RT-PCR: Two-Step Protocol

We will provide both one-step and two-step protocols for RT-PCR. We recommend the two-step protocol for this class.

In the one-step protocol, the components of RT and PCR are mixed in a single tube at the same time. The one-step protocol generally works well for amplifying targets that are reasonably abundant.

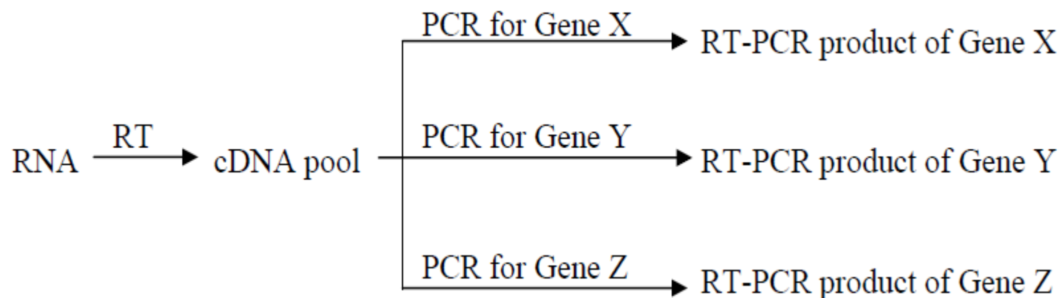
Alternatively, RT-PCR can be done in two steps, first with the reverse transcription and then the PCR. The two-step protocol is usually more sensitive than the one-step method; yields of rare targets may be improved by using the two-step procedure.

One-step RT-PCR:

- Convenient

Two-step RT-PCR:

- Saves RT reagents.
- One RT reaction will provide templates for multiple PCR's can be more sensitive than one-step RT-PCR.



Two-step Protocol

Step One: Reverse Transcription

Note: Wear gloves at all times to avoid RNase contamination

Reagents:

- RETROscript™ kit
- Reverse Transcriptase MMLV-RT (100 units/µl)
- 10X RT Buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30mM $MgCl_2$ 50 mM DTT)
- Random decamers (50µM)
- dNTP (2.5mM each dNTP)
- RNase Inhibitor (10 units/µl)

Before you start:

- Place RNase Inhibitor and Reverse Transcriptase ON ICE directly from the box
- Thaw 10x reaction buffer, random decamers, and dNTP mix quickly in your hands and place ON ICE

Use small 0.25ml PCR tubes.

1) Assemble your reaction as follows on ice. Add the enzyme last.

Component	Stock	Final amount	Experiment (+RT)	Control (-RT)
Total RNA			~1-2 µg	~1-2 µg
Random Decamers	50µM	5µM	2 µl	2 µl
10X RT Buffer	10X	1X	2 µl	2 µl
dNTP mix	2.5mM	0.5mM	4 µl	4 µl
RNase Inhibitor	10 U/µl	10U	1 µl	1 µl
Reverse Transcriptase	100 U/µl	100U	1 µl	0 µl
Nuclease-free water			x µl (to total of 20 µl)	x µl (to total of 20 µl)

2) Mix gently, spin briefly.

3) Incubate in the thermacycler at:

   a. 44°C for 1 hr.
   b. 92°C for 10 min to inactivate the reverse transcriptase.

4) Store reaction at –20°C or proceed to the PCR

Step Two: PCR

A) Primer Preparation

1) Primers are shipped in dry form. Briefly spin the tube before you open the cap to avoid loss of DNA pellet.
2) Dissolve the oligonucleotide in 10mM Tris, pH7.5 to make a primer stock at 100µM concentration.
3) Dilute from this stock 1:20 (in water) to make a working solution at 5 µM for use in setting up PCR reactions.

B) Setting up PCR reactions

Negative Controls

Use 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 is 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.

Reagents:

Taq DNA polymerase

With 10X Buffer (-$MgCl_2$) and 50mM $MgCl_2$

The following table outlines the components needed for PCR. Note: do not use DEPC-treated water.

Component	Working Stock	Final Conc	Experiment	Control 1 (-RT in step1)	Control 2 (notemplate)
RT reaction			1-2 µl	1-2 µl	0 µl
10X PCR Buffer	10X	1X	2.5 µl	2.5 µl	2.5 µl
Forward primer	5µM	0.25µM	1.25 µl	1.25 µl	1.25 µl
Reverse primer	5µM	0.25µM	1.25 µl	1.25 µl	1.25 µl
dNTP mix	2.5mM	0.125mM	1.25 µl	1.25 µl	1.25 µl
Taq Polymerase	5U/µl	1U	0.2 µl	0.2 µl	0.2 µl
dH_2O			Up to 25 µl	Up to 25 µl	Up to 25 µl

For RpS17 control:

Component	RpS17 control (+RT in step 1)	RpS17 control (-RT in step 1)
RT reaction	1-2 µl	1-2 µl
10X PCR Buffer	2.5 µl	2.5 µl
RpS17 forward primer	1.25 µl	1.25 µl
RpS17 reverse primer	1.25 µl	1.25 µl
dNTP mix (2.5mM)	1.25 µl	1.25 µl
$MgCl_2$	0.75 µl	0.75 µl
Taq Polymerase	0.2 µl	0.2 µl
dH_2O	Up to 25 µl	Up to 25 µl

Making Master Mixes:

Consider making master mixes 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 22.5µl of your master mix to each tube. Add 1.25µl of each of the appropriate primer at 5µM working stock concentration.

Component	**Experiment**	**Neg Control 1 (-RT in step 1)**	**Neg Control 2 (no template)**
RT reaction	~1-2*(n+1) µg	~1-2*(n+1) µg	0
10X PCR Buffer	5 *(n+1) µl	5*(n+1) µl	5 *(n+1) µl
dNTP mix	1.25*(n+1) µl	1.25*(n+1) µl	1.25*(n+1) µl
MgCl2	*(n+1) µl	*(n+1) µl	*(n+1) µl
Taq polymerase	0.2*(n+1) µl	0.2*(n+1) µl	0.2*(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:

a) Initial denaturation: 94°C for 4 min
b) 30 cycles: Denature at 94°C for 30 sec

Anneal at 55°C for 20–30 sec**

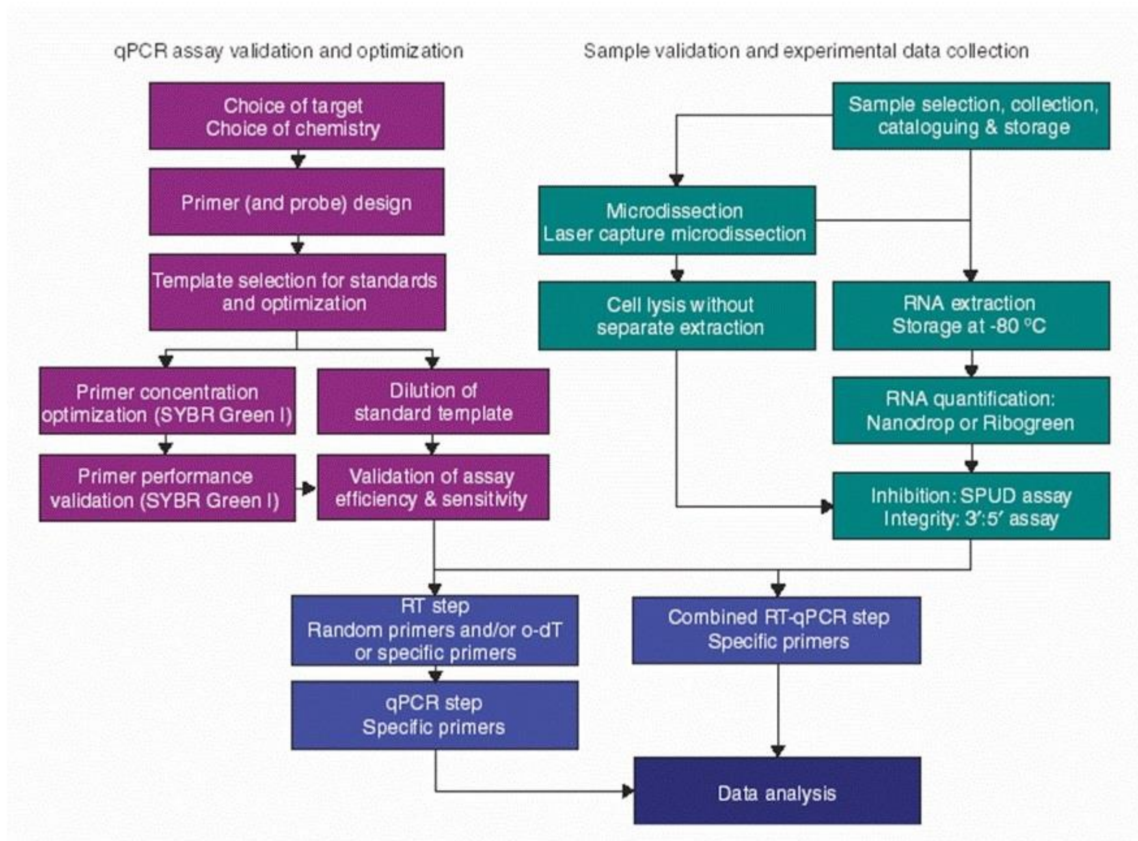
Extend at 72°C for 45 sec ***

c) Final extension: 72°C for 5 min

**Start with the annealing temperature suggested by your primer design software. An annealing temperature of ~55°C used with the cycling times shown is often a reasonable starting point, but the optimal temperature and cycling times for your primer and template combination may need to be determined empirically.

***The rule of thumb is to use an extension time of 1 min per kilobase of target.

Run 15 µl of your reaction on 1-1.5% Agarose gel


qPCR assay validation and optimization
Sample validation and experimental data collection
Choice of target
Choice of chemistry
Primer (and probe) design
Template selection for standards and optimization
Primer concentration optimization (SYBR Green I)
Dilution of standard template
Primer performance validation (SYBR Green I)
Validation of assay efficiency & sensitivity
Sample selection, collection, cataloguing & storage
Microdissection
Laser capture microdissection
Cell lysis without separate extraction
RNA extraction
Storage at -80 °C
RNA quantification:
Nanodrop or Ribogreen
Inhibition: SPUD assay
Integrity: 3′:5′ assay
RT step
Random primers and/or o-dT or specific primers
qPCR step
Specific primers
Combined RT-qPCR step
Specific primers
Data analysis