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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'

   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
1.25	µl	DMSO- for a final concentration of 5%, this helps if the target is GC-rich
11.8	µl	ddH_2O
25	µl	total

If you are using GoTaq Green….
1uL cDNA Template
1uL Forward Primer
1uL Reverse Primer (0.5uL primer puts concentration below recommended for GoTaq)
12.5uL 2X GoTaq Green
9.5uL ddH2O.

PCR program doesn't need to be changed.

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.

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).

Run 2-3 µl of the G50 flow through on a gel to confirm that you have successfully made a riboprobe. It is also advisable to run various amounts of a control sample (i.e. 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/ml, 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. Be sure to take into account how much volume of your probe you have loaded on the gel.

To the remaining G50 flow through, add 450 µl of in situ hybridization solution. You can store this at -20°C indefinitely.