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 37º C instead of 50º (see Quail et al 2008 Nature Methods). Incubate for 30 min, vortexing frequently - make sure gel is completed 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 *Bsh*1236I, *Hpa*II, *Hin*6I, *Ssi*I (from Fermentas) and *HpyCH*4IV (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 37º 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_2O	1
total volume	**50**

incubate in thermal cycler 30 min at 20ºC

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	3
total	**50**

incubate in thermal cycler 30 min at 37ºC

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 ºC.

DNA sample	10 µl
DNA ligase buffer	15

1:10 diluted PE adapter oligo mix	1
DNA ligase	4
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 ºC.

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	13
total	**50**

cycling conditions:

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

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.