In vitro transcription for bisulfite conversion

Adapted from *Malig et al, 2020*

Ethan Holleman

January 4, 2022

Protocol

IVT

1. Determine reaction volume and DNA mass per plasmid. This will be dependent on what intended use for the IVT products is, and the number of reactions per plasmid. Generally, a complete IVT to demonstrate R-loop formation (or lack thereof) via band shift on an agarose gel will have three samples per plasmid; an untranscribed control, a transcribed RnaseH treated and a transcribed untreated. A minimum of 200 ng per sample should be used in order to visualize on an agarose gel.
2. After determining reagent volumes for each sample, assemble reagents for each sample in the following order; npH_20, 10X reaction buffer, NTPs, template DNA. This will serve as a master mix for all reactions utilizing a particular template DNA.[1]
3. Split each sample into individual reactions (typically 2 to 3) of equal volume.
4. Add the appropriate polymerase for your template DNA (usually T7 or T3). 2 μl is suitable for up to 1 μg of DNA.[2]
5. Incubate all samples at 37° C for 20 minutes in a thermocycler.[3]
6. Immediately remove samples and place on ice. Add EDTA to all samples.[4]
7. Thoroughly mix samples and place on ice for 5 minutes.

[1] It can be helpful when dealing with many samples to prepare reactions in a PCR grid. This way the main mix can be created in one well and individual reactions can be split from the main mix into spacial related wells using a multichannel pipette.

[2] If larger masses of DNA are used, NEB's Protocol for Standard RNA Synthesis can be scaled up and used as a guide.

[3] Make sure an aliquote of EDTA is ready after you start the incubation.

[4] Final EDTA concentration should be 5x the concentration of Mg in the transcription buffer. NEB 10x transcription buffer contains 6 mM $MgCl_2$.

Phenol / EtOH precipitation

8. Increase sample volume to 200 μl with TE or 10 mM Tris HCl.
9. Spin down phase lock gel tube at 12,000 x g for 30 seconds. Add 200 μl Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (1 volume) to the tube.
10. Add sample to phase lock tube and vortex until contents are thoroughly mixed.
11. Centrifuge at 12,000 x g for 5 minutes to separate phases.
12. Carefully pipet off nucleic-acid-containing aqueous phase (upper layer) to a fresh tube.
13. Preform standard EtOH precipitation (OpenWetWare has a usefuly guide with images). Add 0.1 volumes 3M sodium acetate pH 5.2, 2.2 volumes 100% EtOH and 2 μl molecular grade glycogen to each sample. Freeze samples occording to your needs. Generally, the lower the concentration of DNA the greater benefit of longer freezing times. If maximum yield is required freeze overnight at -20°C, if time is a greater constraint freeze at -80° for 10 minutes to 1 hour.
14. Spin at full speed in microcentrifuge at 4° for 30 minutes.
15. Carefully vacuum supernatant away. Wash twice with 200 μl 70% EtOH. It can be helpful to spin samples briefly between washes to secure the pellet as it can sometimes be dislodged during the wash. Carefully vacuum away the 70% EtOH between each wash.
16. Air dry pellet for 20-30 minutes.
17. Re-suspend pellet in desired volume of 10 mM Tris HCl. This will be dependent on down-stream use of the sample.

Visualize via agarose gel

18. Prepare a 0.8% 1x TBE agarose gel. Cover in 1x TBE. Do not add ethidium bromide to the gel or running buffer.
19. Aliquote 200-600 ng of each sample into a PCR tube. Increase to suitable volume (usually between 10 and 20 μl depending on well size) and add appropriate amount of loading dye.
20. Run gel at max 60V for minimum time of 2 hours.
21. Post stain gel for 1 hour with ethidium bromide at a concentration of 1 μl ethidium bromide per 100 ml running buffer.
22. De-stain the gel for 10 minutes in np water.
23. Image the gel and record results.