Generation of In Situ Hybridization Probes Using PCR

1. Design PCR primers to your target of interest. Typically, I use the Primer 3 program available on the web from the Whitehead Institute (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Input your sequence of interest (generally by taking mRNA information available from the NCBI data bases). I normally try to avoid the protein coding sequences, as if a gene has high homology to a related gene it could produce a probe that recognizes many targets. 5' and 3' untranslated regions generally make good in situ probes.

   For the product sizes range, I delete all of the options and first try 400-500 (bp). If this does not give you suitable primers or if your UTR is not very big, you can make smaller probes (200-300). In my hands, probes less than 200 bp big do not give very good signals.

   I always use the default parameters for the program, which will usually give you 18-20 bp long primers with a Tm of about 60°C.

2. Once you have primer sequences, add onto the reverse primer a RNA polymerase binding site plus 3 nucleotides 5' to that binding site (I usually use 5'-GAG-3'). The sequences for the RNA polymerase binding sites are as follows:

   SP6: 5'-tatttaggtgacactatag-3'
   T7: 5'-taatacgactcactataggg-3'
   T3: 5'-attaaccctcactaaaggga-3'

   So if I have a primer set selected by Primer 3

   Forward: cagcaaaggcgagctagaac
   Reverse: tgcttcctggtttccatttc

   These can be made into in situ probe good for T3 polymerase by making the reverse primer:
   5'GAG*attaaccctcactaaaggga*tgcttcctggtttccatttc-3'
   T3 R primer

   When the sequence is amplified, it will add the T3 polymerase site to the 3' end, which will allow you to generate an anti-sense riboprobe. You can add one of the other RNA polymerase binding sites to the forward primer if you would like to make sense riboprobes for use as a control in your experiments.

3. Once, designed, the PCR to generate the probe is as follows. Note that in most cases, I use normal Taq polymerase and not high fidelity derivatives, as point mutations in the product should not affect their utility as probes. If you are amplifying a cDNA that you want to also use for expression experiments, be sure to use a high fidelity polymerase to minimize errors in the amplified product. Also note that the protocol below uses Eppendorf Mastermix, but any Taq polymerase should work.
   For each reaction:
   1 µl cDNA template
   0.5 µl forward primer (10 µM stock)
   0.5 µl reverse primer (10 µM stock)- this should have a polymerase binding site in it
   10 µl Eppendorf HotMastermix (2.5x)-contains Taq polymerase, buffer, and dNTPs
   1.25µl DMSO- for a final concentration of 5%, this helps if the target is GC-rich
   11.8µl ddH_2O
   25 µl total

   If you are generating many probes at the same time, it helps to make a mix containing all of the components except the primers. Aliquot this mix into individual tubes and then add the primers. Be sure to mix and spin the tubes briefly before putting them into the PCR machine.

PCR program (we use the Eppendorf Mastercycler Gradient EP machine)

Lid temp- 105°C

1. 95°C, 2'
2. 95°C, 15"
3. 60°C, 30" – you can run a gradient at this step to optimize the primer annealing
4. 72°C, 1' – you may have to use a longer extension time if your product is >700 bp
5. 35 cycles
6. 72°C, 5'
7. Hold at 4°C

4. Run out 2.5 µl of your PCR reaction on a gel to confirm successful amplification.
5. If product looks good, purify the PCR product using the Qiaquick PCR cleanup protocol or if there are multiple bands, you may have to gel isolate.

Qiaquick PCR cleanup

1. Add 5 reaction volumes of buffer PB to your sample
2. Add to Qiaquick column and spin at top speed in a microfuge for 30"-1'. Discard the flow through. If you have more sample then will fit in the column (800 µl is the maximum), just add more of your sample to the column and repeat the spin. Repeat as necessary.
3. Add 750 µl of buffer PE (make sure that it has had the ethanol added to it). Spin 30"-1'.
4. Discard flow through and repeat the spin to remove any last traces of the PE buffer.
5. Move column to a clean eppendorf tube to collect your product.
6. Add 30 µl of buffer EB to the center of the white spot in the column. Spin 1'.
7. Save this elution as it should contain the PCR probe. You can verify this by running a few µl out on a gel. It's worth doing this, as if you have not isolated a good template, you will waste time and reagents trying to make the probe.

6. Generate digoxigenin (dig)-labeled riboprobes using the Roche in vitro transcription labeling protocol.

Dig Probe In Vitro Transcription (scaled for 10 µl reaction final volume)

6.5 µl eluted PCR product containing RNA polymerase binding site
1 µl 10X in vitro transcription buffer (Roche)
1 µl 10X Dig-NTP labeling mix (Roche)
1 µl RNA polymerase (T3, T7, or SP6) (Roche or Promega)
0.5 µl RNase inhibitor (Roche or Promega)
10 µl total

- Incubate for 2 hours at 37°C (I prefer to use the bacterial incubator).
- Add 1 µl DNase I (Roche) and incubate another 15' @ 37°C.
- Stop the reaction by adding 0.5 µl of 0.5 M EDTA (pH 8.0), then put on ice.
- Add 38.5 µl RNase-free dH_2O to reaction and then spin through a G50 microspin column (Amersham).
    i. Vortex column to suspend beads. Spin 2min at 2800rpm to remove liquid. Blot.
    ii. Apply sample to column and spin to elute. Do not disturb surface of column.
- Run 2.5 µl of the G50 flow through on a gel to confirm that you have successfully made a riboprobe. Compare against control sample (the control Dig-labeled RNA included in the Roche Dig-Probe labeling kit (tube #5)) to give you a ballpark estimation of your probe concentration. The control labeled probe is 100 ng/µl, and I load 1 µl of that, along with 1 µl of a 1: 5 and 1:10 dilution of that control probe. Estimate the concentration of your probe by comparing the band intensities.
- To the remaining G50 flow through, add 450 µl of in situ hybridization solution. You can store this at -20°C indefinitely.

Generating DIG Probes through In Vitro Txn

1. Linearize 5ug plasmid (minipreps okay but sub-optimal)
2. Bring digest volume up to 50uL and clean with S200 column, gel purification, or at least purification on Qiaquick spin column, eluting in 30uL.
3. Prepare transcription rxn (you can also scale down to 10 ul reaction by halving the amounts below):

10uL	DNA from S200 spin column (approx 1ug) or 13uL for mini-prep DNA
2uL	10X In vitro txn buffer (Roche #8)
2uL	Dig-NTP labeling mix (Roche #7)
2uL	RNA polymerase (Roche SP6, T7, or T3)
1uL	RNase Inhibitor (Roche or Promega)
0-3uL	DEPC-dH2O, Ultrapure Water
20uL	

4. Incubate transcription rxn 2 hours at 37 degrees.
5. Add 2uL DNase-I (RNase-free, Roche) and incubate 15 min at 37 degrees.
6. Stop the reaction by adding 0.8uL of 0.5M EDTA, pH 8. Put tube on ice.
7. Raise volume to 50uL with DEPC-dH2O or Ultrapure dH2O.
8. Spin volume on G50 column. (Vortex column to suspend, twist off bottom, loosen lid, spin 2 min at 2800rpm, blot with Kimwipe to remove excess, add sample to column, spin through 2 min at 2800rpm)
9. Run 2.5uL solution on gel to evaluate probe concentration, comparing against 1uL, 0.5uL, 0.1uL DIG-labeled RNA standard from Roche.
10. Add 450uL hybridization solution to flow-through and store at -20 degrees.
11. Create working stock from stock using 300ng/mL as starting concentration (200-400 ng/ml is optimal for most probes).

In Situ Reagents (that can be prepared beforehand)

Hybridization Buffer (100mL)
(50% formamide, 5X SSC, 5X Denhardts, 250ug/mL yeast RNA, 500ug/mL salmon sperm)
- 50mL formamide (in fridge)
- 25mL 20X SSC (on bench)
- 5mL 100X Denhardts (in freezer)
- 5mL Sperm DNA (0.05g) (in freezer)
- 0.025g Torula Yeast RNA (in fridge)
- dH2O to 100mL
- Store at -20 degrees.

100X Denhardts Reagent (500mL)
- 10g Ficoll (Type 400, Pharmacia)
- 10g polyvinylpyrrolidone
- 10g Bovine Serum Albumin (fraction V, Sigma)
- dH2O to 500mL
- Filter and store at -20 degrees.

1L 20X SSC
- 175.3g NaCl
- 88.2g Na Citrate
- Dissolve in 800mL dH2O. pH to 7.0
- Bring volume to 1L and filter or autoclave to sterilize.

1L B1 (0.1M Tris, 0.15M NaCl)
- 100mL 1M Tris pH 7.5
- 30mL 5M NaCl
- dH2O to 1L

TE, pH 7.5
- 10mL 1M Tris pH 7.5
- 2mL 0.5M EDTA
- dH2O to 1L

In Situ Hybridization Protocol – Day I – Tissue Preparation and Hybridization

Prepare Fresh Reagents

500mL 4% PFA (per jar of slides)
- Heat hot plate to 60-70 degrees.
- Bring 200mL dH2O to near boil in microwave.
- Add 20g PFA and 8-10 drops 10N NaOH. Stir on hotplate until dissolved. May take 10-30 min.
- Add 125mL PBS.
- Check pH with paper; adjust to pH 7.
- Add 3.75g NaCl, let dissolve.
- Raise volume to 500mL with dH2O
- Filter through 0.2um filter. Chill on ice before use.

250mL Proteinase K (per jar of slides)
(1ug/mL proteinase K, 5mM EDTA, 50mM Tris pH 7.5)

3.125mL 0.5M EDTA
12.5mL 1M Tris pH 7.5
25uL 10mg/mL proteinase K (Roche)
dH2O to 250mL

Tissue Preparation
1. Fix dried slides in 4% PFA for 10 minutes.
2. Wash slides three times in PBS, 3 minutes per wash.
3. Digest slides with proteinase K in specific jar, 5 minutes.
4. Fix digested slides in 4% PFA for 5 minutes.
5. Wash slides three times in PBS, 3 minutes per wash.
6. Submerge slides in 250mL dH2O in jar with stir bar.
    a. Slowly add 3mL triethanolamine. Let dissolve.
    b. Add 650uL acetic anhydride dropwise.
    c. Mix solution by shaking slides up and down gently.
    d. Acetylate for 10 minutes.
7. Wash slides three times in PBS, 5 minutes per wash.

Hybridization
1. Drain PBS off slides and overlay each with 500-1000uL of hybridization solution (pre-hybridization).
2. Incubate in humidified chamber (slide rack) for a minimum of 1hr a RT.
3. While pre-hybridizing, prepare probes.
    a. Heat diluted probes (diluted to working concentration of typically 300ng/mL) to 80 degrees in thermomixer. Leave 5 minutes.
    b. Place probes on ice.
4. Remove hybridization buffer (pour off and blot edges to remove excess).
5. Apply 100uL of hybridization solution containing probe (standard concentration 200-400ng/mL)
6. Carefully coverslip each slide and place in humidified black box. One box per probe. Change gloves and coverslipping needle with each probe to avoid cross-contamination.
7. Seal boxes with lab tape, tape all boxes together, and then seal boxes with Saran wrap.
8. Place boxes in 72 degree hybridization oven overnight.

Day II – Washes and Antibody Staining

Prepare Fresh Reagents

5X SSC (250mL per jar slides)
- 62.5mL 20X SSC
- 187.5mL dH2O

0.2X SSC (750mL per jar slides)
- 7.5mL 20X SSC
- dH2O to 750mL

B2 (for blocking)(B1 + 10% GS)
- 1mL per slide composed of:
- 100uL goat serum
- 900uL B1

B2 (for antibodies)(B1 + 1% GS + 1:5000 antibody)
- 1mL per slide composed of:
- 10uL goat serum
- 0.2uL anti-digoxigenin antibodies (Roche)(fridge)
- 982uL B1

1. Remove slides from black boxes and submerge in 5X SSC (pre-heated to hybridization temperature). Carefully remove coverslips with forceps.
2. Transfer slides to 0.2X SSC wash, preheated to hybridization temperature. Wash 30 minutes.
3. Transfer slides to a second 0.2X SSC wash, preheated to hybridization temperature, wash 30 minutes.
4. Transfer slides to a third 0.2X SSC wash, at RT, wash 5 minutes.
5. Transfer slides to Buffer B1 for 5 minutes at RT.
6. Overlay slides with 1mL of Buffer B2+10% GS and incubate 1hr at RT to block.
7. Remove B2 and blot off excess. Replace with Buffer B2+1% GS and with 1:5000 dilution of AP-conjugated sheep anti-digoxigenin antibody,
8. Incubate slides overnight in humidified chamber at 4 degrees. If RNA expression particularly abundant, can get away with a few hours at RT.

Day III to Eternity – Detection

Prepare Fresh Reagents

B3 (300mL per jar slides)(reserve 50mL for B4)
- 30mL 1M Tris pH 9.5
- 6mL 5M NaCl
- 15mL 1M MgCl2
- dH2O to 300mL

B4 (500uL per slide; may need replacement daily)
- 4.5uL NBT
- 3.5uL BCIP
- 10uL Levamisole (400mM)
- B3 to 1000uL (982uL)

1. In jars, wash slides with 250mL Buffer B1 three times, 5 minutes per wash. More washes may reduce background.
2. Equilibrate slides in Buffer B3 jar for 5 minutes.
3. Prepare 500uL Buffer B4 per slide. Overlay slides and coverslip.
4. Incubate at RT for minutes to days until signal is detectable.
5. Replenish B4 daily and recoverslip to avoid dry-out.
6. Stop reaction by washing slides in TE, pH 8.
7. Dry slide on slide warmer.
8. Place one drop Dako Glycergel onto slide (warm Dako to 50 degrees in water bath) and coverslip.
9. Place on slide warmer until Dako spreads.
10. Let medium harden at RT at least 20 minutes.