Transcription of *In Situ* Probe

This uses Invitrogen reagents – this is not the same protocol that
uses Ambion mMessage mMachine to make injectable mRNA

Use Template that has been cleaned well (using phenol/chloroform/isoamyl-alcohol mix). It should be resuspended at 1 mg/ml in water.

0. Clean your area - use new benchpaper etc. You have to be RNAse-free here!! Use barrier tips for everything, especially when going into the RNA reagents tubes in the freezer.
1. Thaw a tube of 10X transcription buffer at 37 ºC, and vortex it until there are no white flakes floating in it. Thaw the 10X DIG mix and spin it briefly in small (mushroom) centrifuge to bring it down. If we're running out of anything, tell Mike about it. Vortex briefly and spin down your templates also. Then, make sure there is sufficient mix, polymerases, etc. for you to use.

1. For each probe, mix the following in order in a labeled Eppendorf tube:

DEPC H_2O:	36 λ
10X transcription buffer:	6 λ
10X DIG Nucleotide mix:	6 λ
Template (linearized DNA):	6 λ (should = 6 μg total)
RNAse inhibitor:	1.5 λ
RNA Polymerase: (T7, T3, SP6 as needed)	4.5 λ

2. Put at 37 °C for 2 hrs. (can do longer, and some people do overnight; this is especially necessary for SP6 sometimes, because it is lower efficiency than T7 or T3). Put a piece of aluminum foil on top of the tubes and in contact with the heating block, so that the tubes' tops are warm and avoid water condensation. After a couple hours, you can add another 2 λ polymerase. If it is working well, the solution will become cloudy.

3. Run 3 λ from each tube on a 1% agarose gel (tap the bottom of the tube with your finger to make sure it is mixed before you take the 3 λ; use good water in everything, use regular DNA loading dye and markers). Photograph the gel, and look at it - is the size right? (the template DNA should be visible too). If it is all smeared out, don't worry and probably use it anyway. To run it out:
   a) Get new Ependorf tubes, label each with the name of probe.

b) Take 3 λ from each transcription reaction and put it in the corresponding new tube; leave the old ones in the warm block.

c) Add 3 λ of the 5X gel loading dye (since total volume will be 15 λ) to each tube.

d) Add 9 λ of good water to each.

e) Make a gel:

Add 50 ml of the 10X TBE buffer to 450 ml water

Take out 100 ml of that, and put it into a medium-size beaker with 1 g agarose (make sure to choose beaker which will fit into microwave).

Once agarose has melted, swirl, and put it into the gel mold. Leave it to solidify.

2. In the meantime, put 4 λ of ethidium bromide solution into the remaining 400 mls of the buffer. Swirl. That will be the gel buffer you use.

f) Once gel is solid, put it into the gel box, flood with buffer, and (without moving gel box!) load each lane with the contents of corresponding tube. Note which lane is which tube. Before loading, pippet up and down to make sure the contents of tube are mixed well before loading each gel well.

g) Run at 90-100 V, making sure the current is going (bubbles appear); do it until the two blue dyes are about an inch or so apart. Watch it so it doesn't run off the edge (takes about 1 hr to do).

h) Photograph.

While the gel is running, go back to the transcription reaction, to precipitate the RNA. To each tube,

4. Add 1 λ RNAse-free DNAse (this is important – it degrades the template).
5. Put at 37 °C for 15 min to 1 hr.
6. Add 300 λ TE (pH 8), 30 λ 4M LiCl, 900 λ EtOH; make sure cap is sealed tightly, then flip all tubes up and down 5-6 times to resuspend everything.
7. Put them at -20 °C for 30 min. (<u>not</u> on dry ice),
8. Spin (15 min. in cold at max speed), wash with 70% EtOH, air dry. Don't dry it

too much! 5 minutes or so should be enough - once pellet starts to become invisible, resuspend. If you dry too much, mRNA will stick to the wall of the tube.

9. Resuspend in 50 λ of <u>TE + 0.1% SDS</u> by vortexing well.
10. Add 1 ml of heated and mixed pre-hybe mix, and store at -80 °C.
11. To use for in situs, add between 0.1 to 0.5 ml of the mix to 1 scintillation vial with 4-5 ml of hybe, but remember you can re-use probe.