

## *In vitro* Transcription

### *In Vitro* Transcription Reaction (using Ambion Megascript kit)

**Note:** Wear gloves all the time. Take precaution not to contaminate with RNase – do not touch your face with gloved hands, do not spit in the tubes...

**Important:** Make all solutions with DEPC-treated water!

- 1) Thaw the frozen reagents (or aliquots of)

Place RNA Polymerase Enzyme Mix on ice;

Warm 4 nucleotide solutions (ATP, CTP, GTP and UTP) quickly in your hands (wearing gloves) until they are completely in solution, keep on ice;

Vortex the 10x reaction buffer until it is completely thawed, and keep it at **room temperature** while assembling reaction. Note that the reaction buffer contains a precipitate that needs to fully dissolve before the reaction is set up. Make sure to assemble the reaction at room temperature so that the solute in the reaction buffer does not precipitate out.

- 2) Adjust DNA template in DEPC-treated ddH<sub>2</sub>O to final concentration of 0.5 µg/µl. The DNA clones coming from OpenBiosystems are 0.5 µg/µl, so you do NOT need to adjust.
- 3) Add in the following order to assemble the transcription reaction.

DEPC-ddH <sub>2</sub> O	14 µl
10Xbuffer	4 µl
ATP	4 µl
GTP	4 µl
CTP	4 µl
UTP	4 µl
DNA	2 µl
T7 Polymerase	4 µl
Total Volume:	40 µl

Gently flick the tube or pipette the mixture up and down gently and then microfuge tube briefly (2-3 seconds) to collect the reaction mixture at the bottom of the tube.

**Note:** This reaction setup is recommended when the RNA produced will be greater than/equal to 0.5 kb in length.

- 4) Incubate at 37<sup>0</sup>C for 2 hours or overnight (up to 24 hrs).

**Note:** Optimal incubation time will vary depending on size/transcriptional efficiency of your template. For short transcripts, a longer incubation time might be advantageous.

### **LiCl<sub>2</sub> precipitation to purify dsRNA**

- 1) Add 30ul of DEPC treated water to the 40ul IVT reaction
- 2) Add 25ul of the LiCl<sub>2</sub> ppt solution
- 3) Mix thoroughly and place the tube at -20 degrees for 30min-overnight
- 4) Spin for 15min in a table-top centrifuge at 13000rpm at 4 degrees (there are centrifuges in the cold room)
- 5) Carefully pipette off LiCl<sub>2</sub> solution to leave RNA pellet (pellet will be clear and slippery so be careful)
- 6) Add 1ml of 70% ethanol to wash the pellet
- 7) Spin for 15min in a table-top centrifuge at 13000rpm at 4 degrees
- 8) Carefully pipette off ethanol without disrupting clear RNA pellet
- 9) Let tube sit on a clean kimwipe and air dry for 30 minutes or until no residual ethanol remains on the side of the tube (Check with your TA to make sure that your pellet is dry)
- 10) Resuspend the RNA pellet in 40ul of DEPC treated water
- 11) Anneal RNA strands by heating to 65 degrees for 30 minutes in a table-top heat block
- 12) Let the RNA cool off slowly to room temperature by removing the block containing the tube of RNA from the heating element
- 13) Determine the optical density of the RNA (A<sub>260</sub>) by diluting the RNA 1:100 in DEPC-treated water (the cuvette should hold 100ul total volume)
- 14) Calculate dsRNA concentration using the following formula:  
 $A_{260} \times \text{dilution factor (100)} \times 50\mu\text{g/ml} = \text{the concentration of your RNA}$
- 15) Dilute your RNA to a concentration of 1ug/ul (if your RNA is less concentrated than 1ug/ul, ethanol precipitate your RNA and resuspend in a smaller volume of DEPC treated water.)

### **Ethanol precipitation of RNA (to make your RNA more concentrated)**

- 1) Add 1/10<sup>th</sup> volume of 3M Sodium Acetate pH5.2 (in DEPC treated water) to your sample
- 2) Add 2.5x volumes of cold (-20 degrees) 100% ethanol to your sample and mix thoroughly
- 3) Put tube at -20 degrees for 15min-overnight
- 4) Spin 15min in a table-top centrifuge at 13000rpm at 4 degrees
- 5) Pipette off the ethanol and let the tube sit upside down with the lid open on a piece of clean Kimwipe to let air dry for 30min and make sure there is no residual ethanol on the side of the tube (check with your TAs to make sure the pellet looks dry)
- 6) Resuspend RNA pellet in a volume of DEPC treated water such that the RNA concentration will be greater than 1ug/ul

## Run 1% agarose gel to confirm and ensure proper annealing

- 1) Dilute TAE Buffer from 10x stock  
80 mL TAE buffer  
720 mL distilled water, mix well

- 2) Making a 1% Agarose Gel

0.5g agarose

50 mL 1x TAE buffer

Heat 1 minute 20 seconds in the microwave

Let it cool and add 2.5 µl of 10mg/ml Ethidium bromide (in the chemical hood).

Place the gel plate in the running apparatus with the rubber-banded sides in tight contact with the sides of the apparatus to prevent leaking. Put the combs in. Then pour agarose into the gel plates, let it solidify without disturbing.

After the gel is solidified, pour 1x TAE in the running apparatus till the buffer covers the agarose gel.

**Caution: Ethidium Bromide is a carcinogen, so be sure to wear gloves and dispose pipette tips containing ethidium bromide in designated container!**

- 3) Loading the Gel

Add 1 µL 10x loading dye and 1 µL of RNA sample to 8 µL of DEPC-treated water, heat at 65°C for 5 minutes.

Load your RNA sample carefully and do not poke a hole at the bottom of the well.

Let the gels run for 30-45 minutes from negative to positive. (Check with your TAs about the voltage. Generally, for the small apparatus, you can run at 70-80V).

- 4) Examining the Gels

Place the gel on the UV transilluminator and look for orangish, pink bands of RNA. There should be no bands visible in the extraction control lane or the PCR control lane. Record all of your observations on the gel map. Take a picture of the gel to use in the lab. Once you are done with the gel, dispose of it in designated container.