SPRITE-IP (SIP) Protocol (Mnase+NHS version) **Garber Lab**

Last updated April 2019

Non-commercial buffers used in this protocol:

DSG Crosslinking Solution
1X PBS
2mM DSG in DMSO

Scraping Buffer
1 x PBS pH 7.5
0.5% BSA
Store at 4 °C

Cell Lysis Buffer A
50mM HEPES pH 7.5
1mM EDTA
1mM EGTA
140mM NaCl
0.25% Triton-X100
0.5% Igepal CA630
10% Glycerol
(PIC add fresh)

Cell Lysis Buffer B
10mM HEPES pH 7.5
1.5mM EDTA
1.5mM EGTA
200mM NaCl
(PIC add fresh)

Cell Lysis Buffer C1 (MNase)
20 mM HEPES pH 7.5
1.5 mM EDTA
100 mM NaCl
0.1% NaDOC
0.1% Igepal CA630
(PIC add fresh)

10X CaCl2
50 mM in water

RNK-400 Buffer
20mM Tris pH 7.5
400mM NaCl
10mM EDTA
10mM EGTA
0.5% Triton-X
0.2% SDS

2X MNase -> ChRIPA Adjustment Buffer
80 mM HEPES pH 7.5
200 mM NaCl
1.5 mM EDTA
50 mM EGTA
1.8% Igepal CA630
0.9% NaDOC
0.1% SDS
(0.5 mM PMSF add fresh)

ChRIPA Buffer
1X PBS (make from 10X)
1 mM EDTA pH 8
1 mM EGTA pH 8
1% Igepal CA630
0.5% NaDOC
0.1% SDS

Bead Blocking Buffer
0.5% BSA
0.5% Tween 20

RIPA-500
10 mM Tris-HCL pH8
1 mM EDTA
500 mM NaCl
1% Triton X 100
0.1% NaDOC
0.1% SDS

LiCl Wash
10 mM Tris-HCL pH 8
1 mM EDTA
250 mM LiCl
0.5% NP40
0.5% NaDOC

PBS DEB
1x PBS
5 mM EDTA
0.5% SDS
10 mM DTT (add fresh)

M2 Wash Buffer
20mM Tris pH 7.5
50mM NaCl
0.2% Triton-X100
0.2% Igepal CA630
0.2% NaDOC

RLT++ Buffer
1x Buffer RLT
10 mM TRIS pH 7.5
1 mM EDTA
1 mM EGTA
0.2% NLS
0.1% Triton-X 100
0.1% NP-40

DNA Elution Buffer (EB)
10 mM Tris pH 8

1. Cell Cross linking – DSG + 3% Formaldehyde

Reagents:

- Phosphate-buffered saline (PBS) 1X
- PBS 1X + 0.5% Bovine serum albumin (BSA)
- Dimethyl sulfoxide (DMSO)
- 16% Formaldehyde (FA) Solution ampules from ThermoFisher
- Disuccinimidyl glutarate (DSG), 50 mg bottle from ThermoFisher

Procedure:

1. Chill one bottle of PBS 1X + 0.5% BSA. Keep one bottle of PBS 1X at room-temperature. Let 5% BSA 15 mL tube thaw on ice.
2. Add 306 μl DMSO to a bottle of DSG (0.5 M final concentration). Vortex to mix
3. Collect cells from culture in a 50 mL Falcon tube. If cells are adherent or PMA/LPS treated, scrape dish/flask.
4. Wash cells with PBS 1X to remove residual media. Pellet cells from culture (500 *g*, 5 min, room-temperature). Carefully remove media. Re-suspend cells in 10 mL of room temperature PBS 1X.
5. Wash cells again. Pellet cells at 500 *g*, 5 min, room-temperature. Carefully aspirate off media. Re-suspend cells in 10 mL of room temperature PBS 1X. Add 40 μl of 0.5 M DSG (from Step 2) to the 10 mL of PBS 1X to a final concentration of 2 mM (Add 40 μl DSG per 10 mL PBS 1X). Invert to mix.
6. Rotate Falcon tube on Mini Lab roller for 45 min.
7. While waiting on incubation, label the 1.5 mL aliquot tubes, use the colored USA Scientific tubes which are good for freezing. Aim for about 2 million cells per aliquot.
8. Remove DSG solution from cells by PBS 1X wash. Pellet at 500 *g*, 5 min, room-temperature and aspirate off supernatant. Re-suspend with 10 mL room-temperature PBS 1X.
9. Wash cells again. Pellet at 500 *g*, 5 min, room-temperature and aspirate off supernatant.
10. Re-suspend pellet with 10 mL of PBS 1X then add 16% FA from a fresh ampule to the 10 mL of PBS 1X. For 10 mL of PBS 1X, add 2.308 mL of 16% FA for a final concentration of 3% FA.
11. Incubate cross linking reaction at room temperature for 10 min, rotating on Mini Lab roller.
12. Add 6 mL of 1 M Glycine stop solution to quench the 3% FA (500 μl per 1 mL PBS 1X + 3% FA).
13. Incubate cells with Glycine stop solution at room-temperature for 5 min, rotating on Mini Lab roller.
14. Add 5% BSA to solution for final BSA concentration of 0.5%. For 18.308 mL, add 2.034 mL of BSA 5% to achieve a final BSA concentration of 0.5%.
15. Pellet at 1000 *g*, 5 min, 4°C and carefully aspirate off supernatant into FA waste.
16. Re-suspend with 10 mL ice-cold PBS + 0.5% BSA, then pellet at 1000 *g*, 5 min, 4°C. Keep tube on ice.
17. Re-suspend with enough ice-cold PBS + 0.5% BSA so that each 1.5 mL micro centrifuge tube aliquot gets 1 mL with 2 x 10^6 cells (e.g. if there were 20 x 10^6 cells to start, re-suspend with 10 mL ice-cold PBS + 0.5% BSA and then add 1 mL of solution to 10 aliquots. Keep tubes on ice.
18. Wash the sides of the 50 mL Falcon tube with 500 μl of ice-cold PBS + 0.5% BSA per micro centrifuge tube for any remaining cells, then add 500 μl to each of the previous aliquots (e.g. for 10 tubes wash with 5 mL of ice-cold PBS + 0.5% BSA).
19. Pellet cells in table top centrifuge (max speed 15,000 rpm, 1 min, 4°C).
20. Aspirate off supernatant.

21. Flash freeze cells in liquid nitrogen and then store in -80°C.

2. Lysate Preparation and DNA Fragmentation

All steps are performed on ice or in the cold room unless otherwise noted

1. Thaw cell pellet on ice 15-20 min & pre chill centrifuge & pre chill lysis buffers A, B, C1

2. Add fresh PIC to 1X or PMSF to 0.5 mM to lysis buffers

3. Resuspend lysis buffer A to cell pellet (1400 uL/10M cells); incubate on ice for 10 minutes

4. Spin at 4C for 9 minutes at 850g, discard supernantant.

5. Resuspend cell pellet in lysis buffer B; incubate on ice for 10 minutes

6. Spin at 4C for 9 minutes at 850g, discard supernatant.

7. Resuspend cell pellet in 600 ul lysis buffer C1; incubate on ice for 10 minutes

8. Sonicate with Branson microtip sonicator 0.7 seconds ON; 3.3 seconds OFF at 4-5 watts for 1 minute. Return lysate to ice.

9. Set up Mnase test digests on 10 ul lysate aliquots using Mnase diluted in Buffer C1, using the following table as an example. Add CaCl2 last using a multichannel, and mix well. Keep remaining lysate on ice.

Rxn #	Lysate (ul)	Buffer C1 (uL)	1:500 MNase (ul)	10x Cacl2 (50 mM)
1	10	7	1	2
2	10	6	2	2
3	10	5	3	2
4	10	3	5	2
5	10	0	8	2

10. Incubate at 37C on thermomixer shaking at 800 rpm for exactly 20 minutes then place on ice. Quench MNase with 2 uL of 0.5M EGTA and mix.

11. Reverse crosslink for 3 hours or overnight at 65C (in PCR machine) by adding the following to each tube
    1. 74 uL of RNK-400
    2. 4 uL of Proteinase K 20 mg/mL

12. Clean with 2X vol SPRI or Zymo DNA clean and concentrate. Elute in 2 x 9 ul EB (18 ul total)

13. Run 15 ul on a gel to pick a concentration that has a majority of samples mono-nucleosome and bi/tri-nucleosomes

Digest remaining bulk of lysate

14. Calculate the Mnase digestion conditions for the remaining lysate from the test digests. For example, if the remaining lysate is 550 ul (i.e. 55 times as much lysate as the 10 ul test digests) and it was determined that 2 ul 1:500 Mnase should be added to 10 ul lysate, then the bulk digest should be set up as follows:

    <u>SAMPLE BULK DIGEST</u>
    550 uL Lysate in Buffer C1
    15 uL Buffer C1
    75 ul 50 mM CaCl2
    ***Preheat to 37°C for 5 minutes*
    +110 uL 1:500 Mnase in Buffer C1 (55 x 2 ul determined from test)
    Mix well

15. Incubate at 37°C for 20 minutes then place on ice.

16. Immediately quench the reaction with 1/10 volume 0.5 M EGTA and SDS to 0.1% final.

17. Take 2% of the digested lysate from step 17 and preform reverse cross linking as in step 11.

18. Meanwhile, once reverse crosslinking is started, in an appropriately sized tube, add an equal volume of
    2X Mnase->ChRIPA buffer to the digested lysate.

19. Prepare Dynabeads Protein A (or A/G) for pre-clearing. Vortex stock bottle (10 mg/mL) to ensure a uniform suspension. Transfer 60 ul Protein A beads per million cells starting to a fresh tube.

20. Use a magnet to wash Protein A beads twice with ChRIPA Buffer.

21. Resuspend washed Protein A beads in a convenient volume of ChRIPA buffer and add to digested lysate(s) from step 18.

22. Rotate lysate + beads end over end in the cold room for at least 2h.

23. Remove Protein A beads with a magnet and transfer lysate to a fresh tube. *Perform this step twice.*

At this point, digested pre-cleared lysate is ready to use for ChIP in Section 3. Lysate should be stored at 4C up to overnight, or aliquoted (optional) and flash frozen in liquid nitrogen and stored at -80 for less than 6 months.

Lysate QC

24. After reverse crosslinking of the 2% sample collected in step 17, clean with Zymo DNA clean and concentrate column as per manufactures instructions. Elute twice with 10 uL EB (20 ul total)

25. Save at least 2 ul as "Input." Run a Gel/Bio-Analyzer, and measure concentration using Qubit.

26. Calculate and record the total amount and concentration of DNA in lysate. Write on tube.

3. ChIP and Washes

1. Thaw (if necessary) pre-cleared lysate from Step 2.23 above.

2. Aliquot lysate according to scale of IP (e.g. 2-5 ug) and adjust the volume to at least 500 ul using ChRIPA.

3. To each IP, add the appropriate amount of antibody (this must be determined empirically) and rotate O/N at 4C.

4. Meanwhile, separately block a fresh set of Protein A Dynabeads.
    a. Calculate how much total antibody will be used for ChIP. For each ug antibody to be used, transfer 25 ul Protein A Dynabeads (10 mg/mL) to a fresh tube.
    b. Wash beads PBS + 0.5% Tween-20
    c. Resuspend washed beads in 0.5-1 mL Bead Blocking Buffer and rotate end-over-end at 4°C for at least 90 min or overnight.
    d. Capture blocked beads on magnet and wash once with ChRIPA.
    e. Resuspend blocked beads in convenient volume of ChRIPA to divide among IPs in Step 3.

5. Add blocked Protein A beads from Step 3 and rotate at 4°C for 2 hours (preferred) or 1h at room temp.

6. Capture beads/antibody on a magnet. Remove supernatant (* optional: Save as Flowthrough).

7. Resuspend beads in 150 ul ice-cold ChRIPA and transfer to strip tubes. Rinse original capture tube with another 100 ul ChRIPA and pool with beads in strip tubes. Capture and remove sup.

8. Wash beads in strip tubes four times in ice-cold ChRIPA. Wash by adding 200 ul wash buffer and moving strip(s) back and forth on the magnet so that the beads move across the tubes multiple times.

9. Wash beads twice with room temp RIPA 500.

10. Wash beads twice with ice-cold LiCl Wash.

11. Wash beads three times with ice-cold ChRIPA, completely removing the supernatant after the last wash.

12. *If proceeding to SIP/SPRITE:* Elute chromatin from protein A beads by adding 50uL of PBS-DEB + 10 mM fresh DTT and shaking @ 1200 rpm for 5 minutes at 25C. Capture on magnet collect the supernatant in a fresh 1.5 mL tube at room temp.

13. Repeat step 12 another two times, pooling together the total 150 uL euate. Mix well.

(If only performing ChIP-seq and not proceeding to NHS coupling/barcoding, at Step 12 instead resuspend beads in 55 ul PBS DEB + 5 uL Proteinase K (20 mg/mL) and incubate overnight at 65C in a PCR machine with heated lid. Capture beads on magnet and transfer supernatant to a fresh tube. Rinse beads once using 40 ul TE or EB and pool with first eluate. Clean up using 2X vol SPRI or Zymo DNA Clean and Concentrate. Elute twice in 15 ul (30 ul total) EB and skip to ChIP-seq Library Prep, Section 3.19.)

14. Depending on estimated ChIP yield, transfer 5% (QC only) or up to 10-20% (QC + ChIP-seq) of the eluted sample to a fresh tube.

15. Dilute to 95 ul in RNK-400 buffer and add 5 ul Proteinase K (20 mg/mL). Reverse crosslink at 65C for at least 4h to overnight.

16. Flash freeze the remaining ChIP eluate in liquid nitrogen and store at -80 for up to 6 months.

17. Clean up the reverse crosslinked eluate from Step 15 using Zymo DNA clean and concentrate. Elute twice with 10 ul (20 ul total) EB.

18. Run 1 ul cleaned up eluate on bioanalyzer and measure the concentration of 2 ul using Qubit. Calculate the number of molecules per ul in the eluate, and use this number in NHS bead

ChIP SEQ library prep:

19. Start with up to 25 ul cleaned up ChIP eluate and if necessary adjust to 25 ul with water.

20. Add 3.5 ul NEB Next Ultra II End Prep Buffer and 1.5 uL NEB Next Ultra II End Repair Enzyme Mix.

21. Incubate for 30m at 25C then 30m at 65C.

22. To each 30 ul End Repair reaction on ice, add a unique 1.25 ul I96 Barocded Y Adapter (1.5 uM).

23. To each reaction add 15 uL NEB Next Ultra II Ligation Master Mix and 0.5 uL Ligation Enhancer. Mix.

24. Incubate 15 min at 20C.

25. Add 46.75 ul TE (final volume for each sample should now be 93.5). Add 93.5 ul SPRI beads and mix well. Bind and wash as usual. Elute twice with 10 ul EB (20 ul total).

26. To 20 ul sample from Step 25, add and mix:

    30 ul 2X Kapa Hi-Fi PCR Master Mix
    2 ul Broad I96 F/R Primers (25 uM)
    6 ul 10X SYBR green
    2 ul water

27. Transfer 10 ul each PCR reaction to a qPCR plate and store the remainder on ice.

28. Perform qPCR as follows, to determine appropriate number of library amplification cycles.

    98C for 4 min
    Then 20 cycles of
    98C for 20s
    56C for 30s
    72C for 30s (Plate read after this step)

29. Evaluate qPCR curve to determine cycle numbers for each sample that precede the inflection point of the curve. For a robust ChIP, this number is typically 6-12 cycles.

30. PCR amplify the remainder of the reaction volumes for the number of cycles determined in Steps 28-29.

31. SPRI clean the PCR reactions using 0.7X volume SPRI beads. Repeat this step to ensure complete adapter removal. After the second cleanup, elute twice in 8 ul.

32. Quantify library and sequence.

4. **NHS bead coupling** (Note: Read through this entire section before performing it)

✔Using the number of molecules calculated in Section 3.18, determine the volume sample and volume of NHS beads required for the experiment. Fewer molecules from ChIP eluates couple to NHS than with total lysate, so the recommended ratio is 10x109 ChIP'd molecules per 1 mL NHS bead suspension. However, this number should be determined empirically for your particular sample.

✔ Dilute the appropriate volume of thawed/mixed sample in at least 0.5 mL PBS + 0.1% SDS.

✔ Note: All wash steps at 4° C are performed in a cold room. All wash steps above room temperature are performed on an Eppendorf Thermomixer. If a temperature is not specified, it is at room temperature. To wash beads, place the tube containing the beads on a magnetic rack to capture the beads. Wait until the solution is clear and all beads are captured before removing the liquid. Add the wash solution to the beads and remove the tube from the magnet. Invert the tube until all beads are in suspension. If using an Eppendorf Thermomixer, set the thermomixer to shake at 1200 RPM. Briefly centrifuge the tube to remove beads from the lid, then place the tube back on the magnet to capture the beads again. Wait until the solution is clear and all beads are captured before removing the wash liquid. These steps are critical to avoid loss of beads throughout protocol

✔ Note: The protocol can be stopped at any point of the process. To ensure the integrity of the DNA, resuspend the beads in 1 mL RLT++ and store at 4° C until you wish to resume. Wash three times with M2 Buffer to

remove all RLT before proceeding with the protocol to prevent enzyme denaturation in subsequent steps of the protocol.

✔ Note: All steps involving bead pipetting should use low-bind pipette tips.

1. Gently invert the bottle containing the Pierce NHS-activated beads in *N,N*-dimethylacetamide (DMAC) until there is a uniform suspension. Being careful not to introduce water into the bottle, transfer X mL of NHS beads into a clean 1.7 mL lo-bind tube. Place the tube on a magnetic rack to capture the beads.

2. Remove the DMAC and wash beads with 1 mL ice-cold 1mM HCl.

3. Wash beads with 1 mL ice-cold 1 x PBS.

4. Add 1 mL Coupling Buffer to the beads.

5. Add X uL of DNased lysate to the beads.

6. Incubate the lysate and beads overnight at 4° C on a mixer.

7. Place beads on a magnet and remove 500 uL of flowthrough. OPTIONAL: This flowthrough aliquot can be saved to determine coupling efficiency if the DPM QC fails.

8. Add 500uL 1M Tris pH 7.5 (3M ethanolamine pH 9.0 can also be used) to the beads and incubate on a mixer at 4° C for at least 45 minutes. This ensures that all NHS beads will be quenched with protein from bound lysate or Tris, and will not bind enzymes in the following steps.

9. Wash beads twice in RLT++ Buffer.

10. Wash beads three times with M2 Buffer.

11. Spin the beads down quickly in a microcentrifuge and place back on the magnet to remove any remaining liquid.

5.2 Phosphorylation and End Repair

1. Blunt the 5' and 3' ends of the DNA molecules to prevent unwanted ligation by adding the following mixture to the beads:

Stock Solution	**Volume**
H_2O	212.5uL
End Repair Reaction Buffer (10X)	25uL
End Repair Enzyme Mix	12.5uL
Total	250uL

2. Incubate on a thermomixer for 60 minutes at 24° C, 1200 RPM.

3. Wash once with RLT++ Buffer.

4. Wash three times with M2 Buffer.

5. Spin the beads down quickly in a microcentrifuge and place back on the magnet to remove any remaining liquid.

6. Add dATP to the 3' ends of each DNA molecule to allow for ligation of the DPM adaptor by adding the following mixture to the beads:

Stock Solution	**Volume**
H2O	215uL
dA-Tailing Reaction Buffer (10X)	25uL
Klenow Fragment (exo-)	10uL
Total	250uL

7. Incubate on a thermomixer for 60 minutes at 37° C, 1200RPM. If ligating the DPM adaptor barcode on the same day, set up the reaction during this incubation.

8. Wash once with RLT++ Buffer.

9. Wash three times with M2 Buffer.

10. Spin the beads down briefly in a microcentrifuge and place back on the magnet to remove any remaining liquid.

5.3 DPM Adaptor Ligation

✔ Note: There are 96 adaptors that are designed to ligate onto the DNA molecules. These DPM adaptors are kept in a 96-well stock plate at 45uM. The ligation reaction between the adaptors and the DNA occurs in a 96-well plate. The following steps that detail set up are designed for optimum efficiency during the process.

✔ Note: All ligation steps include M2 buffer, which contains detergents to prevent beads from aggregating, sticking to the plastic tips and tubes, and for even distribution of the beads across a 96-well plate. We have verified that these detergents do not significantly inhibit ligation efficiency.

1. Create a dilute M2 Buffer by mixing 1100 uL of M2 Buffer with 792 uL of H_2O.

2. Accounting for bead volume, add the dilute M2 Buffer to the beads to achieve a final volume of 1.075 mL. Ensure that the beads are equally resuspended in the buffer. Distribute the beads equally into a 12-well strip tube by aliquoting 89.6 uL of beads into each well.

3. Make Ligation Master Mix for five rounds of SPRITE (DPM + four extra tags). Split the master mix evenly into each well of a 12-well strip tube by pipetting 260 uL into each well. Keep on ice ✔.

Stock Solution	**Volume**
NEBNext Quick Ligation Reaction Buffer (5X)	1600 uL
Instant Sticky-end Ligation Master Mix (2X)	1000 uL
1,2-Propanediol	600 uL
Total	32000 uL

✔ Note: Ligation Master Mix can be stored overnight at -20° C.

4. Centrifuge the DPM adaptor stock plate before removing the foil seal. Aliquot 2.4 uL from the stock plate of DPM adaptors to a new low-bind 96-well plate ✔. Be careful to ensure that there is no mixing between wells at any point of the process to avoid cross-contamination of barcodes. Use a new pipette tip for each well. After transfer is complete, seal both plates with a new foil seal.

   ✔ Note: This step can be done in advanced, in bulk, so that these plates are ready-to-use.

5. Centrifuge the 96-well plate containing the aliquoted adaptors, and then remove the foil seal.

6. Aliquot 11.2 uL of beads into each well of the 96-well plate that contains 2.4 uL of the DPM adaptors. Be careful to ensure that there is no mixing between wells at any point of the process. Use a new pipette tip for each well. Also be careful to ensure that there are no beads remaining in the pipette tip.

7. Carefully add any remaining beads to individual wells on the plate in 1 uL aliquots.

8. Aliquot 6.4 uL of Ligation Master Mix into each well, mixing by pipetting up and down 10 times. Be careful to ensure that there is no mixing between wells at any point of the process. Use a new pipette tip for each well.

9. The final reaction components and volumes for each well should be as follows:

Stock Solution	**Volume**
Beads + M2 + H_2O Mix	11.2 uL
DPM Adaptor (45uM)	2.4 uL
Ligation Master Mix	6.4 uL
Total	20 uL

10. Seal the plate with a foil seal and incubate on a thermomixer for 60 minutes at 20° C, shaking for 30 seconds at 1600 RPM every five minutes to prevent beads from settling to the bottom of the plate ✔.

    ✔ Note: Ligation time is critical for high efficiency of ligation each round. We have tested ligation at 5, 15, 30, 45, and 60 minute reaction times and 60 minutes ligation time has significantly higher yields over the other times.

11. After incubation, centrifuge the plate before removing the foil seal.

12. Pour RLT++ Buffer into a sterile plastic reservoir, and transfer 60 uL of RLT++ into each well of the 96-well plate to stop the ligation reactions. It is not necessary to use new tips for each well.

13. Pool all 96 stopped ligation reactions into a second sterile plastic reservoir.

14. Place a 15 mL conical tube on an appropriately sized magnetic rack and transfer the ligation pool into the conical. Capture all beads on the magnet, disposing all RLT++ in an appropriate waste receptacle.

15. Remove the 15 mL conical containing the beads from the magnet and resuspend beads in 1 mL M2 Buffer. Transfer the bead solution to a microcentrifuge tube.

16. Wash three times with M2 Buffer.

5.4 QC: Check to Determine Ligation Efficiency of the DPM Adaptor

1. Resuspend the beads in MyRNK Buffer so that the final beads + buffer volume is 1 mL. Remove a 5% aliquot (50 uL) into a separate 1.7 mL microcentrifuge tube.

2. Place the remaining 95% of beads back on the magnetic rack, remove the MyRNK Buffer, and store beads in 1 mL of RLT++ Buffer. Keep beads at 4° C overnight.

3. Elute DNA in the 5% aliquot by reversal of crosslinks through heating and Proteinase K.

Stock Solution	**Volume**
Sample on beads in MyRNK Buffer	50uL
MyRNK Buffer	42uL
Proteinase K	8uL
Total	100uL

4. Incubate at 65° C for two hours minimum.

5. Place the microcentrifuge tube on a magnet and capture the beads. Remove the flowthrough that contains the DNA ligated with DPM adaptor and place in a clean 1.7mL microcentrifuge tube.

6. Pipette 25 uL of H_2O into the tube containing the beads. Vortex, briefly centrifuge, and re-capture the beads. Remove the 25 uL of H2O that now contains any residual nucleic acid and add to the new sample tube. Discard the beads.

7. Clean the DNA by following the protocol provided in the DNA Clean and Concentrator Kit. Elute in 40 uL of H2O.

8. Amplify the DNA molecules that are ligated to the adaptors. The forward primer should prime off the 5' end of the DPM adaptor and the reverse primer should prime off the 3' end of the DPM adaptor.

Stock Solution	**Volume**
Sample (cleaned)	10 uL
DPMQCForward Primer (100uM)	1 uL
DPMQCReverse Primer (100uM)	1 uL
H2O	13 uL
Q5 Hot Start Master Mix	25 uL
Total	50 uL

PCR Program:

1. Initial denaturation: 98° C - 120 seconds
2. 12-16 cycles:
    a. 98° C -10 seconds
    b. 67° C - 30 seconds
    c. 72° C - 40 seconds
3. Final extension: 72° C - 120 seconds
4. Hold 4C

9. Clean the PCR reaction and size select for your target DNA molecules. Our DPM adaptors are 30 base pairs each and our target DNA molecules no less than 100 base pairs. Agencourt AMPure XP beads size select while cleaning the PCR reaction of unwanted products.

    a. Add 1.0 x volume (50 uL) of AMPure XP beads to the sample for a total volume of 100uL and mix thoroughly.
    b. Incubate for 10 minutes at room temperature.
    c. Place the beads on an appropriately sized magnet to capture the beads and the bound DNA. Wait a few minutes until all the beads are captured.
    d. Remove the supernatant and discard.
    e. Wash beads twice with 80% ethanol by pipetting ethanol into the tube while beads are captured, moving the tube to the opposite side of the magnet so that beads pass through the ethanol, and then removing the ethanol solution.
    f. Quickly spin down the beads in a microcentrifuge, re-capture on magnet, and remove any remaining ethanol.
    g. Air-dry beads while the tube is on the magnet.

h. Elute the amplified DNA from the beads by resuspending the beads in 12 uL of H_2O. Place the solution back on the magnet to capture the beads. Remove the eluted amplified DNA to a clean microcentrifuge tube.

10. Determine concentration of DNA with the DPM adaptor in by following directions provided with the Qubit dsDNA HS Assay Kit.

11. Determine the size distribution of DNA with the DPM adaptor in each sample by following directions provided with either the High Sensitivity DNA Kit for the Agilent Bioanalyzer or the D1000 Screentape for the Agilent 2200 TapeStation. The average size should be roughly similar to the average size of the input lysate (around 200-400 base pairs).

12. Calculate the number of DNA molecules in the 5% aliquot by determining molarity from the concentration and average size✖.

    ✖ Critical Point: The 5% aliquot should contain, at the very minimum, 15 million unique DNA molecules in order to proceed with SPRITE. If the aliquot contains less than this number, there will not be enough unique reads to sequence the SPRITE library. If this is the case, troubleshoot what went wrong by assaying coupling efficiency from the flowthrough saved in Step 7 of the NHS Coupling protocol. If lysate was successfully coupled, consider whether a mistake was made during ligation of the DPM adaptor or during one of the critical steps of crosslinking and lysis.

6 SPRITE and Library Preparation Pt. 2

Goal: The SPRITE method provides each DNA-DNA complex in the sample lysate with a unique nucleic acid barcode. When these complexes are decrosslinked, the individual DNA molecules that made up a single complex retain identical barcodes. These DNA libraries are sequenced on an Illumina Next-Generation sequencing platform and analyzed. Any DNA molecules found to have the same barcode interact *in-vivo*.

The SPRITE method works by splitting into a 96-well plate a pooled sample of crosslinked lysate where DNA molecules are ligated to the DPM adaptor. Each well of the 96-well plate contains a unique tag (Odd) to which the DNA molecules are ligated. The ligation reactions are stopped, pooled, and split again into a new 96-well plate containing different, unique tags than the first (Even). If n rounds of tag ligation are performed, 96^n unique barcodes are generated. We typically ligate 5 tags, creating over 8 billion unique barcodes. After all barcodes are ligated, the sample is split again into small m aliquots (100 wells of 1% aliquots up to 10 wells of 10% aliquots are typically used depending on the total material coupled) for PCR amplification. This final splitting of samples effectually sorts the DNA complexes once more, so that the chance that two different non-crosslinked complexes with the same barcode are amplified together is negligible. This last dilution into m wells effectively raises the number of unique tags to each molecule to $m*96^n$. For example, if the sample is aliquoted into 1% aliquots, then over 815 billion unique barcodes are generated.

The first round of SPRITE was already completed with the ligation of 96 unique DPM adaptors that allow for the subsequent ligation of new barcodes. As detailed in the Adaptor and Barcode Creation section, subsequent tag ligations are performed in the following order:

1. ODD Tag Ligation
2. EVEN Tag Ligation
3. ODD Tag Ligation
4. Terminal Tag Ligation

The four barcode ligations listed above are performed in the exact same manner with the only difference being the tag sequence. Thus, the following section will only detail one round of SPRITE.

6.1 SPRITE

1. Do this, but we don't know why. Most likely will delete. But do now anyways.

Stock Solution	**Volume**
H_2O	212.5uL
NEBuffer 2 (10X)	25uL
5' Deadenylase	12.5uL
Total	250uL

2. Incubate at 30° C for 30 minutes at 1200 RPM.

3. Wash once with RLT++.

4. Wash three times with M2.

5. Spin the beads down briefly in a microcentrifuge and place back on the magnet to remove any remaining liquid.

6. Create a dilute M2 Buffer by mixing 1100 uL of M2 Buffer with 792 uL of H_2O.

7. Accounting for bead volume, add the dilute M2 Buffer to the beads to achieve a final volume of 1.075 mL. Ensure that the beads are equally resuspended in the buffer. Distribute the beads equally into a 12-well strip tube by aliquoting 89.6 uL of beads into each well.

8. If frozen, thaw the strip tube containing the Ligation Master Mix made in Step 3 of the DPM Adaptor Ligation protocol. Keep on ice until ready to use.

9. Centrifuge the tag stock plate before removing the foil seal. Aliquot 2.4 uL from the stock plate of tags to a new low-bind 96-well plate ✔. Be careful to ensure that there is no mixing between wells at any

point of the process to avoid cross-contamination of barcodes. Use a new pipette tip for each well. After transfer is complete, seal both plates with a new foil seal.

✔ Note: This step can be done in advanced, in bulk, so that these plates are ready-to-use.

10. Centrifuge the 96-well plate containing the aliquoted tags, and then remove the foil seal.

11. Aliquot 11.2 uL of beads into each well of the 96-well plate that contains 2.4 uL of the tags. Be careful to ensure that there is no mixing between wells at any point of the process. Use a new pipette tip for each well. Also be careful to ensure that there are no beads remaining in the pipette tip.

12. Carefully add any remaining beads to individual wells on the plate in 1 uL aliquots.

13. Aliquot 6.4 uL of Ligation Master Mix into each well, mixing by pipetting up and down 10 times. Be careful to ensure that there is no mixing between wells at any point of the process. Use a new pipette tip for each well.

14. The final reaction components and volumes for each well should be as follows:

Stock Solution	**Volume**
Beads + M2 + H_2O Mix	11.2 uL
Tag (45uM)	2.4 uL
Ligation Master Mix	6.4 uL
Total	20 uL

15. Seal the plate with a foil seal and incubate on a thermomixer for 60 minutes at 20° C, shaking for 30 seconds at 1600 RPM every five minutes to prevent beads from settling to the bottom of the plate ✔.

✔ Note: Ligation time is critical for high efficiency of ligation each round.

16. After incubation, centrifuge the plate before removing the foil seal.

17. Pour RLT++ Buffer into a sterile plastic reservoir, and transfer 60 uL of RLT++ into each well of the 96-well plate to stop the ligation reactions. It is not necessary to use new tips for each well.

18. Pool all 96 stopped ligation reactions into a second sterile plastic reservoir.

19. Place a 15 mL conical tube on an appropriately sized magnetic rack and transfer the ligation pool into the conical. Capture all beads on the magnet, disposing all RLT++ in an appropriate waste receptacle.

20. Remove the 15 mL conical containing the beads from the magnet and resuspend beads in 1 mL M2 Buffer. Transfer the bead solution to a microcentrifuge tube.

21. Wash three times with M2 Buffer.

22. Repeat the process starting at Step 6 for the remaining three SPRITE rounds.

6.2 Final Library Preparation

1. Resuspend the beads in MyRNK Buffer so that the final beads + buffer volume is 950 uL.
2. Remove eight 5% aliquots into clean 1.7 mL microcentrifuge tubes and elute the barcoded DNA from the beads. Keep the remaining 55% of the lysate on beads in RLT++ Buffer at 4° C.

Stock Solution	**Volume**
Beads in MyRNK Buffer	50 uL
MyRNK Buffer	42 uL
Proteinase K	8 uL
Total	100 uL

3. Incubate at 65° C overnight.
4. Place the microcentrifuge tubes on a magnet and capture the beads. Remove the flowthrough that contains the barcoded DNA and place in a clean microcentrifuge tube.
5. Pipette 25 uL of H_2O into the tube containing the beads. Vortex, and re-capture the beads. Remove the 25 uL of H_2O that now contains any residual nucleic acid and add to the new sample tube. Discard the beads.
6. Follow the protocol provided in the DNA Clean and Concentrator Kit. Elute in 20 uL of H_2O.
7. Amplify the barcoded DNA. Refer to section 3.4 for details about the final library amplification step.

Stock Solution	**Volume**
Barcoded DNA (cleaned)	20 uL
First Primer (100uM)	1 uL
Second Primer (100uM)	1 uL
H_2O	3 uL
Q5 Hot Start Master Mix	25 uL
Total	50 uL

PCR Program:

1. Initial denaturation: 98° C - 180 seconds
2. 4 cycles:

    a. 98° C -10 seconds
    b. 68° C - 30 seconds
    c. 72° C - 60 seconds
3. 7 cycles:
    a. 98° C -10 seconds
    b. 70° C - 30 seconds
    c. 72° C - 60 seconds
4. Final extension: 72° C - 180 seconds
5. Hold 4° C

8. Clean the PCR reaction and size select for your target libraries. The total length of our barcode on one amplified product is around 160 base pairs and each target DNA molecules no less than 100 base pairs. Agencourt AMPure XP beads are able to size select while cleaning the PCR reaction of unwanted products.

    a. Add 0.7 x volume (35 uL) AMPure XP beads to the sample for a total volume of 85 uL and mix thoroughly.
    b. Incubate for 10 minutes at room temperature.
    c. Place the beads on an appropriately sized magnet to capture the beads and the bound DNA. Wait a few minutes until all the beads are captured.
    d. Remove the supernatant and discard.
    e. Wash beads twice with 80% ethanol by pipetting ethanol into the tube while beads are captured, moving the tube to the opposite side of the magnet so that beads pass through the ethanol, and then removing the ethanol solution.
    f. Quickly spin down the beads in a microcentrifuge, re-capture on magnet, and remove any remaining ethanol.
    g. Air-dry beads while the tube is on the magnet.
    h. Elute the amplified DNA from the beads by resuspending the beads in 50 uL of H2O.
    i. Repeat the size-select clean up with 0.7 x AMPure XP beads (add directly to the eluted DNA/ bead mix), eluting finally in 12 uL H_2O ✔.

    ✔ Note: To ensure all library material is eluted from beads, elute twice with 6 uL H_2O. Most of the material will be removed in the first elution, and any remaining material will be removed in the second.

9. Determine concentration of the barcoded libraries by following directions provided with the Qubit dsDNA HS Assay Kit. Final libraries are generally between 0.3 ng/uL and 1.5 ng/uL.

10. Determine the size distribution and average size of the barcoded libraries by following directions provided with the High Sensitivity DNA Kit for the Agilent Bioanalyzer. Average sizes are generally around 350-450 base pairs.

11. Calculate the number of DNA molecules in each barcoded library by determining molarity from the concentration and average size. We typically pool together 300 million unique DNA molecules for sequencing.

7 Sequencing and Data Analysis

The Illumina, Inc. HiSeq v2500 platform was employed for next generation sequencing of the generated libraries using a TruSeq Rapid SBS v1 Kit – HS (200 cycle) and TruSeq Rapid Paired End Cluster Kit – HS.

7.1 Computational pipeline

The sequencing data is output as two FASTQ files: one file for read one (forward strand) that contains information about the genomic sequence, and one file for read two (reverse strand) that comprises information about the attached barcodes. In addition to the sequence information, in this case as well as in general, the FASTQ files also contain information about the sequencing quality and the flowcell position for each read, as well as a unique name for each read. This information is retained throughout the following process.

First, the tag sequences within the reads are identified. The process starts with a hashtable containing all barcodes used for the specific experiment. This hashtable maps nucleotide sequence to barcode name, allowing up to two mismatches for the EVEN and ODD tag sequences, but requiring no mismatches for the DPM and the Yshape adapter.

The engine queries the hashtable for subsequences of the reads, first looking at the beginning of read one for a DPM sequence, then "walking down" read two looking sequentially for the Yshape adapter, an ODD barcode, an EVEN barcode, and a final ODD barcode. The output of this process is two FASTQ files identical to the input files, but with each read name adjusted to additionally contain the five identified sequences. (If fewer than five sequences are found, the read name contains all of the found sequences, as well as a "NOT_FOUND" placeholder for each barcode not found.)

The first eleven bases, which should be the DPM sequence, are trimmed from the read-one sequences. The remainder should be wholly genomic DNA, and this is aligned by Bowtie2 (local alignment, default scoring, -D 15 -R 2), first to the mouse mm9 assembly (nucleotide sequences of reference chromosomes and scaffolds), then separately to the human hg19 assembly. Local alignment is chosen over global alignment in case the sequence "reads through" the genomic data into the barcodes on the opposite side. Some sequences may align to both mm9 and hg19, so the two resulting BAM files are compared against each other, and any reads sequences, which align to both assemblies, are removed. Finally, the BAM files are filtered to keep only those reads in which all barcodes were successfully identified. Ultimately, each BAM record contains the following information:

1. Read name
2. Location within the flowcell (lane, swath, tile)
3. Sequencing quality
4. Alignment coordinates (chromosome and nucleotide position)
5. The sequence of identified barcodes
6. The sample identifier (i.e molecule-to-bead ratio tracked indirectly, from the DPM)
7. Alignment score (how well the read aligned)
8. The identification of mismatches/indels

7.2 DNA contact maps

In case of data originating from DNA-DNA barcoding with a pooled sample, each condition was specifically labeled with a subset of DPM adapter barcodes. To de-convolute the different individual samples a script is run that identifies the DPM adapter barcodes in the BAM files resulting from the computational pipeline and generates a file for each DPM adapter barcode subset. This step is not necessary in case of RNA-DNA barcoding. Next, all reads within an individual sample possessing the same barcodes are grouped together and contact frequencies between bins of a given size (for instance 1 Mb) are calculated. Since the number of contacts that each bin participates in is not uniform, we must take this into account in order to accurately compare contact frequencies between different pairs of bins. To do this, we normalize the contact frequencies by calculating the expected number of contacts for each pair of bins based on a uniform distribution of contacts and then dividing the observed number of contacts by the expected number of contacts. This information is then translated into a matrix (contact frequency map) that can plot either the observed/expected ratio or a Z-score of this ratio. The plot can be made for single chromosome and/or a genome-wide contact frequency map. Iterative correction and eigenvector decomposition of Hi-C data can also be performed to normalize DNA contacts as described by Imakaev *et al*. Nature Methods (2012).

The signal to noise level is measured by calculating the percentage of observed over expected contacts containing reads from both specimens (human and mouse) whereby expected is the percent of human-mouse contacts at random.