**Abbreviations**

FA = formaldehyde

SA = streptavidin

QC = Quality control

ProK = Proteinase K

PBS = phosphate buffered solution

MM = mastermix

RT = Room temperature

**High level workflow**

Crosslink cells (Day 0)

**Small scale test experiment**

Generate oligo-labeled beads

1. Biotinylate Protein G beads (Day 0)
2. Make Oligo-SA complex (Day 0)
3. Bind SA-oligo to biotinylated beads (Day 1)
4. QC oligo per bead to ensure oligo/bead ratio is in range (80-160 oligo/bead) (Day 1)

Lyse cells and fragment chromatin using method of choice (Day 1)

1. Verify fragments are correct size before binding antibodies
2. Store at 4C overnight

Bind antibodies to oligo-labeled beads (Day 1)

1. Bind overnight at 4C

Quench beads with excess biotin and IgG-Fc (Day 2)

Wash and pool beads (Day 2)

Add bead pool to chromatin for IP (Day 2)

1. 30 min to 1 hour at RT or 2 hours at 4C

Wash beads

End Repair, dA-tailing and DPM to prepare for split pool (Day 2)

Split pool barcoding (Day 2)

Take small aliquots and reverse crosslink and ProK overnight (Day 2)

Column clean aliquots (Day 3)

Library amplification (Day 3)

Tapestation or BioAnalyzer to QC libraries (Day 3)

Illumina sequencing (Day 4)

Computational demultiplexing (Day 5)

1. Assess chromatin/bead for each target for large scale normalization

**Large scale experiment for full depth**

Generate oligo-labeled beads

1. Biotinylate Protein G beads (Day 0)
2. Make Oligo-SA complex (Day 0)
3. Bind SA-oligo to biotinylated beads (Day 1)
4. QC oligo per bead to ensure oligo/bead ratio is in range (80-160 oligo/bead) (Day 1)

Lyse cells and fragment chromatin using method of choice (Day 1)

1. Verify fragments are correct size before binding antibodies
2. Store at 4C overnight

Bind antibodies to oligo-labeled beads (Day1)

1. Bind overnight at 4C

Quench beads with excess biotin and IgG-Fc (Day 2)

Wash and pool beads (Day 2)

Add bead pool to chromatin for IP (Day 2)

1. 30 min to 1 hour at RT or 2 hours at 4C

End Repair, dA-tailing and DPM to prepare for split pool (Day 2)

Split pool barcoding (Day 2)

Take aliquots and reverse crosslink and ProK overnight (Day 2)

Column clean aliquots (Day 3)

Library amplification (Day 3)

Tapestation or BioAnalyzer to QC libraries (Day 3)

Illumina sequencing (Day 4)

Computational demultiplexing and alignment of chromatin from each antibody to the genome (Day 5)

1. Downstream ChIP-seq analyses

**Equipment**

* Branson needle-tip sonicator (3mm diameter (1/8’ doublestep). Branson ultrasonics 101-148-063)
* Agilent TapeStation 4150 (or Agilent Bioanalyzer)
* Multichannel pipet p20 (12 well)
* Multichannel pipet p200 (12 well)

**Materials and reagents**

* 16% Formaldehyde (w/v), Methanol-Free (Thermo PI28908)
* cOmplete, EDTA-free Protease Inhibitor Cocktail (Millipore Sigma 11873580001)
* EZ-Link Sulfo-NHS-Biotin (Thermo PIA39256)
* DMSO (Sigma-Aldrich D2650-5X5mL)
* Dynabeads Protein G (Life Technologies 10009D) – 2.7\*10^6 beads/uL
* Streptavidin (BioLegend 280302)
* Positive control antibodies
  + H3K4me3 (CST 9751S)
  + CTCF (CST 3418S)
* Q5 2x Mastermix (NEB M0494L)
* SPRI beads (Bulldog Bio CNGS500)
* 96 well PCR plate
* NEBNext End Repair Module (NEB E6050)
* NEBNext dA-tailing Module (NEB E6053)
* IgG Fc (Bio X Cell BE0096)
* Biotin (Sigma Aldrich B4639-5G)
* Proteinase K (NEB P8107S)
* Zymo DNA Clean and Concentrator kit (Zymo 4014)
* Agilent High Sensitivity D1000 ScreenTape (Agilent Technologies 5067-5584)
* Agilent High Sensitivity D1000 Reagent (Agilent Technologies 5067-5603)
* Nunc 96-Well DeepWell Plates with Shared-Wall Technology, Thermo Scientific, Color:Natural, (Thermo Fisher Scientific 260251)
* Instant Sticky-end Ligase Master Mix (ISMM) - 250, rxns (NEB M0370L)
* NEBNext Quick Ligation Reaction Buffer (5X) (NEB B6058S)
* 1,2-Propanediol, ACS reagent, ≥99.5% (Sigma-Aldrich 398039-25ML)
* Bead oligo sample sequence and structure
  + /5phos/TGACTTGNNNNNNNNTATTATGGTAGATCGGAAGAGCGTCGTGTACACAGAGTC/3Bio/
    - Sticky end that ligates Odd barcodes
    - UMI
    - Oligo barcode
    - Illumina primer binding site (i5 primer binding site)
    - Spacer sequence

**Buffers**

|  |  |  |  |
| --- | --- | --- | --- |
| Lysis buffer A | | | |
|  | Stock conc | Final conc | Volume for 10mL solution |
| HEPES 7.4 | 1M | 50mM | 0.5 |
| EDTA | .5M | 1mM | 0.02 |
| EGTA | .5M | 1mM | 0.02 |
| NaCl | 5M | 140mM | 0.28 |
| Triton-X | 10% | 0.25% | 0.25 |
| NP-40 | 10% | 0.50% | 0.5 |
| Glycerol | 50% | 10% | 2 |
| H2O |  |  | 6.43 |
|  |  |  | 10mL |
|  |  |  |  |
| Lysis buffer B | | | |
|  | Stock conc | Final conc | Volume for 10mL solution |
| HEPES 7.4 | 1M | 50mM | 0.5 |
| EDTA | .5M | 1.5mM | 0.03 |
| EGTA | .5M | 1.5mM | 0.03 |
| NaCl | 5M | 200mM | 0.4 |
| H2O |  |  | 9.04 |
|  |  |  | 10mL |
|  |  |  |  |
|  |  |  |  |
| Lysis buffer C | | | |
|  | Stock conc | Final conc | Volume for 10mL solution |
| HEPES 7.4 | 1M | 50mM | 0.5 |
| EDTA | .5M | 1.5mM | 0.03 |
| EGTA | .5M | 1.5mM | 0.03 |
| NaCl | 5M | 100mM | 0.2 |
| DOC | 10% | 0.10% | 0.1 |
| NLS | 20% | 0.50% | 0.25 |
| H2O |  |  | 8.89 |
|  |  |  | 10mL |

|  |  |
| --- | --- |
| SPRITE Instant sticky mastermix | |
| Solution | Volume |
| NEBNext Quick ligation reaction buffer | 1600 uL |
| Instand Sticky-end Master Mix (2x) | 1000 uL |
| 1.2-Propanediol | 600 uL |
|  | 3200 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| MyRNK buffer | | | | | | | | | | | | | | | | | | | |
|  | Stock conc | | | | | Final conc | | | | | Volume for 10mL solution | | | | | | | |  |
| Tris pH 7.5 | 1M | | | | | 20mM | | | | | 0.2 | | | | | | | | 1 |
| NaCl | 5M | | | | | 100mM | | | | | 0.2 | | | | | | | | 1 |
| EDTA | 0.5 | | | | | 10mM | | | | | 0.2 | | | | | | | | 1 |
| EDTA | 0.5 | | | | | 10mM | | | | | 0.2 | | | | | | | | 1 |
| Triton-X | 10% | | | | | 0.50% | | | | | 0.5 | | | | | | | | 2.5 |
| SDS | 10% | | | | | 0.2% | | | | | 0.2 | | | | | | | | 1 |
| H2O |  | | | | |  | | | | | 8.7 | | | | | | | | 43.5 |
|  |  | | | | |  | | | | | 10mL | | | | | | | | 50mL |
| IP Wash buffer 1 (IPWB1) | | | | | | | | | | | | | | | | | | | | | |
|  | | | | | Stock conc, | | | | | Final conc | | | | | Volume for 30mL solution | | | | 50mL |  | 500mL |
| Tris-HCl pH8.0 | | | | | 1M | | | | | 20mM | | | | | 0.6 | | | | 1 | 4.77 | 10 |
| SDS | | | | | 10% | | | | | 0.05% | | | | | 0.15 | | | | 0.25 |  | 2.5 |
| Triton X-100 | | | | | 10% | | | | | 1% | | | | | 3 | | | | 5 |  | 50 |
| EDTA | | | | | .5M | | | | | 2mM | | | | | 0.12 | | | | 0.2 |  | 2 |
| NaCl | | | | | 5M | | | | | 150mM | | | | | 0.9 | | | | 1.5 |  | 15 |
| H2O | | | | |  | | | | |  | | | | | 25.23 | | | | 42.05 |  | 420.5 |
|  | | | | |  | | | | |  | | | | | 30 | | | | 50 |  | 500 |
| IP Wash buffer 2 (IPWB2) | | | | | | | | | | | | | | | | | | | | | |
|  | | | | | Stock conc, | | | | | Final conc | | | | | Volume for 30mL solution | | | | 50mL |  | 500mL |
| Tris-HCl pH8.0 | | | | | 1M | | | | | 20mM | | | | | 0.6 | | | | 1 | 6.87 | 10 |
| SDS | | | | | 10% | | | | | 0.05% | | | | | 0.15 | | | | 0.25 |  | 2.5 |
| Triton X-100 | | | | | 10% | | | | | 1% | | | | | 3 | | | | 5 |  | 50 |
| EDTA | | | | | .5M | | | | | 2mM | | | | | 0.12 | | | | 0.2 |  | 2 |
| NaCl | | | | | 5M | | | | | 500mM | | | | | 3 | | | | 5 |  | 50 |
| H2O | | | | |  | | | | |  | | | | | 23.13 | | | | 38.55 |  | 385.5 |
|  | | | | |  | | | | |  | | | | | 30 | | | | 50 |  | 500 |
| M2 (SPRITE wash buffer) | | | | | | | | | | | | | | | |
|  | | | | Stock conc | | | | | Final conc | | | | | Volume for 50mL solution | |
| H2O | | | |  | | | | |  | | | | | 45.5 | |
| Tris pH 7.5 | | | | 1M | | | | | 20mM | | | | | 1 | |
| Triton-X | | | | 10% | | | | | 0.20% | | | | | 1 | |
| NP-40 | | | | 10% | | | | | 0.20% | | | | | 1 | |
| DOC | | | | 10% | | | | | 0.20% | | | | | 1 | |
| NaCl | | | | 5M | | | | | 50mM | | | | | 0.5 | |
|  | | | |  | | | | |  | | | | | 50 | |
| 2x SA binding buffer | | | | | | | | | | | | | | | | |
|  | | | Stock conc | | | | Final conc | | | | | Volume for 50mL solution | | | | |
| H20 | | |  | | | |  | | | | | 29.4 | | | | |
| Tris pH 7.5 | | | 1M | | | | 10mM | | | | | 0.5 | | | | |
| NaCl | | | 5M | | | | 2M | | | | | 20 | | | | |
| EDTA | | | .5M | | | | 1mM | | | | | 0.1 | | | | |
|  | | |  | | | |  | | | | | 50 | | | | |
| M2 + 75mM EDTA | | | | | | | | | | | | | | | | | |
|  | | Stock conc | | | | | | Final conc | | | | | Volume for 50mL solution | | | | |
| H20 | |  | | | | | |  | | | | | 38 | | | | |
| Tris pH 7.5 | | 1M | | | | | | 20mM | | | | | 1 | | | | |
| Triton-X | | 10% | | | | | | 0.20% | | | | | 1 | | | | |
| NP-40 | | 10% | | | | | | 0.20% | | | | | 1 | | | | |
| DOC | | 10% | | | | | | 0.20% | | | | | 1 | | | | |
| NaCl | | 5M | | | | | | 50mM | | | | | 0.5 | | | | |
| EDTA | | .5M | | | | | | 75mM | | | | | 7.5 | | | | |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| PBSt | | | | |
| reagent | Stock conc, | Final conc | 50mL | 500mL |
| PBS | 1x | 1x | 49.5mL | 495mL |
| Tween-20 | 10% | 0.10% | 0.5mL | 5mL |

|  |  |
| --- | --- |
| Dilute M2 | |
| Reagent | 1x |
| M2 | 7.5mL |
| Water | 7.5mL |

**[Day 0]**

**Crosslinking**

1. Prep work
   1. Chill one bottle of 1x PBS on ice. Keep one at room temperature.
2. Count and wash cells
   1. Count an aliquot of the cells to determine the total number of cells to crosslink. Prior to counting cells, make sure the cells are in a single cell suspension by pipetting vigorously several times.
      1. total cells:
   2. Pellet cells at room temp for 3 minutes at 330xG. Remove media.
   3. Wash 1x by resuspending in 4mL PBS per 10M cells and pellet cells at room temp for 3 minutes at 330xG. Remove PBS solution.
3. 1% FA crosslinking

NOTE: Right before using, premix a 1% formaldehyde (Thermo cat no. 28908) solution in PBS. Use a **fresh** ampule of 16% formaldehyde. Do not use an ampule opened more than ~20 min before crosslinking the cells.

|  |  |  |
| --- | --- | --- |
|  | 1% FA | |
|  | 1x (10M cells) | 10x (100M cells) |
| 16% FA | 250 | 2500 |
| PBS | 3750 | 37500 |
|  |  | 40000 |

* 1. Prepare a 50mL conical centrifuge tube with 3.75 mL PBS for each 10M cells to be crosslinked.
  2. Use 1mL of PBS solution prepared in 3.a. to resuspend cell pellet
  3. Crack open 16% FA ampule and transfer to a labeled 15mL centrifuge tube
  4. Add 250uL 16% FA solution per 4 mL PBS solution from 3.a. to get a ~1% FA solution. Invert to mix
  5. Add resuspended cell pellet to the ~1% FA tube to make a tube of 1% final concentration FA in PBS
  6. Rock gently at room temperature for **exactly** 10 minutes
  7. Immediately add 200uL of 2.5M glycine stop solution per 1mL of 1% formaldehyde solution directly to the tube to quench the crosslinker. Mix well.
     1. Glycine calculation:
  8. Rock gently at room temperature for 5 minutes.

1. Wash and aliquot crosslinked cells
   1. Spin down cells at 4 °C for 4 minutes at 1000xG. Discard FA solution in the FA liquid waste in the fume hood. From here onward, keep cells on ice.
   2. Resuspend pellet in cold 1x PBS (4mL PBS per 10M cells)
   3. Spin down cells at 4 °C for 4 minutes at 1000xG. Discard solution in FA waste.
   4. Repeat the wash step.
   5. Resuspend cells in 200uL of PBS per 10M cells.
   6. Aliquot cells into microcentrifuge tubes (5M-20M cells each) and spin at 4 °C for 5 minutes at 2000xG. Remove supernatant carefully without disrupting the pellet.
2. Flash freeze in liquid nitrogen or proceed to cell lysis
3. Prepare and QC SA-Oligo and biotinylated protein G beads as outlined in “Starter guide to ChIP DIP bead labeling”

**Day 1**

**Prepare chromatin**

1. Cell lysis (for up to a 20M cell aliquot)
   1. Resuspend in 700uL Lysis Buffer A with 14uL 50x PIC
   2. Incubate 10 min on ice
   3. Spin down at 4C for 8 min at 850G
   4. Remove supernatant
   5. resuspend each sample in 700uL Lysis Buffer B with 14uL 50x PIC
   6. Incubate 10 min on ice
   7. Spin down at 4C for 8 min at 850G
   8. Remove supernatant
   9. Resuspend each sample in 550uL Lysis buffer C with 22uL 50x PIC
   10. Incubate 10 min on ice
2. Sonicate (mESC and K562)

**NOTE: sonication times and power will vary depending on cell type and crosslinking condition and will need to be empirically optimized.**

* 1. Sonicate each aliquot with 20% power (3-4 watts) at 4C for 2.5 min (.7sec on, 3.3sec off)
  2. Check size distribution of chromatin:
     1. reverse crosslink 10uL-20uL at 80C for 30 minutes
     2. clean up with 6x DNA binding buffer and IC Zymo column
     3. elute in 20uL H2O
     4. Run on a 1% gel to check the chromatin fragment sizes (desired fragment size in 200bp-500bp)
     5. If the size range is 200bp-500bp for most fragments, save this as your input. If not, go back to sonicated sample and sonicate more. Repeat once if needed.
  3. ***Take 20uL input and reverse crosslink overnight***
  4. Proceed to ChIP DIP

**Bind unique oligos to Protein G beads**

1. Aliquot 10uL of biotinylated Protein G beads (2.7\*10^6 beads/uL = 27\*10^6 beads per IP) per IP
2. Wash biotinylated Protein G twice with 1mL PBSt
3. quick spin and put on magnet to remove excess PBSt
4. Resuspend in 100uL PBSt per 10uL of beads
5. Add 100uL of 2x SA binding buffer per 10uL beads. This is the bead master mix (MM).
6. Split bead MM into deep well plate, 200uL per well.
7. Add XuL (optimized volume – It is ~14uL for me) of 5.67nM SA-PC50 to corresponding wells
8. Shake at RT for 30 minutes
9. Quick spin for 30sec at 100G
10. Wash 2x with 200uL M2
11. wash 2x with 200uL PBSt
12. Keep on ice while prepping chromatin

**QC oligos/bead from bead labeling reaction**

1. Take 2uL worth of beads (40uL of 200uL) from the last well of each row for representative assessment of coupling for that row
2. Resuspend beads in 40uL of dilute M2. (Dilute M2 is 50% M2 and 50% water)
3. Prepare the following reaction for each condition

|  |  |
| --- | --- |
| **Reagent** | **1x (uL)** |
| Beads in Dilute M2 | 40 |
| Term Ligates Even | 12 |
| Instant Sticky MM | 32 |
| total | 84 |

1. Shake at 25C for 15 minutes, 1200RPM
2. Wash 3x with 200uL M2
3. Resuspend in 42uL water
4. Take 1/2 of sample (21uL, 1uL of beads) and PCR amplify 10 cycles

|  |  |  |
| --- | --- | --- |
| Reagent | 1x (uL) | 10x |
| Beads in water | 21 |  |
| 2pbc (25uM) | 2 |  |
| 2puni (25uM) | 2 |  |
| 2x Q5 MM | 25 |  |

* 1. 2uL 2pbc (25uM), 2uL 2puni (25uM), 21uL beads in water, 25uL of 2x Q5 MM

|  |  |  |
| --- | --- | --- |
| **Temp (C)** | **Time (s)** | **Cycles** |
| 98 | 60 | 1 cycle |
| 98 | 15 | 4 cycles |
| 69 | 15 |
| 72 | 90 |
| 98 | 15 | 6 cycles |
| 72 | 90 |
| 72 | 2min |  |

1. 1.2x SPRI
   1. Elute in 20uL
2. Run each sample on tapestation to quantify molarity
3. Use unique molecule calculator to back calculate number of oligos/bead
   1. Aim for condition that gives 70-100 oligos/bead

**Bind Antibodies to Labeled Protein G Beads**

1. resuspend beads in 200uL PBSt
2. Add 2.7ug of antibody to each well (if antibody is very dilute, remove some beads such that beads are fully loaded with amount of antibody available)
   1. The antibody binding capacity is 0.24 ug human IgG per 1 uL beads
3. Shake at 4C overnight
   1. 10 seconds shaking at 1200 RPM, every 15 minutes

**Day 2**

**1hr RT IP**

1. Shake beads at RT at 1100rpm while preparing reagents and lysate
2. Prepare lysate
   1. Add 500uL PBSt to each tube
   2. 30uL 50x PIC
   3. add XuL of IgG Fc (2.5ug per 10uL beads)
   4. Add 12uL 1M biotin (10mM biotin final)
3. Wash beads 2x with 200uL PBSt
4. Resuspend in 200uL PBSt + 4mM biotin + 2.5ug Human IgG Fc per well
   1. This quenches any free protein G and SA to prevent mixing of labels or antibody
5. Shake at RT for 15 minutes

|  |  |  |
| --- | --- | --- |
|  | 1X | 48X |
| PBSt | 200 | 9600 |
| Biotin (1M) | 0.8 | 38.4 |
| Human IgG (8.6ug/uL) | 0.3 | 14.4 |

1. Pool all beads with volumes outlined in excel sheet to equalize coverage
   1. Normalize number of beads to evenly distribute sequencing coverage for each antibody
      1. **Normalize to the beads with the antibody that pulls down the least amount of chromatin. For example, if antibody A pulls down 100chrom/bead and antibody B pulls down 10chrom/bead then there would be 10x less beads from antibody A compared to antibody B added to the bead pool.**
2. Wash beads 2x with 1mL PBSt+4mM biotin
3. resuspend beads in 200uL PBSt
4. Add beads to prepared lysate
5. rotate on hula mixer at RT for 1 hour or 2 hours at 4C

**Wash beads post IP**

1. Wash beads 2x with 1mL IPWB1
   1. Put on magnet and remove supernatant between washes
2. Wash beads 2x with 1mL IPWB2
   1. Put on magnet and remove supernatant between washes
3. Wash beads 2x with 1mL M2
   1. Put on magnet and remove supernatant between washes
4. quick spin and remove all excess M2

**End Repair and dA-tailing**

**Note:** It is important to not allow beads to dry out. Prepare mastermixes ahead of time before removing all buffer from beads.

End Repair

1. Remove all liquid before preparing proceeding to the following reaction

|  |  |
| --- | --- |
| Stock Solution | 1x |
| H2O | 211.5 |
| 1M biotin | 1 |
| End Repair Reaction Buffer (10X) | 25 |
| End Repair Enzyme Mix | 12.5 |
| Total | 250 |

1. Add 1ug human IgG1 Fc for every 10uL of beads
2. Shake at 20C for 15 min, 1400RPM
3. Quench with 500uL PBSt+100mM EDTA
4. Wash 2x with 1mL M2
   1. Put on magnet and remove supernatant between washes
5. Quick spin and put on magnet, remove all residual solution

dA-tailing

|  |  |  |  |
| --- | --- | --- | --- |
| Stock Solution | 1x |  |  |
| H2O | 214 |  |  |
| 1M biotin | 1 |  |  |
| dA-Tailing Reaction Buffer (10X) | 25 |  |  |
| Klenow Fragment (exo-) | 10 |  |  |
| Total | 250 |  |  |

1. Add Master Mix to each sample
2. Add 1ug human IgG1 Fc for every 10uL of beads
3. Shake at 37C for 15 min, 1400RPM
4. Quench with 500uL PBSt+100mM EDTA
5. Wash 2x with 1mL M2
   1. Put on magnet and remove supernatant between washes
6. Quick spin and put on magnet, remove all residual solution

**Ligate DPM to chromatin**

|  |  |  |
| --- | --- | --- |
| Reagent | 1x (per well) |  |
| Beads in dilute M2+4mM biotin + Protein G | 11.2 |  |
| DPM (4.5uM) | 2.4 |  |
| ISMM | 6.4 |  |
|  | 20 |  |

1. Resuspend each aliquot of beads in 135uL of dilute M2 + 4mM biotin + Protein G
2. Distribute into 12 wells
3. Add unique DPM to each well
4. Add ISMM with multichannel pipet in volume outlined on reaction table. Pipet up and down to mix
5. Shake at 20C for 20 minutes
6. Quench with 30uL PBSt+100mM EDTA
7. Pool all beads into a single tube
8. Wash 2x with 1mL M2
   1. Put on magnet and remove supernatant between washes
9. Quick spin and put on magnet, remove all residual solution

**Split pool**

1. 6 rounds of 24 well barcoding (barcode combinations)
   1. Odd (Rows A, B)
   2. Even (Rows A, F)
   3. Odd (Rows C, D)
   4. Even (Rows C, D)
   5. Odd (Rows E, F)
   6. Term Lig Odd (Rows F, G, A)

|  |  |
| --- | --- |
| Stock solution | Volume |
| beads + dilute M2 + 4mM biotin + Protein G | 11.2 |
| barcode (4.5uM) | 2.4 |
| Ligation master mix | 6.4 |
| total | 20 |

1. Resuspend beads in 270uL dilute M2 + 4mM biotin
2. Distribute into 24 wells
3. Prepare the outlined reaction for each well
   1. Add barcode with multichannel pipet in volume outlined on reaction table
   2. Add ISMM with multichannel pipet in volume outlined on reaction table. Pipet up and down to mix
4. incubate at RT for 5 minutes
5. Quench with 30uL PBSt+100mM EDTA
6. Pool into 2mL tube and place on magnet
7. Wash 2x with 1mL M2 using magnet
   1. Put on magnet and remove supernatant between washes
8. Quick spin and put on magnet, remove all residual solution
9. Repeat 2-9 5 more times
10. wash 2x extra 1mL washes before resuspending in MyRNK
11. resuspend in 1mL MyRNK
12. Take aliquots and reverse crosslink overnight
    1. 92uL of MyRNK (this includes the aliquot) + 8uL of Proteinase K
    2. Example of a 1% aliquot: 10 uL aliquot in MyRNK + 82 mL MyRNK + 8 uL Proteinase K
13. 50C for 2 hours, 65C overnight

**Day 3**

**Clean up samples**

1. Put beads on magnet and transfer supernatant to a new tube, this contains your IP’d chromatin fragments
2. Add 700uL of DNA binding buffer to sample and add to Zymo IC column
   1. Wash 2x with 200uL RNA/DNA wash buffer, 20 sec spin at >10,000 RPM
   2. Empty spin of column in empty collection tube
   3. Put column in a new, labeled 1.5mL tube
   4. Add 21uL H2O to elute from column, incubate at RT for 3-5 minutes
   5. Spin down 60 sec at > 10,000 RPM
   6. This eluate contains purified DNA fragments

**Amplify samples to add sequencing index barcode**

1. Prepare the following reaction to amplify **all** of the aliquot

|  |  |
| --- | --- |
| Reagent | 1x (uL) |
| Purified DNA | 21 |
| I5 (12.5uM) | 2 |
| I7 (12.5uM) | 2 |
| 2x Q5 MM | 25 |
| Total | 50 |

1. Amplify with the following PCR program

|  |  |  |
| --- | --- | --- |
| **Temp (C)** | **Time (s)** | **Cycles** |
| 98 | 60 | 1 cycle |
| 98 | 15 | 4 cycles |
| 69 | 15 |
| 72 | 90 |
| 98 | 15 | 6 cycles |
| 72 | 90 |
| 72 | 2min |  |

1. Clean up with 1.2x SPRI
   1. Add 60uL SPRI beads and mix with pipet
   2. Incubate at RT for 10 minutes
   3. Wash beads 2x with 200uL 80% **freshly prepared** EtOH
   4. Quick spin and put on magnet to remove residual EtOH
   5. Remove excess EtOH
   6. Add 30uL H2O to elute
   7. Incubate at 37C for 5 minutes
   8. Transfer supernatant away from SPRI beads into a fresh tube
      1. This contains the amplified library
2. Proceed to Tapestation analysis or freeze for later
   1. Quantify library and number of unique molecules per aliquot with tapestation
      1. Run tapestation or BioA, following manufacturers protocol for each aliquot
      2. Use molarity generated by tapestaion software, volume of sample, and number of cycles of PCR to back calculate number of unique molecules for oligo fraction (spike on the trace), and chromatin fraction (wide distribution trace). See library complexity calculator for more information on formulas used to calculate number of unique molecules.
   2. Gel cut oligo and chromatin to remove PCR primers.
      1. **Optional:** gel cut chromatin and oligo separately. This is helpful if you want to sequence more chromatin than oligo or vice versa.
   3. Quantify molarity for sequencing