

Materials needed

25:24:1 Phenol:Chloroform:Isoamyl alcohol
125:24:1 Citrate-saturated phenol:chloroform:isoamyl alcohol
24:1 chloroform:isoamyl alcohol
Promega Ribomax IVT kit
Nuclease-free HOH

General Considerations

- All buffers should be nuclease-free. See “RNase removal” for details.
- All tubes should be nuclease-free. These typically come certified, but may be further treated according to “RNase removal” instructions
- All tips should be certified nuclease-free. Use ART tips.
- NEVER handle something with bare hands. Keep a clean, sterile environment

Step 1: Plasmid digestion

Due to the “run-on” nature of IVT, the length of the transcript is determined by corresponding template length. This is controlled via restriction enzyme plasmid digestion.

** Avoid the use of restriction enzymes that produce 3’ overhangs, as they produce extraneous transcripts. Convert all 3’ overhangs to blunt ends. **

- Measure plasmid concentration @ A=260 nm ($\epsilon=50 \mu\text{g/ml}$). Spin to dryness, reconstitute in nuclease-free HOH to ca. $1 \mu\text{g}/\mu\text{l}$
- Digest with restriction enzyme. Typical reaction composition is as follows:

50 μg plasmid DNA
5 U restriction enzyme
1 X enzyme buffer
1 X BSA
Nuclease-free HOH

Step 2: Digested DNA cleanup

I have found that digested DNA must be further treated before IVT to ensure an efficient reaction. In lieu of this treatment, a commercial cleanup kit (Qiaquick, Promega Wizard, etc.) can be used in place with no loss in activity.

- Heat denature restriction enzyme according to specs (usually 65°C , 15min)
- Perform phenol:chloroform extraction
 - o Add 1 eq. 25:24:1 to DNA. Vortex for 30 sec. Centrifuge at max speed for 2 min. Collect top phase
 - o Add 1 eq 24:1 to DNA. Vortex for 30 sec. Centrifuge at max speed for 2 min. Collect top phase
- Perform EtOH precipitation
 - o Add 0.1 eq. 3M sodium acetate, mix.

- Add 2 eq ice cold absolute ethanol, mix, place on ice 20-30 min to allow DNA pellet to form
- Centrifuge at 4°C, max speed for 15 min. Decant off liquid phase.
- Add 1-1.5ml 70% EtOH (diluted with DEPC-treated HOH). Vortex, place on ice ~10 min to reform DNA pellet
- Centrifuge at 4°C, max speed for 15min. Decant off liquid phase. Allow to dry, or dry with nitrogen purge.
- Reconstitute DNA pellet in nuclease-free HOH to ca. 1 µg/µl
- Test purity with agarose gel electrophoresis

Step 3: In Vitro Transcription

Follow Ribomax protocol, scaling up or down as necessary. My recommendations:

- Use 0.25 µg/µl template DNA for maximum transcription product formation
- Allow reaction to proceed at 37°C for the full 4 hours
- Before termination, the solution may become cloudy. This is likely dissolution of pyrophosphate, but it does not impact yield, provided DNA has been sufficiently cleaned up

Step 4: IVT product cleanup

- Perform DNase treatment (0.2 U/µg template DNA, 15 min, 37 °C)
- Perform phenol:chloroform extraction
 - Add 1 eq 125:24:1 to reaction mixture. Vortex for 30 sec. Centrifuge at max speed for 2 min. Collect top phase
- Perform chloroform extraction
 - Add 1 eq 24:1 to reaction mixture. Vortex for 30 sec. Centrifuge at max speed for 2 min. Collect top phase
- Perform EtOH precipitation
 - Add 0.1 eq. 3M sodium acetate, mix.
 - Add 2 eq ice cold absolute ethanol, mix, place on ice 20-30 min to allow DNA pellet to form

IVT product may be stored overnight at this stage

- Centrifuge at 4°C, max speed for 15 min. Decant off liquid phase.
- Add 1-1.5ml 70% EtOH (diluted with DEPC-treated HOH). Vortex, place on ice ~10 min to reform DNA pellet
- Centrifuge at 4°C, max speed for 15min. Decant off liquid phase. Allow to dry, or dry with nitrogen purge.
- Reconstitute pellet in nuclease-free HOH, back to original IVT volume

Step 5: Product identification and further purification (if necessary)

- Cast denaturing TBE-acrylamide gel. % acrylamide guidelines:

RNA size	% acrylamide
1000-2000	3.5
80-500	5
60-400	8

40-200	12
--------	----

- Dilute ca. 2-5 μ l IVT product in TBE-urea loading buffer (total loading volume = 10 μ l). Heat mixture to 95°C (denature) for 5-10 min, immediately place on ice
- Run sample in gel (150V). Include denatured DNA ladder for length reference
- Stain with EtBr, destain with water, image

If sample is full length and homogeneous:

- Remove rNTPs with Microcon spin filter (cutoff determined by product length)
- Dilute in appropriate CE buffer (should contain EDTA)

If sample is heterogeneous:

- Run entire IVT reaction on denaturing acrylamide gel
- Briefly stain with EtBr (Alternative: backshadow)
- Excise full length band with Xacto knife, place in 1-2 microcentrifuge tubes
- Perform RNA isolation with crush and soak method (Molecular Cloning, 2nd ed, Chapter 5, protocol 12)
- Dilute in appropriate CE buffer (should contain EDTA)

This protocol borrows heavily from the following sources:

- Molecular Cloning, 2nd edition (several chapters)
- Promega Technical Bulletin, "RiboMAX Large Scale RNA Production Systems – SP6 and T7", Revised 6/06