IVT followed by bisulfite conversion for SMRF-seq samples not requiring untranscribed controls

1. Begin with 2000 ng of DNA substrate resuspended in 20 ul 10 mM Tris Hcl.
2. Add 58 ul npH20, 10 ul 10x NEB transcription buffer, 2 ul NEB rNTPs, 10 ul DTT to each transcribed sample.
3. Add 1 ul NEB T7 RNA Pol / 400 ng template DNA to the transcribed fraction; 5 ul.
4. Incubate samples @ 37C for 20 minutes. Stop transcription reaction by adding 0.5 M EDTA to a final concentration of ~25mM (5.25 ul).
5. Place samples on ice. Immediately remove 600 (33.1 ul) and increase fraction volume to 40 ul using npH20. Split sample into 20 ul fractions and treat each with 130 ul Zymogen lightening conversion reagent and incubate at 37C for 2 hours with rotation.
    1. If sample is linear, you can save 10 ul to run on an agarose gel. Since this is pre-RnaseA digestion the presence of an RNA smear will confirm transcription occurred.
6. For the remaining transcribed fraction, add 1 ul 1:1000 dilution RnaseA / 200 ng DNA template; 7 ul.
7. Incubate samples for 10 mins at room temperature.
8. Add 1 ul protinease K / 200 ng DNA: 7 ul. Incubate samples for 10 minutes at room temperature.
9. Increase sample volume to 200 ul using TE buffer. Preform phenol chloroform extraction using phase lock tubes.
10. Re-suspend samples in 20 ul mM Tris-HCL.
11. In the manner of step 5 remove 8.57 ul (600 ng) of sample and increase volume to 40 ul using npH20. Split sample into 20 ul fractions and treat according to step 5.
12. Save remaining transcribed de-protienized sample for analysis on agarose gel to confirm transcription.

IVT followed by bisulfite conversion for SMRF-seq samples requiring transcribed controls

1. Begin with 2000 ng DNA re-suspended in 20 ul 10 mM Tris Hcl.
2. Add 58 ul npH20, 10 ul 10x NEB transcription buffer, 2 ul NEB rNTPs, 10 ul DTT to each transcribed sample.
3. Remove 15 ul of the sample to use as untranscribed control. Increase sample volume to 20 ul using npH20 and add to 130 ul Zymogen lightening conversion reagent. Treat as previously described.
4. To the remaining sample add 1 ul NEB T7 RNA Pol / 400 ng template DNA to the transcribed fraction; 4.25 ul.
5. Incubate samples @ 37C for 20 minutes. Stop transcription reaction by adding 0.5 M EDTA to a final concentration of ~25mM (4.46 ul).
6. Place samples on ice. Immediately remove 300 ng (18.53 ul) and increase fraction volume to 30 ul using npH20. Treat with 130 ul Zymogen lightening conversion reagent and incubate at 37C for 2 hours with rotation.
7. For the remaining transcribed fraction, add 1 ul 1:1000 dilution RnaseA / 200 ng DNA template; 7 ul. Incubate at room temperature for 10 minutes.
8. Add 1 ul protinease K / 200 ng DNA to each sample: 3.5 ul. Incubate samples for 10 minutes at room temperature.
9. Increase sample volume to 200 ul using TE buffer (148 ul) and preform phenol/chloroform extraction using phase lock tubes.
10. Re-suspend in 40 ul 10mM Tris-Hcl. Split the sample into two fractions.
11. Digest one of the two transcribed fractions with RnaseH1I Add 10 ul 10x NEB RnaseH buffer, 2 ul RnaseHI and 68 ul npH20. Digest sample at 37C for 30 mins.
12. Preform EtOH precipitation on RnaseHI digested fraction. Resuspend in 20 ul 10mM Tris Hcl.
13. Remove 8.57 ul of each sample and increase volume to 20 ul with 11.4 ul npH20. Treat with 130 ul Zymogen lightening conversion reagent and incubate at 37C for 2 hours with rotation. Save remainder of each sample for analysis on agarose gel to confirm transcription and RnaseH activity.