DNaseI hypersensitive site (for DNase-seq) protocol

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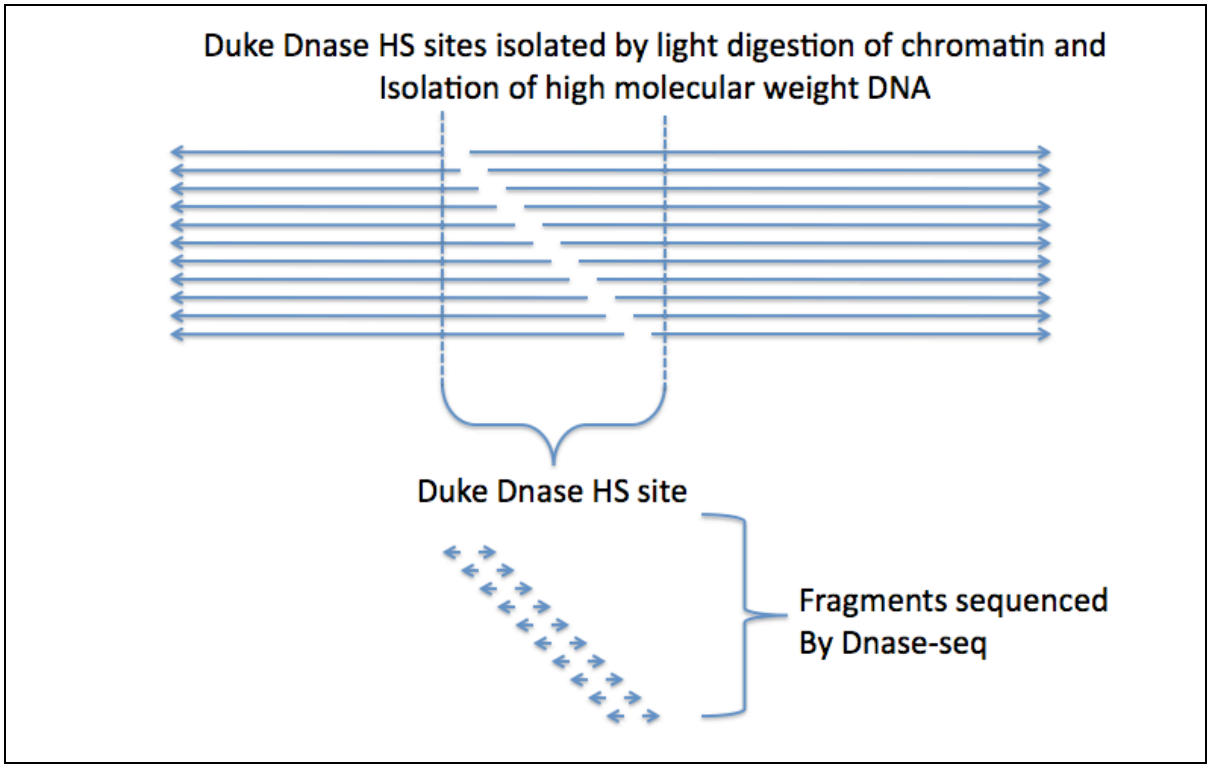

Step 1: DNaseI digestion of nuclei to isolate high molecular weight DNase-treated DNA (20 million cell protocol)

Before starting protocol, make sure you…

- Melt low melt gel agarose at 75 degrees, and keep melted at 55 degrees (InCert high purity low melt
- 1% Incert low melt gel in 50 mM EDTA melted at 75 degrees
- 55 degree heat block or water bath
- Water bath at 37 degrees
- LIDS Buffer made and filtered
- PBS, RSB, and RSB+NP40 chilled on ice
- DNase dilutions made (make sure mixed thoroughly) and aliquoted into 7 x 1.5ml tubes on ice

1) Spin down **20x 10^6** cells at **900 RPM x 5** minutes; use vacuum or pipet (do not pour off supernatent) to carefully remove the supernatant. Since pellet is loose, leave a 1 mm-thick supernatant layer over the pellet during washes to reduce cell loss. In residual liquid flick to resuspend cell pellet
2) Wash 2X in cold PBS (50 ml PBS for each washing). If start with two or more than two tubes of cell culture, combine cells into one tube for the 1st wash. Resuspend final pellet in **500** ul cold RSB (NO NP40!) by gentle flicking and transfer to 15 ml conical tube (keep on ice). Wash the 50ml-tube with **500** ul cold RSB for one more time, pipet it into **500** ul cell suspension.
3) Gentle lysis with cold RSB + 0.1% NP40 (total of 14 mls)

---some cell types become overlysed with 0.1% NP40 (try 0.05%, 0.01%, etc.)----
---check cells after lysis with trypan blue and microscope----
---if overlysed (e.g., material clumps together into a goopy mess), use less NP40---
Pour the lysis buffer into 15 ml tube that has resuspended cells (no clumps) at bottom
Invert 5-10 times and spin (NO Pipeting!). Look to make sure no precipitate at this step

4) Spin down immediately at **500 x G** for **10** min at 4 deg to pellet nuclei, remove sup completely. DON'T leave any supernatant on the pellet.
5) During spin, add various concentrations of DNAse to **5** eppendorf tubes (1.5ml) and keep on ice. When making DNase dilutions, make sure each dilution is mixed thoroughly. Pipet/flick dilutions extensively (but do not vortex)
6) Resuspend cells in **720** ul cold RSB buffer (mix by flicking). Pellet should appear white and fluffy and should resuspend completely.
7) Pipet **120** ul/tube (using wide bore tip…tip clipped off with razor or scissors) into each of #1, #2, #3(1/1000 Dnase), #4(1/300), #5(1/100), #6(1/30). Mix nuclei with DNase concentrations by gentle, but thorough, flicking (NO PIPET or VORTEX). Resuspend nuclei by occasional flicking. Might not need highest DNase concentration if using less cells
8) Incubate at 37 deg for 10 minutes (**except keep tube #1 on ice)**. water bath is preferable
9) Add **160** ul of 50 mM EDTA/tube into #1, #2, #3, #4, #5 and #6 to stop the reaction (no SDS). Invert 5 times to mix (no pipeting!). Keep at Room temp until ready to embed (but work quickly)
10) Melt InCert low melt gel agarose (1% in 50 mM EDTA) at 75 degrees and keep melted at 55 degrees (Incert agarose from Lonza, Cat # 50121)
11) Equilibrate DNased DNA to 55 degrees for 1 minute
12) Pipet **280** ul/tube of low melt gel agarose to #1, #2, #3, #4, #5, and #6 nuclei/DNase tubes. invert 4 times to mix (no pipeting) . With wide bore pipet; pipet into Bio-Rad plug molds (cat#1703706) ~80 ul/plug. let set at 4 degrees for approx 5 minutes to solidify
13) Release plugs into in 15 ml LIDS buffer (15 ml conical tube) (Do not shake the tubes after releasing plugs! it may break them) and incubate for 1-2 hours at room temp while gently shaking (keep on side) (~50-60 RPM)

Use CHEF Screened Plug Caps to keep plugs in tube while washing (Biorad Cat# 1703711)

14) Incubate with fresh LIDS buffer O/N at 37 degrees (not shaking, but on the side)
16) Wash plugs in 0.05 M EDTA pH8, 5 x 35 mls for 1 hour each (room temp shaking ~60 RPMs). Wash the filters and lids with destilled water for twice at the 4th and 5th washing. Make sure that no detergent-caused bubbles left in tubes after 5th washing.
17) Store indefinitely at 4 deg in 0.05 M EDTA (at this point, plug are stable to be shipped in EDTA)
18) Run Pulsed field gel (PFG). See Figure 1. Optimal concentrations for DNase-chip are in bold. For DNase-seq, I have mostly used the smearing pattern that looks like 0.4 U DNase concentration. We load 1/3 of plug into each lane, and slide the plug into the side of each well using two metal spatulas.

Conditions (20-60 second switch time for 18 hours)
6 Volts/cm (180 volts)
1% Agarose in 0.5X TBE
Keep 0.5X TBE running buffer chilled at 16 degrees
Use NEB yeast chromosome PFG marker (NEB catalog # NO345S)

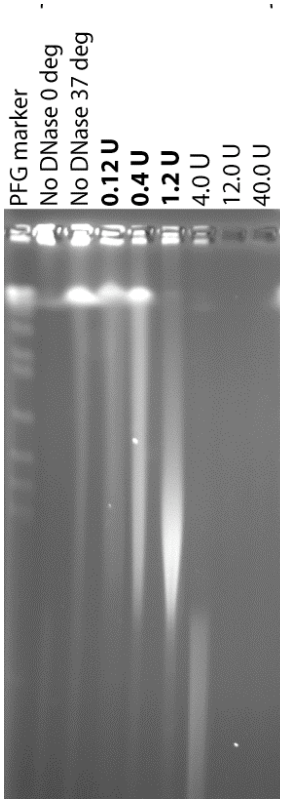

Figure 1. Pulsed field gel. This is what a good PFG should look like.,,gradual and consistent changes in fragment sizes as you increase DNase concentrations.

<u>Step 2:</u> Blunt ending DNase treated ends for DNase-chip or DNase-seq

1) Wash with T4 DNA pol buffer to remove EDTA (2 X 50 mls X 1 hour each)
2) Remove all liquid from 50 ml conicals and push plugs to bottom of tube
3) Polish ends with T4 DNA polymerase (do this in 50 ml conical use for washing)

DNA plug	80 ul
NEB Buffer 10X	12 ul
dNTPs (10 mM)	5 ul
T4 DNA Pol	6 ul (NEB Catalog # M0203L)
DH20	99.2 ul
<u>BSA (100x)</u>	<u>2 ul</u>
Total:	200 ul

let go for 4 hours at room temp (mix occasionally)
do a quick rinse with T4 buffer to remove most of enzyme

4) Add 500 ul of 10mM Tris pH8 containing 100mM NaCl. Heat at 65 deg for 15min to melt
Flick every few minutes to make sure agarose is dissolving
phenol, phenol:chloroform, chloroform
ETOH ppt with 1 ul glycogen

5) Pellet DNA at 4 degrees, wash 1x with 70% ETOH, spin and remove all residual liquid

6) Let dry FOR NO MORE THAN 4 minutes. Any longer, and the high molecular weight DNA will not resuspend.

7) Resuspend in 40 ul of suitable buffer (10 mM Tris for DNase-seq OR TE for DNase-chip)

Step 3: DNase-seq protocol

1) Anneal adaptor I and adaptor II.

2) Adaptor I ligation to blunted DNase-treated DNA:

Blunt DNA (3ug)	12 ul
10x ligation buff	5ul
T4 DNA ligase (10U)	2 ul
adaptor I (150pmole)	6 ul of 25 pmol/ul
water	
Total Volume	50ul

Incubate tubes in PCR machine, 20 degrees C, overnight

3) Remove unligated linkers by gel purification. Run the above ligated reactions on 0.8% EB-containing low melt agarose gel for 30-50 min at 80V. Make the low-melt gel with 10 mM EDTA-1XTBE. Make sure that the running buffer TBE contains 10 mM EDTA too.

- Cut out high molecular weight bands (leave linker-only band behind), put the gel into 500 ul of 10 mM Tris(pH8) + 100 mM NaCl; heat at 65 degree for 15 min to melt

- Gently flick every few minutes to make sure agarose is dissoving
- Extract with 500 ul phenol, phenol:chloroform, and chloroform.
- ETOH precipate. Add 1 ul glycogen, 1/10 volume of 3M NaAc, 1ul pellet paint (Novagen: 70748-4) and 325ul EtOH precipitate the eluted material

4) MmeI digestion:

DNA	75ul	
10x NEB4	10ul	
10xSAM(500uM)	10ul	Be sure to dilute the 32 mM SAM stock to 500 uM before use
Mmel	5ul	
Total Volume	100ul	

- Incubate 37C 1.5 hr
- add SAP (3ul)
- 37C 1hr
- Clean up product: 100 ul phenol/chloroform; chloroform clean, precipitate DNA by using 1 ul glycogen, 1/10 volume of 3 M NaAc, 1 ul pellet blue. Wash the pellet with 70% ethanol. respend the pellet with 50ul H2O

5) Invitrogen Dynal M-280 beads binding:
each reaction requires 100ul dynal bead (Invitrogen). Before use, wash beads 1ml 1XTE 2 times, followed by bead wash with 1ml 1XB&W 1 time.

DNA	50ul	100ul(1mg) dynal beads bind 5pmol 2-4 kb, or 200pmol oligo
2XB&W	50ul	See dynal protocol for B&W buffer composition
Total:	100ul	

Add mixture to washed Dynal beads

- 30C for 30 min, flick the tubes every 10 min.
- remove supernatant
- wash beads 1ml 1XTE 5 times.

6) Adaptor II ligation

Dynal beads	10ul	
10x ligation buffer	10ul	
Adaptor II	150pmole	6 ul of 25 pmol/ul
ligase 10U	2ul	roche (5U/ul)
H2O		
Total Volume	100ul	

- Rotate at room temp. 4 hr

7) Alkaline treatment

- Wash beads 1ml 1XTE 1 time.
- add 500ul of 0.15N NaOH directly to beads
  - 0.15N = 100ul 10N NaOH bring up to 6.6ml in H2O
- Room temp 5 mins
- Wash bead 1ml 1XTE 5 times.
- Resuspend bead in 25ul EB EB=10mM Tris pH8.0

8) PCR

Dynal bead	10ul	
5x buffer	10ul	
primer1(25uM)	0.5ul	Gex PCR Primer 1
	5' CAAGCAGAAGACGGCATACGA	
primer2(25uM)	0.5ul	Gex PCR Primer 2
	5'AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA	
10mM dNTP	1.25ul	
phusion taq	0.5ul	
H2O	28ul	
Total Volume	50ul	

Cycling conditions

1. 98C 30sec
2. 98C 30" 10sec
3. 60 20" 30sec
4. 72C 15sec
5. Go to 2, 12 cycles total
6. 72C 7'

9) PAGE purification

- Load 1μL of 10bp DNA marker into one well and 50μL of PCR products into another 2 wells of 4-20% TBE PAGE gel (Bio-rad). Electrophorese ~2h at 120V.
- Pry apart cassette, and stain the gel in TE /ethidium bromide in a clean container for 2-3 minutes.

- Cut out ~90 bp band with a clean razor blade (You only cut about ~3mm, just around the band), and put band into a 0.5mL Eppendorf tube whose bottom has been punctured by a 21 gauge needle.

- Set this tube into a 2mL round-bottom Eppendorf tube, and spin the gel throμgh the hole into the 2mL tube (2 min spin at full speed in microfuge).
- Add 100μL of 1x NEB2 to the gel, and elute the DNA by rotating the tube gently at room temperature for 2 hours.
- Transfer the eluate and the gel debris onto the top of a Spin-X filter (Fisher Cat # 8162). Spin the filter in the microfμge for 2 minutes at full speed..
- Add 1μL of Pellet Paint, 1 ul glycogen, 10μL of 3M NaOAc and 325μL of –20°C EtOH, spin at 14K for 20 mins.
- Wash with 500μL of RT 70% EtOH, dry in the air for 5 min, and resuspend in 6-11μL of EB solution.
- Run 1 ul of recovered DNA on 4-20% TBE gel and stain with Ethdium Bromide
- Bioanalyzer quantification showed ca. 5 ~100nM is a typical concentration of the final product.

REAGENTS, BUFFERS, AND LINKERS/PRIMERS

DNase Concentrations (Roche- DNase I RNase-free, cat # 776 785): always freshly made.

Tube #	Amount DNase	Dilution (in RSB)		Amount added
1	0	0		0 (Keep at 4 degrees on ice)
2	0	0		0
3	0.12U	1/1000	30 ul 0.4U + 70 ul RSB	12 ul
4	0.4U	1/300	30 ul 1.2U + 70 ul RSB	12
5	1.2U	1/100	30 ul 4U + 70 ul RSB	12
6.	4U	1/30	2 ul Dnase+ 60 ul RSB	12

RSB Buffer:	Amount per 1 Liter
10 mM Tris pH 7.4	
10 mM NaCl	
3 mM MgCl2	

LIDS Buffer:	Amount per 1 Liter
1% (w/v) LIDS (lauryl sulfate, lithium salt: Sigma L 4632)	10g
10 mM Tris-Cl	10 ml of 1 M (pH8)
100 mM EDTA	200 ml of 0.5 M (pH8)
H20	to make 1 liter

DNA Polymerase Buffer:	Amount per 1 liter
50 mM NaCl	10 ml of 5 M stock
10 mM Tris-Cl	10 ml of 1 M Tris pH 8.0
10 mM MgCl2	5 ml of 2 M MgCl2
H2O	to make 1 liter
1 mM dithiothreitol (DTT)	1 ml of 1 M stock

PRIMERS and LINKERS: HPLC purify all oligos and make sure linkers are annealed properly

adapter I (contains MmeI site): Stock linkers are a concentration of 25 pmol/ul. Linkers are annealed in 1x NEB Buffer 2, heat at 95 deg for 5 min and slow cool to RT

MmeI site
5’-Bio-ACAGGTTCAGAGTTCTACAGTCCGAC
3’-Amm- CAAGTCTCAAGATGTCAGGCTG-P

adapter II: Stock linkers are a concentration of 25 pmol/ul. Linkers are annealed in 1x NEB Buffer 2, heat at 95 deg for 5 min and slow cool to RT

5’- P-TCGTATGCCGTCTTCTGCTTG
3’- NNAGCATACGGCAGAAGACGAAC

Sequencing primer for DNase I sample:
5’ CCACCGACAGGTTCAGAGTTCTACAGTCCGAC