

5xMRE-seq library construction protocol v5 Costello Lab November 2010

Notes:

- A. For all Qiagen gel extraction steps (Qiaquick and MinElute), melt gel slice at 37° C instead of 50° (see Quail et al 2008 Nature Methods). Incubate for 30 min, vortexing frequently - make sure gel is completely melted before proceeding.
- B. For Qiagen Qiaquick and MinElute column purifications (not gel extractions) use Qiagen buffer PB, not PBI (newer Qiagen kits don't even have PBI).
- C. For size-selection after adapter ligation, be aware that the adapters are only partially double-stranded so adapter-ligated fragments might run at slightly different position than expected.
- D. All gel purifications are performed with 1xTAE agarose gels. We add 1:10000 Sybr-Safe to gel after microwaving (instead of EtBr).
- E. The Illumina Genomic DNA Sample kit manual specifies Bio-Rad certified low-range Ultra Agarose for gel extraction steps; the ChIP-seq Sample kit manual does not specify a particular agarose. I have been using the Bio-Rad Ultra Agarose.
- F. Minimum amount of starting DNA is currently 2.5 µg.

PROTOCOL:

1. Start with high-quality, high molecular weight gDNA. It is very important to prevent random shearing of DNA. Isolate DNA using the Costello lab's "genomic DNA extraction from frozen tissue" protocol. Include overnight proteinase K digestion and a **1 hour**, 37 degree DNase-free RNase treatment (Roche catalog# 11 119 915 001) (final RNase concentration = 40 µg/ml). Perform 2 PCI and 2 chloroform extractions using 2 ml "Light" phase lock gels. Be careful at all steps not to shear DNA – always use wide-bore tips and pipet gently. Resuspend DNA in TE (not water). Store DNA at 4° C.

2. Digest DNA using the methylation-sensitive enzymes *Bsh1236I*, *HpaII*, *Hin6I*, *SsiI* (from Fermentas) and *HpyCH4IV* (from NEB). Each digest is performed separately; use 0.5 µg DNA per digest. Use 10 units of enzyme per µg of DNA. Add half of enzyme, incubate 3 hrs at 37 ° C, then add rest of enzyme, incubate additional 3 hrs at 37° C. Total digest time = 6 hrs.

Setup for each digest (0.5 µg DNA each : USE WIDE-BORE FILTERED TIPS)

10X buffer	5 µl
Restriction enzyme, 10 U/µl	0.25 µl, then additional 0.25 µl after 3 hrs
DNA in TE	add volume for 0.5 µg
Illumina ultrapure water	total volume to 50 µl

mix reaction gently – stir, tap gently, or pipet gently with wide-bore tips

3. Clean up digest and resuspend in smaller volume. (Use **wide-bore filtered tips**). Add 1 volume of phenol/chloroform/isoamyl alcohol to each digest and mix. Next, combine digests into 1 phase lock gel tube (MREs should be inactivated by addition of PCI). Continue with PCI extraction followed by one chloroform extraction, using phase lock gel. Precipitate DNA by salt/ethanol precipitation (1/10 volume of 3M sodium acetate, pH 5.2; 2.5X volume of 100% EtOH) with 1 µl glycogen. After addition of EtOH, place in -20 overnight. Resuspend pellet in 12 µl Qiagen EB buffer. Nanodrop 2 µl to check concentration and purity. Save remainder of digests at -20.

4. Make a 50 ml, 2.5% 1xTAE gel (Owl small gel; use thick 10-well comb). **Using wide-bore filtered tips** mix DNA with 4X tris/EDTA/sucrose loading buffer (does not contain dyes). Loading gel: skip first and last wells. Load combined digests on one side of gel; load 5 µl of 100 bp ladder and 3 µl of NEB Low MW ladder on other side. Load a lane next to marker with 0.5 µl dye only (BPB and XC) to help monitor gel running. Run at 120 V for 1 hr or longer, depending on size-selection.

5. Size selection of digest: Cut out 50-300 bp (and additional size fractions if wanted) with clean scalpel. Be careful to exclude fragments <50 bp. Minimize exposure to UV, and take a picture before and after excising gel slice. Try to make cut as horizontal as possible, and minimize the size of each gel slice. Purify each gel slice with a single Qiagen Qiaquick column (for gel slices < 400 mg) or two Qiagen MinElute columns (for gel slices > 400 mg). Melt gel at 37° C for ~15-30 min with frequent mixing. Elute DNA in a total of 32 µl of Qiagen EB buffer. Concentration will be low. After this step, wide-bore tips are not necessary.

6. Repair reaction. Use all 30 µl of size-selected digest. In contrast to standard Illumina Genomic DNA kit protocol, **exclude** T4 DNA polymerase and T4 PNK. Enzymes and buffers are from Illumina Genomic DNA kit. Make Klenow DNA polymerase dilution: 1 µl + 4 µl Illumina water. Discard unused diluted Klenow after use.

Illumina water	12 µl
DNA sample	30
T4 DNA ligase buffer with 10mM ATP	5
dNTPs	2
Klenow DNA polymerase, diluted 1:5 in H ₂ O	<u>1</u>
total volume	50

incubate in thermal cycler 30 min at 20°C

7. Clean up reaction with Qiagen **Qiaquick** column, as per Qiagen instructions. Elute in 32 µl EB.

8. Addition of 3' A (using Illumina reagents)

DNA sample	32 µl
Klenow buffer	5
dATP	10
Klenow 3'-5' exo minus	<u>3</u>
total	50

incubate in thermal cycler 30 min at 37°C

9. Clean up with Qiagen **MinElute** column, elute in 10 µl EB.

10. Adapter Ligation. Use 1 µl of 1:10 dilution of PE adapters. Dilute 1 µl Illumina adapter oligo mix in 9 µl Illumina Ultrapure water. Store extra diluted adapter oligo mix at -20 °C.

DNA sample	10 µl
DNA ligase buffer	15

1:10 diluted PE adapter oligo mix	1
DNA ligase	<u>4</u>
total	30

incubate 15 min at room temperature

11. Clean up with Qiagen **MinElute** column, elute in 20 µl EB.

12. Size-select adapter-ligated fragments. Follow Illumina Chip-seq kit protocol for making and loading 50 ml 1xTAE gel (Owl small gel; use thick 10-well comb).

Mix 20 µl DNA with 6 µl 4X tris/EDTA/sucrose loading buffer (does not contain dyes). Loading gel: skip first and last wells. Load all 26 µl sample on one side of gel; load 5 µl of 100 bp ladder and 3 µl of NEB Low MW ladder on other side of gel. Load a lane next to markers with 0.5 µl dye only (BPB and XC) to help monitor gel running. Run at 120 V 1 hr. Cut out gel slice corresponding to original size selection + adaptors. Optional: excise an additional gel slice of the same size from a lane without sample; use this as negative control for PCR. Gel-purify each slice with a single Qiagen **Qiaquick** column (for gel slices < 400 mg) or two or more Qiagen **MinElute** columns (for gel slices > 400 mg). Elute in 30 µl EB. Use 1/3 (10 µl) for PCR; save rest at -20 °C.

13. PCR enrichment of adapter-modified fragments. Can use water and/or blank gel slice as negative control.

DNA	10
Illumina Phusion DNA polymerase	25
Illumina PE PCR primer 1.0	1
Illumina PE PCR primer 2.0	1
Illumina Ultrapure water	<u>13</u>
total	50

cycling conditions:

30 sec 98 °C
15 cycles of:
 10 sec 98 °C
 30 sec 65 °C
 30 sec 72 °C
 Then: 5 min 72 °C
 Then: hold at 4 °C

14. Size select 170-420 bp by Qiagen **MinElute** gel purification. Elute in 16 µl EB.

- 2 µl for nanodrop
- 1 µl for Bioanalyzer
- ? µl for subcloning and Sanger sequencing (optional)

15. If subcloning: use Invitrogen pCRII-Blunt Topo cloning kit. Try for 3:1 ratio of insert:vector for ligation. Sequence ~ 25 colonies per sample.