

## One-Step RT-PCR

### Reagents:

- RETROscript™ kit from Ambion, Catalogue # 1710
  - Reverse Transcriptase M-MLV-RT (100 units/μl)
  - 10X PCR Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>)
  - Random decamers (50μM)
  - dNTP (2.5mM each dNTP)
  - RNase Inhibitor (10 units/μl)

Taq polymerase: from Invitrogen, Catalogue #: 10342-020 (5U/μl)

**Notes:** Use small 0.25ml PCR tubes supplied by your TAs  
Wear gloves at all times to avoid RNase contamination

### Before you start:

- Thaw 10x reaction buffer, random decamers, and dNTP mix quickly in your hands and place ON ICE;
- Place RNase Inhibitor and Reverse Transcriptase (M-MLV-RT) ON ICE directly from the box
- Prepare working primer stock solution as described in the two-step RT-PCR protocol

There should be two negative controls among the PCRs.

- I. The minus-RT control from the previous step, or alternatively, untreated RNA can simply be subjected to PCR.
- II. A minus-template PCR. It should have all the PCR components, but use water as template instead of an aliquot of the cDNA (RT reaction). This control will verify that none of the PCR reagents are contaminated with DNA.

### *Positive Control*

- I. Perform PCR to amplify a cDNA that corresponds to a basal/housekeeping transcript, e.g., ribosomal protein S17 (RpS17). The TA's will provide each team with RpS17 primers. For your reference, these are the primer sequences:

Forward RpS17: 5' - cga acc aag acg gtg aag aag - 3'

Reverse RpS17: 5' - cct gca act tga tgg aga tac c - 3'

Expected RT-PCR product size: 211bp

The primers are located on different exons that are separated by a 59bp intron. If genomic DNA is amplified, the product size would be 270bp.

- II. Use genomic DNA isolated from S2 cells as template. If your primers span intron(s), note the size of the expected PCR product and if necessary, adjust annealing temperature of the PCR program.

The components needed for each reaction are outlined in the following table:

<b>Component</b>	<b>Stock conc</b>	<b>Final amount</b>	<b>Experiment</b>	<b>Neg Control 1 (-RT in step 1)</b>	<b>Neg Control 2 (no template)</b>
Total RNA			~1-2 µg	~1-2 µg	0
Ambion 10X PCR Buffer (with Mg <sup>2+</sup> )*	10X	1X	10 µl	10 µl	10 µl
dNTP mix	2.5mM	0.16mM	6.5 µl	6.5 µl	6.5 µl
Random Decamers	50µM	5µM	2 µl	2 µl	2 µl
Forward PCR primer	5µM	0.25µM	5 µl	5 µl	5 µl
Reverse PCR primer	5µM	0.25µM	5 µl	5 µl	5 µl
RNase Inhibitor	10U/µl	10U	1 µl	1 µl	1 µl
Taq polymerase	5U/µl	2U	0.4 µl	0.4 µl	0.4 µl
Reverse Transcriptase	100U /µl	100U	1 µl	0	1 µl
Nuclease-free water			Variable	Variable	Variable
Total Volume			100 µl	100 µl	100 µl

\*Use the 10X PCR Buffer in the Ambion Retroscript kit. This buffer contains 15mM MgCl<sub>2</sub>.

#### Making a Master Mix:

Consider making a master mix if you are testing multiple sets of primers at once. A master mix will contain everything except the PCR primers. If you are testing n sets of primers, make a master mix enough for n+1 tests.

Mix the components gently but thoroughly. Aliquot 90µl of your master mix to each tube. Add 5µl of each the appropriate primer.

#### Master Mixes:

<b>Component</b>	<b>Experiment</b>	<b>Neg Control 1 (-RT in step 1)</b>	<b>Neg Control 2 (no template)</b>
Total RNA	~1-2 *(n+1) µg	~1-2*(n+1) µg	0
Ambion 10X PCR Buffer	10 *(n+1) µl	10*(n+1) µl	10 *(n+1) µl
dNTP mix	6.5 *(n+1) µl	6.5 *(n+1) µl	6.5*(n+1) µl
Random Decamers	2*(n+1) µl	2*(n+1) µl	2*(n+1) µl
RNase Inhibitor	1 *(n+1) µl	1 *(n+1) µl	1*(n+1) µl
Taq polymerase	0.4 *(n+1) µl	0.4 *(n+1) µl	0.4*(n+1) µl
Reverse Transcriptase	1*(n+1) µl	0	1*(n+1) µl
Nuclease-free water	Variable	Variable	variable
Total Volume	100 *(n+1) µl	100*(n+1) µl	100*(n+1) µl

Assemble reactions on ice.

Incubate in Thermacycler at:

- 1) 44°C for 1 hr for reverse transcription
- 2) 94°C for 5 min to inactivate the RT and denature reaction product
- 3) 94°C for 30 sec to denature strands
- 4) 55°C for 30 sec to anneal strands\*\*
- 5) 72°C for 45 sec to extend\*\*\*
- 6) repeat steps 3 to 5, 29 more times
- 7) 72°C for 5 min for a final extension

\*\* Start with the annealing temperature suggested by your primer design software, usually use  $(T_m - 5)^\circ\text{C}$ . An annealing temperature of  $\sim 55^\circ\text{C}$  used with the cycling times shown may be a reasonable starting point, but the optimal temperature and cycling times for your PCR may need to be determined empirically.

\*\*\* The rule of thumb is to extend for 1 min per kb of target in the PCR cycle.

Run 10  $\mu\text{l}$  of your reaction on 1-1.5% Agarose gel to check the PCR product.