Generation of Template DNA for RNA probes by RT-PCR using sense and T7-antisense primers

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I. Primer Design

1. Design primer sets for PCR. I usually design them to amplify approximately 800bp including 3'-end of ORF and 3' UTR. For this, I first select the candidate sequences by eye and then examine whether they are valid (i.e. Tm is around 60 °C, no primer dimers or secondary structures) by DNA Calculator on Sigma Genosys homepage (http://www.sigma-genosys.com/calc/DNACalc.asp).
2. Add T7 promoter sequence **5'- GCGGTAATACGACTCACTATAGGGC -3'** at 5'-end of the antisense primer.

II. Reverse Transcription

1. Add the following reagents in an RNase-free microcentrifuge tube.

Total RNA	1 μg
Oligo d(T)18 mRNA primer ($5A_{260}$ units; NEB)	3 μl
10 mM dNTP mix	1 μl
Sigma H_2O (nuclease-free)	to 12 μl

2. Heat at 65°C and then chill on ice quickly. Collect the contents of the tube by brief centrifugation and add:

5x First-Strand Buffer	4 μl
0.1 M DTT	2 μl
RNaseOUT (40 units/μl; Invitrogen)	1 μl

3. Mix contents of the tube gently. Incubate at 42°C for 2 minutes.
4. Add 1 μl (200 units) of SuperScript II RT (Invitrogen) and mix gently by pipetting gently up and down.
5. Incubate at 42°C for 50 minutes.
6. Inactivate the reaction by heating at 70°C for 15 minutes.
7. The cDNA now can be used as a template for PCR.

III. PCR

I preferably use Phusion Hot Start DNA Polymerase (Finnzyme; sold by NEB) for PCR.

1. Add the following components into a 0.2 ml PCR tube:

32.5 μl	nuclease-free H_2O (Sigma)
10 μl	5x HF Buffer
1 μl	10 mM dNTP mix
2.5 μl	10 μM forward primer
2.5 μl	10 μM reverse primer
1 μl	1st strand cDNA
0.5 μl	Phusion Hot Star DNA Polymerase

2. Set the PCR program as below:

Initial activation (1 cycle): 98°C for 30 min

Touchdown PCR (10 cycles):

Denatue	98°C for 10 sec.
Annealing*	70-61°C for 20 sec

*Start with 70°C and decrease the temperature by 1°C each cycle.

Extension	72°C for 30 sec.

Normal PCR (25 cycles):

Denature	98°C for 10 sec.
Annealing	60°C for 20 sec.
Extension	72°C for 30 sec.

Final extension (1 cycle): 72°C for 10 min.

3. Place PCR tubes on the thermal cycler and start PCR.
4. Check the PCR products by agarose gel electrophoresis. If the yield is too low, take 1 μl of the PCR product and run the second round of PCR. For the second PCR, it may be better ro use T7 primer instead of T7-antisense primer as a reverse primer.
5. Purify the PCR products by spin column (Gel-purification is usually not necessary). Elute the PCR products by 10 mM Tris-HCl pH 8.0.
6. Use 5 μl purified PCR product for a 20 μl *in vitro* transcription reaction.