

## ***in vitro* RNA preparation for injecting into zebrafish embryos**

(updated July, 2010)

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### **Before Starting**

Order:

- mMESSAGE mMACHINE® SP6 Kit from Ambion, Cat # AM1340
- 0.5-10 Kb RNA Ladder from Invitrogen, Cat # 15623-200

Common lab reagents and equipment needed:

- EtOH, RNase blocking spray, DEPC treated water, TE
- Microfuge, incubator at 37 C, pipetters (RNase free)

Cloning:

- The region to be transcribed need to be cloned into a vector (such as pCS2+) to be downstream of a promoter (such as SP6) and with a 3' polyA signal. You will need a miniprep of the construct to start with.
- Gateway compatible vectors for cloning are described in Villefranc, J.A., Amigo, J., and Lawson, N.D. (2007). Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev Dyn* 236, 3077-3087.
- See also <http://lawsonlab.umassmed.edu/reagents.html> for vectors that will be useful for making tagged proteins and using the Gateway system.

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### ***Day 1: Transcribe and precipitate***

- Everything except the template is included in the mMESSAGE mMACHINE® SP6 Kit
- Work RNase free: take all necessary precautions (gloves, Rnase free water and containers, new pipette tips, avoid talking while operating) to avoid contamination.

#### **A. Preparation of Template DNA**

1. Linearized plasmid with a restriction enzyme downstream of the insert to be transcribed.
2. Terminate the restriction digest by adding the following:
  - 1/20th volume 0.5 M EDTA
  - 1/10th volume of 3 M Na acetate or 5 M NH<sub>4</sub> acetate
  - 2volumes of ethanol
3. Mix well and chill at -20°C for at least 15 min.
4. Pellet the DNA by spinning for 15 min in a microcentrifuge at top speed.
5. Remove the supernatant, and re-spin the tube for a few seconds, and remove the residual fluid with a very fine-tipped pipet.
6. Resuspend in dH<sub>2</sub>O containing DEPC or in TE buffer at a concentration of 0.5–1 µg/µL.

## B. Assemble the Capped Transcription Reaction

1. Thaw the frozen reagents and store as follows:

- RNA Polymerase Enzyme Mix on ice, it is stored in glycerol and will not be frozen at  $-20^{\circ}\text{C}$ .
- Vortex the 10X Reaction Buffer and the 2X NTP/CAP until they are completely in solution.
- Once thawed, store the ribonucleotides (2X NTP/CAP) on ice, but keep the 10X Reaction Buffer at room temperature while assembling the reaction.
- *All reagents should be microfuged briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube.*

2. Assemble transcription reaction at room temp

- The spermidine in the 10X Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.
- Add the 10X Reaction Buffer after the water and the ribonucleotides are already in the tube (i.e. add reagents in order from top (water) to bottom (enzyme mix)).
- The following amounts are for a single 20  $\mu\text{L}$  reaction.

<u>Amount</u>	<u>Component</u>
to 20 $\mu\text{L}$	Nuclease-free Water
10 $\mu\text{L}$	2X NTP/CAP
2 $\mu\text{L}$	10X Reaction Buffer
0.1–1 $\mu\text{g}$	linear template DNA
2 $\mu\text{l}$	Enzyme mix

3. Mix thoroughly. Gently flick the tube or pipette the mixture up and down gently, and then microfuge tube briefly to collect the reaction mixture at the bottom of the tube.

4. Incubate at  $37^{\circ}\text{C}$  for 2 hours.

5. Add 1  $\mu\text{L}$  TURBO DNase and mix well.

6. Incubate 15 min at  $37^{\circ}\text{C}$ .

## C. RNA recovery via Lithium Chloride (LiCl) precipitation

1. Stop the reaction and precipitate the RNA by adding 30  $\mu\text{L}$  Nuclease-free Water and 30  $\mu\text{L}$  LiCl Precipitation Solution.

2. Mix thoroughly. Chill for  $\geq 30$  min at  $-20^{\circ}\text{C}$ .

3. Centrifuge at  $4^{\circ}\text{C}$  for 15 min at maximum speed to pellet the RNA.

4. Carefully remove the supernatant. Wash the pellet once with  $\sim 1$  mL 70% ethanol.

If the pellet comes off the bottom of the tube, respin for 5 minutes at maximum speed.

5. Carefully remove the 70% ethanol, add another 1 ml 70% ethanol, flick the tube to loosen the pellet from the bottom of the tube. Store in  $-20^{\circ}\text{C}$  overnight.

**Day 2: RNA re-suspension and quality control.**

- Start in the morning.

1. Take the tube containing the pellet from freezer, centrifuge at 4°C for 15 min at maximum speed to pellet the RNA.
2. Carefully remove the 70% ethanol, air dry the pellet
3. dissolve in 20 µl RNase free water (use the one come with the mMessage mMachine kit).
4. Aliquot the 20 µl into individual RNase free tubes, ~2 ul per tube.
  - Keep 1 µl for quality control in step 3
  - put all the rest ASAP in -70 as stock for future applications.
5. Dissolve the 1 ul from step 2 into 4 ul using RNase free water.
6. Use 2 ul on a nanodrop or equivalent to determine the concentration.
7. Run the remaining 3 uL on an RNA gel, with a RNA ladder (0.5-10 Kb RNA Ladder from Invitrogen, Cat # 15623-200 protocol comes with the kit) to check the quality of the transcription.
  - You will often see 2 bands or the band may not be sharp. *This is ok.*
  - It is NOT ok to see a blob of EtBr staining material at the bottom of the gel.

*If your RNA is intact, you can use for injecting into zebrafish embryos!*

- Avoid freeze-thaw in the future applications of the RNA stock.
- Use one tube for only once and through away any leftover.
- The stock could last for over a year if properly stored. However, do trash the whole batch away if any signs of RNA degradation (such as failed control experiment) were observed in two consecutive applications.
- Carry out a titration of the amount of your RNA that can be tolerated by your embryos (start at 50 ng/ul).