Chédin sDRIP Protocol

Day 1 / / :

I) DNA Extraction and Restriction Digestion for DRIP

1. Transfer the contents of a 10cm plate of 90% confluent cells (roughly $5x10^6$ to a 15 ml falcon tube and pellet cells gently @ 1000 rpm for 2 mins)
2. Wash and resuspend cells with **10 ml 1x DPBS** and pellet again. Remove supernatant.
3. Resuspend cells in:
   - **1.6 ml of TE**.
   - **5 µl of Proteinase K 20** mg/ml (Roche Life Sciences)
   - **50 µl of 20% SDS**

   Gently invert the tube a few times until solution becomes viscous. Incubate overnight at 37°C. (**no crosslinking!**).

Day 2 / / :

I) DNA Extraction and Sonication

1. Spin a 15 ml Phase lock tube (Maxtrack Qiagen) for 1 minute @ 1500 xg to pellet the gel
2. Add your DNA from previous step by pouring it directly into phase lock gel tube.
3. Add 1 volume (1.65 ml) of Phenol / Chloroform / Isoamylalcohol (pH 8) invert gently, spin down @ 1500 xg for 5 min.
4. Meanwhile prepare new 15 ml tube and add:
   - Add 1/10 (165 µL) volume 3M NaOAc pH 5.2
   - 2.4 (~5 mL) volumes 100 % EtOH
   - pour in the DNA (top aqueous phase) from phase lock tube.
5. Invert gently until the DNA precipitates, spool DNA with cut tips, transfer to 2 ml tube. (May take up to 10 mins; should see a little white cloudy blob) ** Do NOT centrifuge to pellet DNA!
6. Wash DNA 2-3 times with 70% EtOH (add 1.5 ml, invert gently, let it stand for 10 min, discard EtOH, redo, don't spin between each wash).
   - Wash 1
   - Wash 2

   *Carefully remove as much EtOH as possible by pipetting after last wash.
7. Allow to air dry completely while inverting tubes (it can take up to 1-2 hours per the amount of DNA) and resuspend in TE (usually 125 µl). Resuspend DNA gently while on keeping tubes on ice for a minimum of 1 hr.
8. Sonicate DRIP DNA using Bioruptor from Diagenode.
   - Perform 15 cycles of 15 sec ON / 60 sec OFF (spin after 6 cycles to ensure homogeneous sonication).
   - If needed, run sonicated DNA on an Agilent BioAnalyzer chip to confirm the distribution of sonicated material. The peak distribution of fragment sizes should be around 300 bp.

II) DRIP on Genomic DNA

1. Dilute 4.4 ug (4400 ng) of digested DNA in 500 µl TE.
   ** For DRIP-seq, perform 3 IPs in parallel to obtain enough material for library construction. 1 tube can also be used to prepare an input library if needed.
   This means to prepare 3 tubes of 4.4 ug of digested DNA in 500 µl TE.
2. Save 50 µl of each tube as input for DRIP-qPCR, this will allow you to calculate recovery as % input.
3. Add 50 µl of 10x binding buffer to each tube
4. Add 10 µl of S9.6 antibody to each tube
5. Incubate 16 hours at 4°C while gently inverting on a rotisserie shaker.
6. **For a negative control,** treat one tube of DNA with 3 µl Ribonuclease H (NEB, Cat# M0297) overnight at 37°C.
   - 10 µg of digested DNA
   - 3 µl of RNase H
   - 1 µl of RNase H buffer

     Total Volume 20 µl

   ***Store Remaining Samples at -80C in the meantime.

Day 3 / / :

II) DRIP on Genomic DNA

1. For each tube, wash 50 µl of Agarose beads **2**-3 times with 500 µl of 1x binding buffer (10x diluted in TE). Each wash is 10 min with gentle shaking, spin down the beads after each wash at 800 xg.
    - Wash 1
    - Wash 2
2. Add DNA/antibody complexes to prewashed beads and incubate 2 hours at 4°C on a rotisserie shaker.
3. Spin down beads, for 1 min @ 800 xg, discard supernatant
4. Wash the beads:antibody complexes by adding 700 µl of 1x binding buffer, invert 15 minutes at room temperature on a rotisserie. Spin down as in step 7 and discard supernatant.
5. Repeat step 8(4) twice (3 washes total). (2 washes sufficient for qPCR)
    - Wash 1
    - Wash 2
    - Wash 3
6. Add 250 µl Elution buffer and 7 µl Proteinase K 20 mg/ml and incubate at 55°C for 45 minutes with inversion (parafilm your tubes)
7. Pellet tubes at 5,000g for one minute. DO NOT PICK UP ANY OF THE BEADS!
1. Spin a 2 ml Phase lock tube (Maxtrack Qiagen) for 1 minute @ max speed 13000 rpm to pellet the gel; add your DNA from previous step by pouring it directly into phase lock gel tube.
2. Add 1 volume (250 µL) of Phenol / Chloroform / Isoamylalcohol (pH 8), invert gently, spin down @ Max speed xg for 15 min.
3. Meanwhile prepare a new 1.5 ml tube and add:
    - 1.5 µL Glycogen
    - 30 µL 3M NaOAc pH 5.2
    - 750 µL 100 % EtOH
    - gently pipette in the DNA (top aqueous phase) from phase lock tube.
4. Incubate at -20C for at least 1 hr
5. Spin at 16,000 xg for 35 min at 4ºC
6. Discard supernatant, add 200 µL 70% EtOH, spin 10 min @ 4°C, discard supernatant).
    - For ***DRIPs-qPCR***: resuspend each tube in 50 µl of 10 mM Tris-HCl pH 8. Leave on ice for 15 min.
    - For ***DRIPs-seq***: resuspend each tube in 15 µl of 10 mM Tris-HCl pH 8. Leave on ice for 15 min and combine all three tubes.
    - Note: for DRIP-seq, withdraw 5 µl out of the 45 µl (3 DRIPs combined), add 5 µl of 10 mM Tris HCl pH 8 and use 2 µl per locus

II) DRIP-qPCR on Genomic DNA - Enrichment Validation by qPCR

1. Used the following primers:
    - EGR1 (negative control)
    - RPLI3A (positive control at 10% enrichment)
    - TFPT (positive control at 5% enrichment)

    Note: For sonication these number tend to be lower
2. Reaction

	vol. (µL)
10 uM primer F+R	2
template	1
2x SYBR mix	10
dH20	7
total	20

Day 4 / / :

Note: sonication essentially breaks off the non-template ssDNA strand of an R-loop. Therefore the IP leads to the recovery of 2-stranded RNA:DNA hybrids. For library construction, the first step is to convert these hybrids back to dsDNA through a second strand synthesis step.

III) Building Sequencing Libraries

1. Use the 3 DRIP tubes combined previously (40 µl)
2. Sonicate DRIP DNA using Bioruptor from Diagenode.
   - Perform 12 cycles of 15 sec ON / 90 sec OFF (spin after 6 cycles to ensure homogeneous sonication).
   - If needed, run sonicated DNA on an Agilent BioAnalyzer chip to confirm the distribution of sonicated material. The peak distribution of fragment sizes should be around 300 bp.

Second Strand Synthesis

5X Buffer

Final Concentration	Stock	Stock added (mL)
200 mM Tris-Cl pH 7	1M Tris-Cl pH 7	2 mL
22 mM	1M $MgCl_2$	220 uL
425 mM	3M KCl	1.42 mL
	npH_2O	6.36 mL
a total of 10 mL		**10 mL**

Protocol:

Add the following components to sDRIP reaction (40 µl) on ice:

	vol. (uL)
DNA (3 IPs combined)	40
5X Second Strand Buffer	20
10 mM dNTP Mix (replace dTTP for dUTP if you want strand specificity)	5
16mM NAD	1
Np H_2O	32
total	98

►Mix well and incubate on ice for 5 minutes.

	vol. (uL)
DNA Mixture	98
DNA polymerase I (10 units)	1
RNase H (1.6 units)	0.3
E. coli DNA ligase	0.5
total	100

►Incubate at 16°C for 30 min (mix well)

Bring Ampure beads to room temp before using

* Make Fresh 80% ethanol and store at -20C until needed

1. Add 165 of Ampure beads to each sample and mix bead and incubate for 15 minutes at room temperature (Bind on beads everything)
2. Place samples onto magnet and remove supernatant
3. wash with 500 µl of 80% ethanol for 30 seconds
4. Remove supernatant and repeat ethanol wash
5. Allow samples to air dry on magnet (~15 mins) do not let the beads crack
6. Remove from magnent
7. Add 40.5 µl of 10 mM Tris pH8 and incubate for 5 minutes at room temperature
8. Place sample back on magnet (~2 minutes)

9. Transfer supernatant to new tube
10. Continue with Library construction

Library Construction

1) End repair

Mix:

	vol. (uL)
DNA (3 IPs combined)	40
NEB enzyme mix	2.5
NEB 10x end repair module buffer	5
ATP	2
total	50

► Incubate 30 minutes at room temperature.

2) Clean up using MiniElute column from Qiagen or AMPURE

Elute in 34 µl 10 mM Tris pH8. (add 250 of Buffer PB if using Qiagen) (1.65X Ampure ratio)

3) A-base addition

Mix:

	vol. (uL)
DNA	34
NEB buffer 2	5
1mM dATP	10
NEB Klenow exo	1
total	50

►Incubate 30 minutes at 37°C.

4) Clean up using MinElute column from Qiagen or AMPURE

Elute in 12 µl 10 mM Tris pH8. (add 250 of Buffer PB) (1.65X Ampure ratio)

5) Ligation of adapters

	vol. (uL)
DNA	12
Index adapters	1
NEB 2x quick ligation buffer	15
NEB quick ligation ligase	2
total	30

►Incubate 15 minutes at room temperature.

6) Clean up using AMPure (1:1 Ratio)

*Bring Ampure beads to room temp before using

* Make Fresh 80% ethanol and store at -20C until needed

11. Add 10 mM Tris pH 8 to each sample to bring up volume to 100 µl
12. Add 100 of Ampure beads to each sample and mix bead and incubate for 15 minutes at room temperature

13. Place samples onto magnet and remove supernatant
14. wash with 500 µl of 80% ethanol for 30 seconds
15. Remove supernatant and repeat ethanol wash
16. Allow samples to air dry on magnet (~15 mins) do not let the beads crack
17. Remove from magnent
18. Add 20.5 µl of 10 mM Tris pH8 and incubate for 5 minutes at room temperature
19. Place sample back on magnet (~2 minutes)
20. Transfer supernatant to new tube

Note: At this point you can use 0.5 µl of your elution to do a qPCR test using the PCR enrichment primers. This will allow you to estimate the number of cycles you will have to perform in order to amplify your library in the next step.

	vol. (uL)
DNA	0.5
Forward Primer	1
Reverse Primer	1
SyberGreen	10
npH_20	7.5
total	20

7) Generate Strand Specific Libraries

- Add 1.5 µl of UNG (removes any uracil incorporated into the DNA) to each sample and incubate for 20 minutes at 37°C

8) Enrichment – Amplifying DRIP-seq Libraries

	vol. (uL)
DNA	10
Forward Primer	1
Reverse Primer	1
Phusion 2X HF master mix	15
npH_20	3
total	30

► Run on a thermal cycler with the following program:

98°C 30 sec followed by **10-15** cycles of:
10 sec @ 98°C
30 sec @ 60°C
30 sec @ 72°C
Follow by 5 min @ 72°C and a 4°C hold.

9) Clean up using AMPure (0.6:1 Ratio followed by 1:1 ratio)

*Bring Ampure beads to room temp before using
* Make Fresh 80% ethanol and store at -20C until needed

1. Add 70 µl 10 mM Tris pH8 to each sample to bring up volume to 100 µl
2. Add 65 µl of Ampure beads to each sample and mix bead and incubate for 15 minutes at room temperature *This removes fragments >500 bp)
3. Place samples onto magnet and transfer supernatant 165 µl to new tube
4. Add 100 ul of 10 mM Tris pH8 to the supernatant to bring up volume to 265 µl (200 µl of sample to 65 µl of Ampure)
5. Add 135 µl of Ampure (removes fragments <200bp) to bring up volume to 400 µl (200 ul of sample to 200 µl of Ampure – 1:1 ratio)
6. Incubate for 15 minutes
7. Place samples onto magnet for 2 minutes
8. Discard supernatant and wash with 185 µl of 80% ethanol for 30 seconds
9. Remove supernatant and repeat ethanol wash
10. Allow samples to air dry on magnet (~15 mins) do not let the beads crack

11. Remove from magnet
12. Add 12 µl of 10 mM Tris pH8 and incubate for 5 minutes at room temperature
13. Place sample back on magnet (~2 minutes)
14. Transfer supernatant to new tube

Use 1µl to check your library on an Agilent BioAnalyzer
Use 1µl to test for qPCR enrichment: add 9 µl of 10 mMTris HCl pH8 to 1 µl of library and use 2 µl per locus (use the same loci you checked after DRIP).