Probe generation for *In Situ* Hybridization: DNA template generation by PCR

Gene expression is detected with digoxygenin- or fluorescein-labelled RNA probes that are generated by *in vitro* transcription (IVT) from DNA templates. The core does the IVT but requires DNA templates to be provided by the customer.

The DNA templates are generated by PCR using a cDNA as the initial PCR template. The cDNA should be made from RNA from tissues expressing the gene of interest. The PCR is using primers consisting of 20-25 nt of gene-specific sequence linked to T3 or T7 polymerase promoter sequences.

*Please provide a minimum of 2 µg DNA template in no more than 20 µl (TE or DEPC water), however the higher the DNA concentration the better the yield of labelled **RNA**.*

1. RNA isolation (to be used for cDNA generation)

Isolate the total RNA from mouse-brain (e.g. P0, P7) and mouse-embryos (e.g. E 10.5, E 14.5,) with the RNAzolB kit (Qiagen RNeasy Mini Kit also works) or the tissue where your gene of interest is highly expressed. Mix the isolated RNA in the same ratio (on a mass basis) for subsequent cDNA synthesis.

2. Reverse Transcription to generate cDNA

Synthesize cDNA with ThermoScript™RT-PCR System, Invitrogen, Cat. No: 11146-024

Mix 1:

Oligo(dT) primer	1 µl
RNA	2 µl (1µg/µl)
DEPC-treated water	7 µl

Denature the RNA and the primer by incubating for 5 min at 65°C, then place PCR vial on ice.

Mix 2 (for one aliquot of cDNA):

5x cDNA synthesis buffer	4 µl
0.1M DTT	1 µl
RnaseOut™ (40 U/µl)	1 µl
DEPC-treated water	1 µl
10 mM dNTP-Mix	2 µl

ThermoScript™RT (15 U/µl) 1 µl

Gently vortex and combine Mix 1 and Mix 2. Incubate for 60 min at 50°C in a thermocycler and stop the reaction by heating to 85°C for 5 min. Add 1 µl RNaseH and incubate for 20 min at 37°C. Place on ice. In this form, the cDNA can be frozen and kept at -80°C for >1 year.

3. Primer design and order

Design gene specific oligonucleotide primers using a primer selection program or your own experience/strategy (manuals on PCR usually deal with this issue; GC content in the range of 40 to 60 %, the end at which the polymerase will extend should have a GC or CG, avoid repeats, no homology to other genes in the genome [run a BLAST search], no long hairpin structures, no dimer formation).

Use primers in the range of 18-20 bases that border the desired probe sequence. We recommend a probe length of 500-1000 bp, if necessary the length can be reduced to 250bp.

Once you have selected a gene specific primer pair, add **different** RNA polymerase promoters to the 5´end of the forward and reverse primers. The sequence of the T3 promoter is GCGAATTAACCCTCACTAAAGGG and for the T7 promoter it is GCGTAATACGACTCACTATAGGG. **Please note**, 3 extra bases (underlined!) are attached to each of the promoters for better binding of the RNA polymerase.

These primers are synthesized by external suppliers (10 nMol is sufficient). Please let the core know which polymerase sequence you put on which primer.

4. PCR - small

The annealing temperature depends on the sequence of the gene specific part of the primer because the T7 or T3 sequences do not bind to the cDNA. Therefore, paste only the gene specific part of the primer in any program to determine the optimal annealing temperature.

To determine the best annealing temperature, it's advisable to run an analytical (gradient) PCR with 6 tubes in a range of 20°C (PCR efficiencies may change according to various thermocyclers).

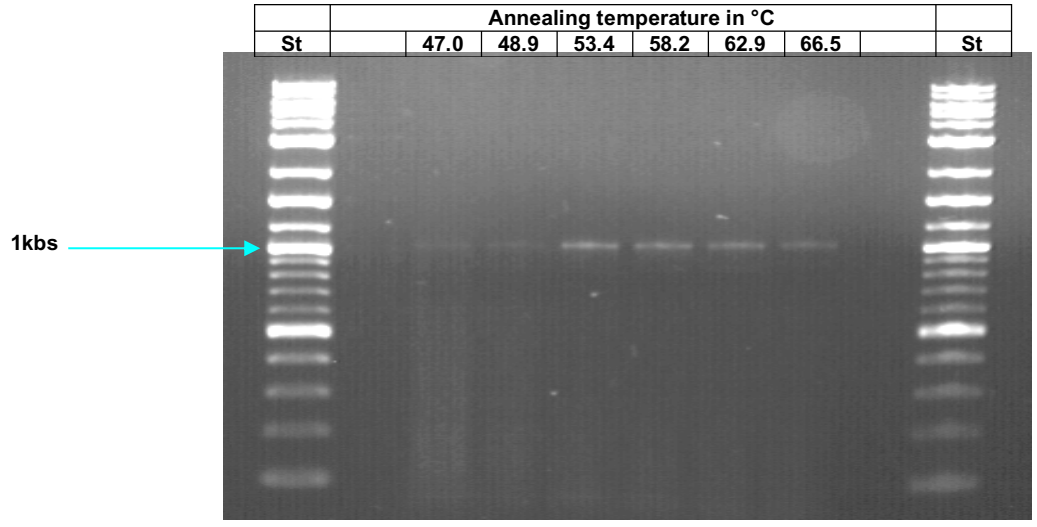
Example of one small PCR (20µl) using RT-product as template:

H_2O	6.9-7.9 µl
10x Buffer	2 µl
Q (enhancer)	4 µl
dNTPs (2 mM)	2 µl
Primer F (5 pmol/µl)	2 µl
Primer R (5 pmol/µl)	2 µl
cDNA (RT-product)	1-2 µl
Enzyme	0.1 µl

The amount of cDNA to be used depends on the abundance of the transcript. The volume of 1 to 2 µl refers to the cDNA synthesis protocol described above which yields 21 µl of cDNA.

For the gradient PCR, carry out 20 µl reaction for about 35 cycles. 5 µl of each reaction is used for electrophoretic analysis. Those PCRs that show the desired band only (see figure below) are pooled and purified with a PCR purification kit.

If multiple bands are present, pool all material, re-run on an agarose gel and cut out and extract the desired band using a gel extraction and purification kit.



The yield of DNA depends on how strongly the gene of interest is expressed. In the gel shown here we have a gene that was weakly expressed. We pooled the last 4 PCR reactions and, following purification, used them as template for the second, large scale PCR (see below).

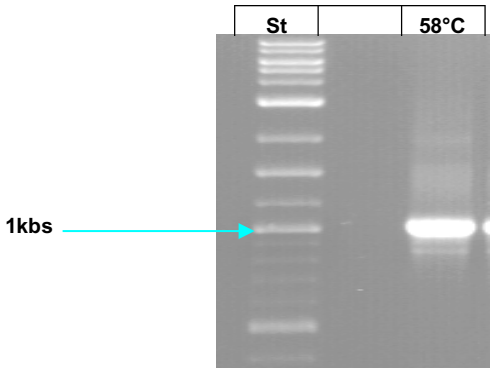
5. Large scale (preparative) PCR

In order to prepare enough template for the riboprobe synthesis, a preparative 50-100 µl PCR is done using *the purified PCR product* as template. Use the same primers that were used to generate the first PCR product from the cDNA.

Use 2 PCR tubes to carry out the 100 µl preparative PCR (i.e a 50 µl reaction in each). Taq-polymerase, the buffer and enhancer are from Qiagen, dNTPs are from Roche. Use the annealing temperature which showed the best result in the first PCR.

H_2O	37.5 µl
10x Buffer	10 µl
Q (enhancer)	20 µl
dNTPs (2 mM)	10 µl
Primer F (5 pmol/µl)	10 µl
Primer R (5 pmol/µl)	10 µl
Template (product from small PCR)	2 µl (20-50 ng)
Enzyme	0.5 µl

Analyze 5 µl on an agarose gel (see gel below for a typical result) and purify the rest (95 µl) with a PCR purification kit. Elute with 25 µl of buffer (10 mM Tris, pH 8.5), 1-3 µl of which are used to measure the concentration and purity of the DNA. Typically, the yield is 200-400 ng/µl.



After you quantify your DNA confirm that you have the correct PCR product by sequencing! Normally 50 ng of PCR product are used for each sequencing reaction. The T7 and T3 primers could be the same primers used for the PCR.