In Vitro Transcription (IVT) and Labelling
AJ 21.02.24

Getting DNA Template

- Use your preferred PCR method to amplify the DNA that codes for the RNA you want. Make sure you add a T7 polymerase binding site to your forward primer (if the site is not already in the DNA template).
    - I use KOD; you can test DMSO and MgSO4 conditions
- T7 Sequence is: TAATACGACTCACTATAGGG
- I've tried gel extracting the amplified DNA or precipitating with GlycoBlue, and I much prefer GlycoBlue:

1. Add 1 ul of GlycoBlue carrier
2. Add 0.1 volumes of 3 M sodium acetate, pH 5.2 and 3 volumes of ethanol.
3. Incubate at -20 deg C for 30 min (or longer is fine)
4. Centrifuge at maximum speed in a cold microcentrifuge - 8min
5. Aspirate the supernatant, avoiding the pellet
6. Wash with 70% ethanol.
7. Centrifuge again for 1 min, and thoroughly aspirate the supernatant, avoiding the pellet.
8. Redissolve the pellet in the desired volume of water or 1x TE.

- If there's not a T7 binding site in the original plasmid, you shouldn't have to worry about the full plasmid still being there.

In Vitro Transcription

Produce the RNA using this T7 kit:

1. Mix X uL nuclease free water, 10 uL NTP buffer mix, 2 uL T7 polymerase, and X uL template DNA (1ug total) for a 20uL reaction volume
2. Incubate at 37 for 2 hr. (Optimal incubation time may vary, but 2hrs has worked for me on 1kb and 5kb RNAs.)

To recover the RNA, you can use Trizol cleanup or an RNA cleanup kit (like these ones). Both have worked for me. Trizol may have higher recovery, but it's also more of a pain - I prefer the cleanup kits, personally.

- RNA cleanup kits come with their own instructions
- Trizol (more details here):

1. Add 0.75 mL of TRIzol™ Reagent per 0.25 mL of sample
2. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.
3. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, then securely cap the tube.
4. Incubate for 2–3 minutes.
5. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C. The mixture separates into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase.

6. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.
7. (Optional) If the starting sample is small (<106 cells or <10 mg of tissue), add 5–10 µg of RNase-free glycogen as a carrier to the aqueous phase. Note: The glycogen is co-precipitated with the RNA, but does not interfere with subsequent applications.
8. Add 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis.
9. Incubate for 10 minutes.
10. Centrifuge for 10 minutes at 12,000 × g at 4°C. Total RNA precipitate forms a white gel-like pellet at the boJom of the tube.
11. Discard the supernatant
12. Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis. Note: The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C.
13. Vortex the sample br' efl¢ then centrifuge for 5 minutes at 7500 × g at 4°C.
14. Discard the supernatant with a m' crop' peJorƷ
15. Vacuum or air dry the RNA pellet for 5–10 minutes. IMPORTANT! Do not dry the pellet by vacuum centrifuge. Do not let the RNA pellet dry, to ensure total solubilization of the RNA. Partially dissolved RNA samples have an A230/280 ratio <1.6.
16. Resuspend the pellet in 20–50 µL of RNase-free water, 0.1 mM EDTA, or 0.5% SDS solution by p' peJ' n up and down. IMPORTANT! Do not dissolve the RNA in 0.5% SDS if the RNA is to be used in subsequent enzymatic reactions.
17. Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.
18. Proceed to downstream applications, or store the RNA at –70°C.

Labeling RNA

I use this kit (dye binds to the nucleotide backbone but doesn't disrupt structure, and the kit is super easy to use)

- Protocol here
- I'm not happy with the recovery over the spin columns; I always use the EtOH precipitation method

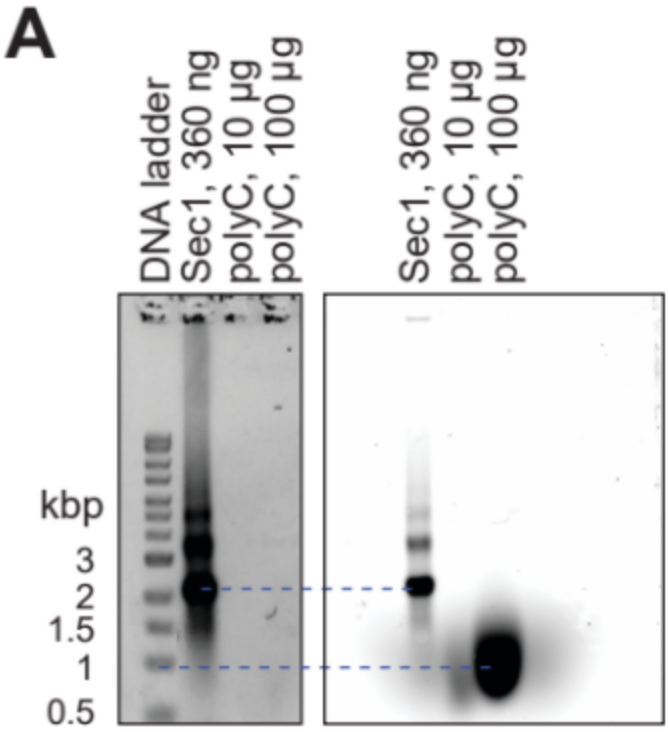
Run the final product on an agarose gel to confirm there is a single clean band.

- You may notice some laddering - apparently this is normal; the T7 polymerase can "wrap" around the DNA template and keep going in the other direction. These bands should be fainter, though.
- Technically you should run the RNA on a denaturing gel using this protocol; I've never actually done that though

Results

For a 1.2 kb RNA sequence, did 4 T7 reactions (80 uL) and used RNA cleanup kit; final was 50 uL at 2.26 uM ~113 pmol (did this a couple of times)

In this figure, Sec1 is a 5kb IVT RNA:



Agarose gel with a DNA ladder. Left shows GelRed; right is Cy3. As you can see, there is a slight laddering effect, but the band appears crisp (few degradation products)