minutes

6. 69–40°C go down 1 degree per 10 seconds
7. 4°C forever

Annealed adapter is now at 250 µM .
To validate that annealing has been successful, run 0.1 µl of annealed primer on a pre-cast, pre-stained agarose gel such as a Lonza FlashGel, along with a non-paired (single oligo) control lane. Annealed oligo's (dsDNA) are much brighter than single stranded control (ssDNA) due to the binding properties of SYBR green DNA gel stain. The annealing step has a large impact on the efficiency of the protocol. We find that paired oligos (top and bottom strand) that have not been subject to the annealing reaction are nevertheless spontaneously partially annealed, hence they can not serve as a negative control for the success of the annealing protocol; use instead a single stranded oligo as your negative control. After validating successful annealing, aliquot the DS DNA adapters at 5 ul per tube in strip tubes. Do not subject adapters to repeated cycles of thaw - freeze, since the performance of adapters so treated has been observed to fall over time, presumably due to loss of double strandedness.

[2] A single primer with a random hexamer at the 3' end to prime cDNA synthesis. We typically purchase this at the 250 nM synthesis scale. We solubilize this at 500 uM. This oligo is called CBE100-SBS3-RT in the attached Excel spread sheet.

[3] PCR Primers to convert the cDNA to your barcoded DS DNA Illumina sequencing library. These are the CBE2XX series oligos in the attached Excel spread sheet. Please note, we have supplied designs to support both single index and dual index Illumina sequencing.

If performing single index sequencing, each PCR Primer from the CBE2XX series is solubilized at 100 uM and then mixed 1:1 with oligo PvG900-P5_SBS3, also at 100 uM. The following volumes are to be used: 25 ul CBE2XX, 25 ul PvG900, and 200 ul TE. The result is 250 ul of primer mixture, with each primer present at 10 uM. This primer mixture may be subject to repeated rounds of thaw - freeze, but we prefer to aliquot it into several strip tubes to lessen the necessary handling.

If performing dual index sequencing, each PCR Primer from the CBE2XX series is solubilized at 100 uM and then mixed 1:1 with the correspondingly named CBE2XX-Dual-P5 oligo. Volumes are exactly as specified for single index primer pair preparation. Please note, these primer designs are intended to achieve "unique" dual indices, meaning that each I5 index is only used in combination with a single I7 index, and vice versa. This is recommended for Illumina technologies with patterned flowcells, such as the NovaSeq.

Detergents and Buffers. In addition, before you start, you will need to pre-mix and have on hand stocks of 2x MINT Lysis Buffer, MINT Lysis Dilution Buffer, Low Salt RIPA, High Salt RIPA, LiCl Wash Buffer, and ChIP Elution Buffer. Recipes for all of these are supplied herein. Other necessary solutions are prepared in small volumes to suit the experiment underway on the days of the experiment.

Planning and duration. Please note, this protocol takes 3 days to complete. The first day involves preparation of the cells, cell lysis, Mnase digestion, adapter ligation, and the start of overnight immunoprecipitation. The second day involves recovery of ChIP DNA and the start of overnight *in vitro* RNA synthesis. The third day involves the recovery and purification of IVT RNA, reverse transcription, cDNA purification, PCR amplification, and final DNA purification.

Day 1

1

Preliminary Steps

Prechill microfuge to 4°C.

Thaw the chromatin adapters needed per your experimental design and hold on ice.

When preparing the chromatin adapters, make single use aliquots in strip tubes; avoid thaw - freeze cycles.

Prepare the following three master mixes and hold on ice, before taking cells from the freezer.

a. 2X MNase/Lysis Mastermix:

The following yields a 2X lysis buffer with 60 U MNase per 20 ul, which is in routine use in our lab. We typically make excess MNase/Lysis Mastermix, because the MNase is highly concentrated and thus we avoid handling miniscule amounts of MNase. The sodium butyrate can be omitted if no acetyl marks are being ChIPed. The MNase can be used in a range from 30 U to 150 U per 20 ul; the MNase volume can be adjusted accordingly for lower or higher MNase amounts. The 100X Protease Inhibitor cocktail stock is made by dissolving 2 Roche 4693132001 tablets in 1 ml of molecular biology grade water. Store unused 100X stock at 4C and discard unused portion after 2 weeks.

MNase/Lysis Mastermix	
Component	**Volume to add (uL)**
Sodium Butyrate (1M stock)	9.60
Protease Inhibitors (100x)	19.20
2x Lysis Buffer	929.8
Mnase stock (2000 units / ul)	1.44
Total Volume (uL)	**960**

2X MNase/Lysis Master Mix, for 60 Units MNase per 20 ul

b. T7 Adapter Ligation Mastermix for 8 reactions:

When performing 8 reactions, we make sufficient mastermix (MM) for 9 reactions, to accommodate any small losses that may occur during pipetting.

Component	Kit	Per Reaction (uL)	9xMM (ul)
1x End-Repair Buffer (10x stock)	End-It DNA End-Repair Kit or Fast-Link DNA Ligation Kit	8	72
1.5 mM ATP (10mM stock)	End-It DNA End-Repair Kit or Fast-Link DNA Ligation Kit	12	108
1x dNTP Mix (10x stock)	End-It DNA End-Repair Kit	8	72
End Repair Enzyme Mix	End-It DNA End-Repair Kit	1.6	14.4
Fast-Link DNA Ligase	Fast-Link DNA Ligation Kit	1.6	14.4
5mM Sodium butyrate (1M stock)		0.2	1.8
1x Protease Inhibitor (100x)		0.4	3.6
Total		31.8	286.2

Chromatin Adapter Ligation Master Mix

c. Ligase Quenching Solution [sufficient for 25 reactions]:

Component	Volume (uL)
5mM Sodium butyrate (1M stock)	10
1x Protease Inhibitor (100x)	20
Lysis Dilution Buffer	1970
Total	2000

Ligase Quenching Solution

Starting the Protocol.

1. Suspend cells in 20 ul of ice cold PBS (typically 50K to 100K per antibody in use).

2. Achieve lysis by adding 20 ul of 2X MNase/Lysis Mastermix, mix by using a few gentle cycles of pipetting.

3. Hold on ice for 20 minutes [to achieve cell lysis].

4. Transfer to a pre-warmed thermal cycler at 37^{O}C for 10 minutes [to achieve MNase digestion].

5. Immediately place samples on ice and add 4.8 uL of 0.5 M EGTA to each sample [to stop MNase activity].

In the Van Galen *et al* (2016) protocol, EGTA was included in the ligation master mix. In contrast, we suggest you add EGTA alone at the conclusion of MNase digestion, as indicated.

6. Use 1 uL of digested cells to measure the DNA concentration with the dsDNA Qubit kit.

A typical range in concentration for 50,000 cells / 40 uL lysate is 15 to 20 ng/ul. If you are multiplexing multiple chromatin preparations together, this measurement helps evaluate the equivalence (between lysates) of the number of cells in use. It is crucial that mixtures of chromatin have reasonably similar contributions from each component, because this drives the yield of ChIP DNA, the yield of library DNA, and the yield of reads from library sequencing. Imbalanced mixtures will lead to imbalanced yields from library sequencing.

A further constraint on chromatin mixing can potentially arise in experiments involving treatment of cells with modulators of the activity of chromatin modifying enzymes, such as HDAC inhibitors. Chromatin from cells that have been *extensively* treated with such a drug cannot be combined with chromatin from untreated cells for the purpose of ChIP with an antibody recognizing the affected histone modification, when the global abundance of the modification has been *highly* perturbed by the treatment; in this case, the yield of ChIP DNA would also be correspondingly imbalanced between the treated and untreated samples. In effect, when multiplexing chromatin, you lose the opportunity to characterize the genomic landscape of the modification from a sample in which the modification is significantly less abundant -- either because there are many fewer cells, or because there are many fewer modifications per cell.

T7 Adapter Ligation:

1. On ice, add 4 uL of 250 uM annealed chromatin adapter to the cell lysate. The EGTA should protect the dsDNA adapters from residual MNase activity. If multiplexing distinct chromatin preparations together, be sure to use a different adapter barcode with each to enable subsequent demultiplexing. The following table summarizes 8 different barcodes we have found perform well; full adapter sequences and instructions for ordering are attached to this protocol.

#	Barcode	Sequence
1	CBE135P.PT3-Ad_BC35	GCCATGAT
2	CBE136P.PT3-Ad_BC36	AGGTCATC
3	CBE139P.PT3-Ad_BC39	AGCTGCAT
4	CBE142P.PT3-Ad_BC42	TCGGATCA
5	CBE143P.PT3-Ad_BC43	CGAATCGT
6	CBE147P.PT3-Ad_BC47	CACTGGAT
7	CBE152P.PT3-Ad_BC52	TGAGTCAC
8	CBE155P.PT3-Ad_BC55	TACGGCAT

Suggested T7 Adapter barcodes.

2. Add 31.8 uL of T7 Adapter Ligation Mastermix and immediately mix contents well. The total volume should be about 80 uL at this point.

3. Cover the PCR plate with a foil cover, and incubate at room temperature for 2 hours [to ligate adapters to chromatin].

4. After 2 hours, end the T7 Adapter Ligation reaction by adding 80 uL of the Ligase Quenching Solution (recipe above) to each sample. The total volume should be 160 uL at this point. The 160 uL is made up of cells, MNase/Lysis buffer mastermix, the T7 Adapter Ligation mastermix, and the Quenching solution.

Chromatin Multiplexing (Optional) and ChIP Construction: Because each sample has a unique Mint Chromatin Adapter, multiple chromatin samples can optimally be mixed together at this point. Follow one of the following two procedures to complete Day 1.

a. Mint-ChIP without Chromatin Multiplexing:

1. If you are using an input control in your experimental design, reserve 3% of the volume PRIOR to adding antibody. Hold the input control in a sealed tube at 4°C until **Day 2**.

2. Add 1-5 uL of antibody of interest to each sample and incubate rotating end over end overnight at 4^{O}C. Depending on the antibody, the ideal volume of antibody to chromatin ratio will differ, and should be optimized empirically. We typically find that 1-2 ug of antibody works well for a range of antibodies and cell numbers.

The sample can be divided prior to antibody addition, if using multiple antibodies, but typically if we have not combined multiple chromatin preps, then we don't split the sample. If you are planning to split the sample, keep in mind the effective cell number per chromatin prep per ChIP at this point. We get excellent results with 50,000 to 100,000 cells per chromatin prep per antibody, and acceptable results with as few as 10,000 cells per antibody, depending on the antibody. If splitting a non-pooled sample (total volume = 160 ul), you may need to supplement the volume with a suitable buffer cocktail to enable gentle mixing during end-over-end rotation. We have standardized our operations to perform ChIPs in a minimum volume per ChIP of 100 ul, typically in a foil covered deep well plate or in individual microfuge tubes. Regardless of the plasticware used, as stated elsewhere, you must have a suitable method to magnetize the sample to wash the ChIPs on day two. If the chromatin volume is under 100 ul, we supplement the chromatin with a 1:1 mixture of Ligase Quenching Solution and 1X MNase/Lysis Mastermix **with the MNase omitted**.

If no chromatin multiplexing is performed, and only a single chromatin adapter is in use, you will obtain a monotemplate (the T7 Adapter barcode) during the first 8 cycles of Illumina read 2. We tend to avoid this by designing experiments with more than one chromatin adapter. If using only one adapter, you may be able to compensate by adding Illumna's PhiX174 control DNA during sequencing, and by using your Illumina flowcell at a lower loading density.

b. Mint-ChIP With Chromatin Multiplexing:

1. Mix up to 8 samples with distinct barcodes in a 1.5 ml microfuge tube (we typically use only 2 to 4 barcodes in a mixture).

2. If you are using an input control in your experimental design, reserve 3% of the mixture PRIOR to adding antibody. Hold the input control in a sealed tube at 4°C until **Day 2**.

3. Whatever the total volume of the mixture, divide it evenly across the number of antibodies in use. Please see the comment above about the effective cell number per ChIP. In the case of a pooled sample, the relevant number is the total number of cells per chromatin prep per ChIP, not the total cell number over-all per ChIP. If the chromatin volume is under 100 ul, we supplement the chromatin with a 1:1 mixture of Ligase Quenching Solution and 1X MNase/Lysis Mastermix **with the MNase omitted**.

4. Add 1-5 uL of antibody of interest to each sample and incubate rotating end over end overnight at 4^{O}C. Depending on the antibody, the ideal volume of antibody to chromatin ratio will differ, as described above.

The samples are typically in 1.5 ml microfuge tubes. You must have a suitable way to subject the tubes to a magnetic field, such as a Promega MagneSphere Magnetic Separation Stand, catalog # Z5342.

*****This is the END of Day 1*****

Day 2

2 Day 2: Starting with chromatin-antibody complex and ending by set-up of *In Vitro* Transcription (IVT)

Overview of Day 2:
1. Protein A/G Binding
2. ChIP Wash
3. 1X SPRI Cleanup
4. *In Vitro* Transcription (Overnight)

1. Protein A/G Bead Binding:

1. Remove Protein A/G Magnetic Beads from 4^{O}C and leave on rotator for ~30 minutes at room temperature so that beads are thoroughly and evenly mixed.
2. Calculate total Protein A/G bead slurry needed. The total total slurry needed is equal to 1/10 of the total combined volume of the chromatin - immune complexes (i.e. 20uL per 200uL chromatin - antibody complex).
3. Wash an appropriate volume of Protein A/G beads with two volumes of lysis dilution buffer, by immobilizing beads on magnet, and removal of clear fluid. Then resuspend the washed beads in the same volume of lysis dilution buffer that was used to wash the beads.
4. Add washed beads to each sample (10 ul of reuspended beads per 100 ul of immune complex) and incubate on rotator for 1 hour at 4^{O}C.
5. While the samples are incubating, supplement ChIP Elution Buffer with Proteinase K (1.25 ul of 20 mg/ml Proteinase K per 100 ul of ChIP Elution Buffer; we call this CEBPK). You will require 100 ul of CEBPK per ChIP. Note, CEBPK tends to precipitate SDS if held on ice, so maintain at room temperature until used.

2. ChIP Wash: After the samples have incubated with Protein A/G beads for an hour, the ChIPs are ready to be washed. **When washing, resuspend, magnetize, and aspirate as quickly as possible. Never leave for more than a minute.**

1. Magnetize samples and remove the supernatant (chromatin plus non-adsorbed antibodies). As a precaution you may save the supernatant as a backup at -20°C. Do not let samples dry while on magnet. Proceed immediately to ChIP wash.

2. Pipette mix the beads with 200 ul per well of ice cold RIPA Low Salt Buffer. Transfer the beads as suspended in Low Salt Buffer mixture to a 96 well plate. Take pains to minimize bead loss during transfer.
1. Magnetize the plate and wait for the beads and Low Salt Buffer to separate, no more than 1 minute
2. Remove and discard the supernatant once it is cleared.
3. Remove the plate from the magnet
4. *Optional: Resuspend the beads in another 200 uL of ice cold RIPA Low Salt Buffer*
5. *Magnetize the plate and wait for the beads and Low Salt Buffer to separate, no more than 1 minute*
6. *Remove and discard the supernatant once it is cleared.*
7. *Remove the plate from the magnet*

3. Mix the beads with 200 uL of ice cold RIPA High Salt Buffer
1. Magnetize the plate and wait for the beads and High Salt Buffer to separate
2. Remove and discard the supernatant once it is cleared.
3. Remove the plate from the magnet

4. Mix the beads with 200 uL of ice cold LiCl Wash Buffer
1. Magnetize the plate and wait for the beads and LiCl Wash Buffer to separate
2. Remove and discard the supernatant once it is cleared.
3. Remove the plate from the magnet
4. *Optional: Resuspend the beads in another 200 uL of ice cold LiCl Wash Buffer*
5. *Magnetize the plate and wait for the beads and LiCl Wash Buffer to separate, no more than 1 minute*
6. *Remove and discard the supernatant once it is cleared.*

5. Mix the beads with 200 uL of ice cold TE
1. Magnetize the plate and wait for the beads and TE to separate
2. Remove and discard the supernatant once it is cleared.
3. Remove the plate from the magnet
4. Resuspend the beads in another 200 uL of ice cold TE Buffer
5. Magnetize the plate and wait for the beads and TE to separate, no more than 1 minute
6. Remove and discard the supernatant once it is cleared.

6. Resuspend the chromatin/antibody beads using 100 uL of room temperature CEBPK made earlier.

7. At this point, we resume processing the input control chromatin (or chromatin mixture) that was reserved on **Day 1**. Mix the reserved chromatin extract (typically about two microliters) with CEBPK to a total volume of 100 uL.

8. Seal the plate with a foil cover and hold at 62^{O}C for 1 hour in a thermocycler.

9. While Proteinase K treatment is underway, remove SPRI beads from 4°C and resuspend by rotating at room temperature for at least 30 minutes

3. 1x SPRI Cleanup: At Room Temperature

1. Remove the plate from the thermocycler and hold at room temperature

2. Magnetize the plate and wait for the beads and ChIP Elution Buffer to separate.
   a. Transfer the supernatant -- which contains your eluted ChIP DNA, and should be 100uL -- to new wells in the plate

3. Remove the plate from the magnet and add 100 uL of suspended SPRI beads to your saved supernatant, to achieve a 1X SPRI cleanup. This should retain fragments over 100 BP in size.
1. Mix the supernatant and SPRI beads by pipetting up and down several times
2. Incubate at room temperature for 10 minutes

4. Place the plate on the magnet, loosely cover with the plastic cover from a pipette tip box, and magnetize for 5 minutes
   a. After 5 minutes, discard the supernatant, which represents sub-100 BP DNA fragments and diverse proteolysis byproducts.

5. Wash with 200 uL of freshly prepared 75% ethanol (and always prepare fresh 75% ethanol for each experiment)
1. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
2. Leave on the magnet for 1 minute and then discard the ethanol.

6. Wash AGAIN with 200 uL of 75% ethanol
1. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
2. Leave on the magnet for 1 minute and then discard the ethanol.

7. Air-dry at room temperature for exactly 5 minutes, and no longer; do not allow to over-dry.

8. Elute DNA in 15 uL Elution buffer (EB).
1. Remove plate from magnet and resuspend beads in 15 uL EB
2. Let rest for 1 minute off the magnet and then 2 minutes on the magnet
3. Once the beads are on the sides of the wells, remove and SAVE the supernatant; this is your purified ChIP DNA

9. Use 2 uL for Qubit measurement, to gain some insight into the success of your ChIP so far. We typically recover 0.50-2.00 ng/ul of ChIP DNA at this point. However, regardless of the ChIP DNA concentration, 8 ul of it will go into the IVT as shown in the box below. The remaining 5 ul is saved as a backup in case of a downstream failure.

4. *In Vitro* Transcription (Overnight):

1. In a new plate, prepare the following mixture of ChIP DNA and IVT Mastermix:

Component	Kit	Per Rxn (uL)	Per 8 Samples (uL)
DNA	-	8	-
NTP Buffer Mix (2x)	T7 Quick High Yield Synth	10	90
T7 RNA Pol. Mix	T7 Quick High Yield Synth	2	18
Total MM		12	108

T7 In vitro transcription master mix; total reaction volume is 20 ul per IVT.

2. Cover plate with a foil cover and incubate at 37^{O}C for 16 hours (overnight).

*****This is the END of Day 2*****

Day 3

3 Day 3: Starting with RNA Isolation and ending with Library Construction

Overview of Day 3:

1. RNA Isolation with Silane Beads
2. Reverse Transcription (RNA to cDNA)
3. 1X SPRI Cleanup
4. Library PCR to Enrich Adapter-Modified DNA Fragments
5. Final SPRI Cleanup

1. RNA Isolation with Silane Beads:

1. Remove Silane Beads and SPRI Beads from 4°C and incubate on a rotator for at least 30 minutes at room temperature, or until beads are thoroughly and evenly mixed.

2. Add 1 uL of DNase to IVT RNA samples and incubate at 37°C for 15 minutes.

3. Using a 96 well plate, magnetize 20 uL of Silane Bead suspension per IVT reaction. Remove clear supernatant, and wash with four volumes of RLT Buffer (i.e. 80 uL RLT for 20 uL of beads), and resuspend washed beads in 4 volumes of RLT.

4. Transfer 60 uL of the washed beads to the 20 uL IVT reaction. The 60 uL will contain 15 uL of beads which will bind up to 3 ug of RNA. The remaining 20 ul of beads is discarded.
a. Incubate at room temperature for 1 minute

5. Add 48 uL of 100% ethanol and mix well by pipetting
a. Wait for 2.5 minutes, mix, and wait an additional 2.5 minutes (for a total of 5 minutes).

6. Magnetize the plate
a. Remove and discard the supernatant once the solution looks clear. The RNA is adsorbed to the beads.

7. Wash with 200 uL of FRESH 75% ethanol
a. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
b. Leave on the magnet for 1 minute and then remove the ethanol

8. Wash AGAIN with 200 uL of FRESH 75% ethanol
a. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
b. Leave on the magnet for 1 minute and then remove the ethanol
c. It is critical to wash thoroughly to eliminate all DNase prior to the reverse transcription step.

9. Air-dry at room temperature for 2 minutes. Make sure not to over dry.

10. Elute RNA with 9 uL TE
a. Remove plate from magnet and resuspend beads in 9 uL TE. Make sure to get all the beads off the sides of the wells
b. Let rest for 1 minute off the magnet and then 2 minutes on the magnet
c. Once the beads are on the sides of the wells, remove and SAVE the supernatant; it is your IVT RNA prep.

11. OPTIONAL: Measure 2 ul on RNA Qubit
a. If below detection limit (4 ug/ml), the libraries are likely to be very low in complexity.
b. Total amount of RNA should be higher than input DNA for IVT reaction; typically the yield of RNA is 50 ng to 100 ng.

2. Reverse Transcription (RNA -> cDNA):

1. Combine IVT RNA with random primer and annealing buffer and incubate mixture at 65°C for 5 minutes on the thermocycler, then immediately cool to 4°C in the thermal cycler (i.e. ramp as fast as the instrument will allow).

Component	Kit	Per Rxn (uL)	Per 8 Samples (uL)
RNA from IVT	-	8	-
50 uM primer (CBE100-SBS3-RT)		3	27
Annealing buffer	SuperScript III First-Strand Synthesis Supermix	1.5	13.5
Total MM		4.5	40.5

2. Once the program cools to 4^{O}C, remove the plate and place immediately on ice, for at least one minute.
3. Supplement the primer annealed IVT RNA with the master mix described below and return the plate to the thermal cycler. The thermal profile should be as follows:

10 minutes at 25^{O}C

50 minutes at 50^{O}C

5 minutes at 85^{O}C

4^{O}C indefinite.

We employ a single PCR program with a pause in the middle to accomplish both the annealing and elongation steps.

Component	Kit	Per Rxn (uL)	Per 8 Samples (uL)
RNA with annealed primer	-	12.5	-
2x First-Strand Rxn Mix	SuperScript III First-Strand Synthesis Supermix	15	135
SSIII Enzyme	SuperScript III First-Strand Synthesis Supermix	3	27
Total MM		18	162

4. At the conclusion of the cDNA synthesis step, add 1 uL RNase to each sample, mix thoroughly, and incubate at 37^{O}C for 15 minutes in a PCR machine, then return to 4C. At this point, you have eliminated the IVT RNA, and should only have cDNA.

3. 1X SPRI Cleanup:

1. Add 68 uL of water to cDNA so that the volume is easier to work with manually. The total volume is now 100 ul per well.

2. Add 100 uL of SPRI Beads (suspended at the start of Day 3) and mix by pipetting
a. Incubate at room temperature for 10 minutes

3. Magnetize plate for 5 minutes so that the beads separate out
a. Remove and discard the supernatant

4. Wash with 200 uL of FRESH 75% ethanol
a. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
b. Leave on the magnet for 1 minute and then remove the ethanol

5. Wash AGAIN with 200 uL of FRESH 75% ethanol
a. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
b. Leave on the magnet for 1 minute and then remove the ethanol

6. Air-dry at room temperature for 5 minutes

7. Elute cDNA in 24.5 uL TE
a. Remove plate from magnet and resuspend beads in 24.5 uL TE.
b. Let rest for 1 minute off the magnet and then 2 minutes on the magnet
c. Once the beads are on the sides of the wells, remove and SAVE the supernatant; it is your cDNA.

8. Use 1 ul of cDNA to measure cDNA concentration with ssDNA Qubit (this is not optional, as will be apparent immediately below).

4. Library PCR to Enrich Adapter-Modified DNA Fragments:

We put a maximum of 200 ng of cDNA into the PCR, so if the yield of cDNA exceeds 200 ng, then the cDNA needs to be diluted prior to PCR. Using more than 200 ng input cDNA impairs the yield of DS DNA with suitable ends for Illumina sequencing. If the yield of cDNA is under 200 ng, we use the entire 200 ng volume in the PCR reaction. If the yield of cDNA exceeds 200 ng, remove the appropriate volume of cDNA (containing up to 200 ng) and add it to Elution Buffer so that the combined volume sums to 22.5 ul.

Prepare the following PCR enrichment reaction and place on thermal cycler for the following times and temperatures:

Component	Per Rxn (uL)
cDNA (200ng max)	22.5
10uM forward primer	1.25
10uM reverse primer	1.25
PFU Ultra II HS 2x MM	25

PCR Enrichment Reaction Components

Time	Temperature (Celsius)	
3 min.	95	
15 sec.	95	4x
30 sec.	65	
30 sec.	72	
15 sec.	95	10-12x
60 sec.	72	
5 min.	72	
End	4	

PCR Cycling Conditions

5. Post PCR Final SPRI Cleanup:

1. Add 50 uL of molecular biology grade water and check that the volume is exactly 100 uL

2. Add 60 uL of SPRI beads (0.60×) and mix by pipetting. The beads will bind DNA fragments of >700 bp. The purpose of this step is to remove these large fragments.
a. Incubate at room temperature for 5 minutes

3. Magnetize plate for 5 minutes to separate the beads out
a. After 5 minutes, transfer the supernatant (about 160 uL) to clean plate wells. The supernatant should contain DNA fragments of <700 bp. It is your library, now cleared of high molecular weight fragments.

4. Add 35 uL of SPRI beads (0.95×) and mix by pipetting. The beads will bind to DNA fragments of >200 bp, thus excluding unincorporated PCR primers, which remain in solution.
a. Incubate at room temperature for 5 minutes

5. Magnetize plate for 5 minutes to separate the beads out
a. After 5 minutes, remove and discard the supernatant

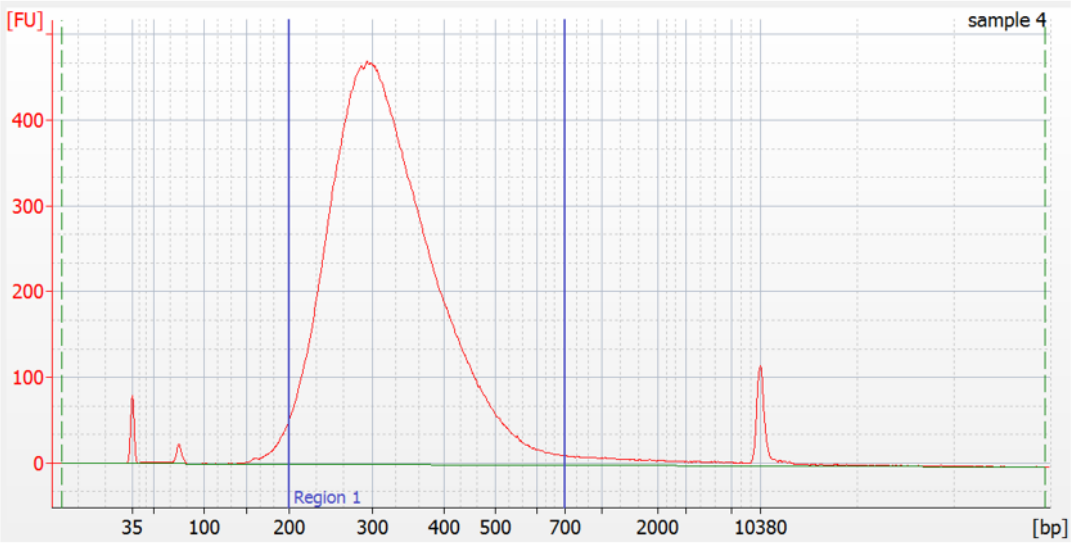
6. Wash with 200 uL of FRESH 75% ethanol
a. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
b. Leave on the magnet for 1 minute and then remove the ethanol

7. Wash AGAIN with 200 uL of FRESH 75% ethanol
a. Mix by moving the plate from the center of the magnet to off center of the magnet. You will see the beads move from one side of the well to the other side of the well.
b. Leave on the magnet for 1 minute and then remove the ethanol

8. Air-dry at room temperature for 5 minutes

9. Elute DNA in 40 uL Elution Buffer.
a. Remove plate from magnet and resuspend beads in 40 uL EB.
b. Let rest for 1 minute off the magnet and then 2 minutes on the magnet
c. Once the beads are on the sides of the wells, remove and SAVE the supernatant. The supernatant is your PCR product enriched for fragments in the size range of 200-700 bp.

10. Analyze samples by dsDNA Qubit for concentration and BioAnalyzer for fragment size. An ideal BioAnalyzer trace is included here:



An ideal BioAnalyzer trace showing the PCR product after SPRI bead cleanup.

*****This is the END of Day 3*****