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Preparation of labeled RNA probes:

I think one key to making a good fluorescent *in situ* is having a smoking hot probe. With enzyme-based (alkaline phosphatase) detection, a mediocre probe can still give you a nice stain because you just let the reaction go longer. But if you don't have very good probes, you might be disappointed with the results of this protocol. What is a very good probe on the alkaline phosphatase reaction-time scale? A probe whose signal is well-developed after 10-20 minutes reaction time, and over-developed by 30 minutes. Truly flaming hot probes are over-developed by 10 minutes. I'm following this fairly standard protocol for making probes.

Preparing and Quantitating DNA Template:

1. Linearize 20 μg cDNA plasmid with 5' enzyme (sense strand) in 100 μl. Only 1 μg of linearized template is required in the transcription reaction, so you can digest a lot less. I start with this large digest because I'm making several probes from each template and also like to have a back-up supply.
2. Run out 2 μl on a gel to check for complete cutting
3. Increase volume to 200 μl with TE and add 20 μl 3 M sodium acetate, pH 5.2
4. Perform a phenol/chloroform extraction as you like to do it. Either
5. Extract 1x with phenol and 1x with chloroform OR
6. Extract 2x with phenol/chloroform

****RNase-free begins here: tips, tubes, reagents, and take a shower before work!**

7. Precipitate DNA with 2x volume ethanol
8. Freeze at -20° C. for >30 minutes, then spin at maximum speed for 15 minutes in a microfuge
9. Wash pellet with 200 μl 70% ethanol, spin at maximum speed for 2 minutes, drain completely and air dry
10. Resuspend in 30 μl RNase-free ddH_2O
11. For accurate quantitation by spectrophotometer, dilute 2 μl resuspended template in 100 μl TE, and take the Abs_{260} reading in a microcuvette

Synthesizing Labeled RNA:

1. Heat template DNA to 55° C. for 2 minutes, then put back on ice
2. Set up this reaction on ice in a RNase-free tube:

Component	Volume(μl)
~1 μg template DNA	X
RNase-free ddH_2O	Y
10x transcription buffer	1.5
10x hapten-U NTP mix	1.5
RNase inhibitor	1.0
RNA polymerase(T7, T3)	1.5
Total	15

**** Note that the reaction recipe on the Roche product information sheets calls for greater volume of reagents, but the same amount of template: 20 μl total, instead of 15 μl as shown here; many protocols call for 10 μl.**
3. Mix thoroughly with p20 and incubate at 37° C. for 2-2.5 hours.
4. Add 11 μl RNase-free ddH_2O
5. Take 1 μl out to put on a gel, then store the reaction at -20° C. or proceed to the fragmentation step
6. Check the reaction product by running it on a regular 0.9% TAE/ethidium bromide gel with a RNA marker. Your RNA product should run in a tight band at about the predicted size and should be at least 10-fold stronger than the DNA template band.

Fragmentation and Precipitation:

1. Add 25 μl 2x carbonate buffer
2. Mix and incubate at 65° C. for 20-40 minutes (vary to control average probe fragment size)
3. Add 50 μl stop solution ... optional: take out 3-5 μl to see the fragmented probe sizes on a gel
4. Add 10 μl 4 M lithium chloride
5. Add 5 μl 20 mg/ml tRNA (phenol/chloroform extracted, ethanol precipitated)
6. Add 300 μl ethanol
7. Vortex and freeze at -20° C. for >30 minutes
8. Spin at maximum speed for 20 minutes in a 4° C. microfuge
9. Wash pellet with 300 μl 70% ethanol, spin at maximum speed for 2 minutes, drain completely and air dry
10. Dissolve pellet in 200 μl hybridization solution. Don't let the probe pellet dry for too long, it might become hard to resuspend. Let the probe dissolve on ice for a while, then mix it thoroughly by pipetting with a p200 and vortexing. Probe stocks should be stored at -20° C.