GENE EXPRESSION ANALYSIS BY QUANTITATIVE REVERSE TRANSCRIPTION PCR (RT-qPCR)

Quantitative real time reverse transcription-polymerase chain reaction (RT-qPCR) is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as the template for the qPCR reaction. RT-qPCR is used in a variety of applications including gene expression analysis, RNAi validation, microarray validation, pathogen detection, genetic testing, and disease research.

One-step vs. Two-step RT-qPCR

RT-qPCR can be performed in a one-step or a two-step assay. One-step assays combine reverse transcription and PCR in a single tube and buffer, using a reverse transcriptase along with a DNA polymerase. One-step RT-qPCR only utilizes sequence-specific primers. In two-step assays, the reverse transcription and PCR steps are performed in separate tubes, with different optimized buffers, reaction conditions, and priming strategies.

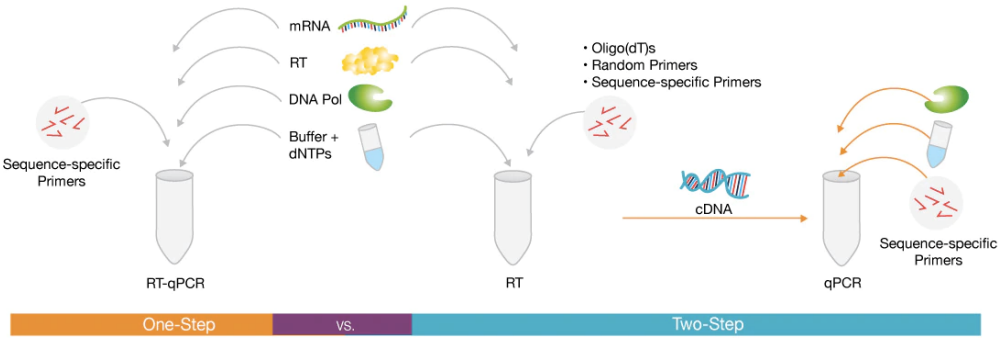


Table 1. Advantages and Disadvantages when using one-step versus two-step assays in RT-qPCR

	Advantages	Disadvantages
One-step	• Less experimental variation since both reactions take place in the same tube • Fewer pipetting steps reduces risk of contamination • Suitable for high throughput amplification/screening • Fast and highly reproducible	• Impossible to optimize the two reactions separately • Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions • Detection of fewer targets per sample
Two-step	• A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions • The target and reference genes can be amplified from the same cDNA pool without multiplexing • Optimized reaction buffers and reaction conditions can be used for each individual reaction • Flexible priming options	• The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination Time consuming • Requires more optimization than one-step

We will perform the Two-Step RT-qPCR

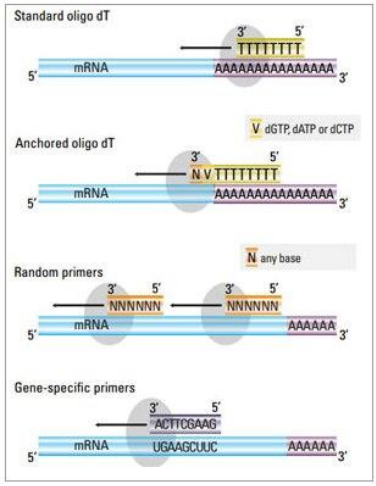
1. Reverse Transcription in RT-qPCR

Choosing total RNA vs. mRNA

When designing a RT-qPCR assay it is important to decide whether to use total RNA or purified mRNA as the template for reverse transcription. mRNA may provide slightly more sensitivity, but total RNA is often used because it has important advantages over mRNA as a starting material. First, fewer purification steps are required, which ensures a more quantitative recovery of the template and a better ability to normalize the results to the starting number of cells. Second, by avoiding any mRNA enrichment steps, one avoids the possibility of skewed results due to different recovery yields for different mRNAs. **Taken together, total RNA is more suitable to use in most cases since relative quantification of the targets is more important for most applications than the absolute sensitivity of detection.**

Primers for Reverse Transcription

Three different approaches can be used for priming cDNA reactions in two-step assays: oligo(dT) primers, random primers, or sequence specific primers. Often, a mixture of oligo(dT)s and random primers is used. These primers anneal to the template mRNA strand and provide reverse transcriptase enzymes a starting point for synth.



Reverse Transcriptase Enzymes

Reverse Transcriptase is the enzyme that makes DNA from RNA. Most reverse transcriptases used in molecular biology are derived from the pol gene of avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV).

The AMV reverse transcriptase was one of the first enzymes isolated for cDNA synthesis in the lab. The enzyme is a 170-kDa heterodimer with an optimal reaction temperature range of 42–48°C. The AMV reverse transcriptase possesses strong RNase H activity that degrades RNA in RNA:cDNA hybrids, resulting in shorter cDNA fragments (< 5 kb).

The MMLV reverse transcriptase became a popular alternative due to its monomeric structure, which allowed for simpler cloning and modifications to the recombinant enzyme. MMLV reverse transcriptase is a 75-kDa enzyme with an optimal reaction temperature around 37°C. Although it is less thermostable than AMV reverse transcriptase, MMLV reverse transcriptase is capable of synthesizing longer cDNA (<7 kb) at a higher efficiency, due to its lower RNase H activity.

To further improve cDNA synthesis, MMLV reverse transcriptase has been engineered for even lower RNase H activity (i.e., mutated RNase H domain, or RNaseH–), higher thermostability (up to 55°C), and enhanced processivity (65 times higher). These attributes result in increased cDNA length and yield, higher sensitivity, improved resistance to inhibitors, and faster reaction times

EQUIPMENT

Fixed Equipment

- Thermal Cycler
- Centrifuge
- Pipettes suitable for measuring 10, 200 and 1000 µl, respectively

Consumables

- Polypropylene microcentrifuge tubes, 0.2 µl
- Filter pipette tips 10, 200, 1000 µl

REAGENTS

- RNA samples extracted day 1
- Oligo(dT)$_{18}$ Primer
- Random Hexamer Primer
- M-MuLV Reverse Transcriptase (200u/ul) (RT)
- RiboLock RNase Inhibitor (20u/ul) (RNAase Inbib)
- 5X Reaction Buffer (RB 5X)
- 10 mM dNTPs Mix
- H_2O, nuclease-free

1.1 Mix A

- After thawing, mix and briefly centrifuge the components of the reaction. Store in ice.
- Add the following reagents into a sterile tube, previously signed with an indelible marker.

REAGENTS	RNA	VOLUME
RNA Sample	500 ng	?
Oligo(dT)$_{18}$ Primer		1 ul
Random Examer Primer		1 ul
H_2O nuclease free		to 12 ul

- Mix gently, centrifuge briefly and put the sample in the thermal cycler at 65°C for 5 Minutes.

1.2 Mix B

- Take the sample from the thermal cycler
- Add the following reagents:

REAGENTS	FINAL CONCENTRATION	VOLUME
5X Reaction Buffer	1X	?
RNase Inhibitor		1 ul
Reverse Transcriptase		1 ul
10 mM dNTPs MIX	2mM	?

Consider a final volume of 8 ul

- Mix gently, centrifuge briefly and put the sample in the thermal cycler with the following program:

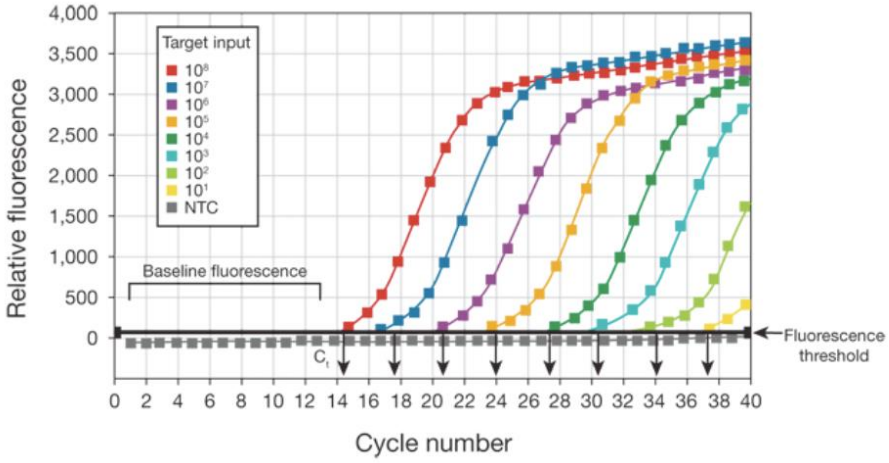
  25°C- 5 minutes
  42°C- 15 minutes
  70°C- 5 minutes

- Dilute the cDNA 1:3 in a final volume of 60 µl.

- The reverse transcription reaction product can be directly used in Real Time reaction or stored at -20°C.

2. Real Time qPCR in RT-qPCR

In real-time PCR, the amount of DNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated. Data collected in the exponential phase of the reaction yield quantitative information on the starting quantity of the amplification target. Fluorescent reporters used in real-time PCR include double-stranded DNA (dsDNA)- binding dyes, or dye molecules attached to PCR primers or probes that hybridize with PCR product during amplification.
The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the



accumulation of product over the duration of the entire PCR reaction.

Real-time PCR steps

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 40 cycles.

1. **Denaturation:** High temperature incubation is used to "melt" double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.
2. **Annealing:** During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).
3. **Extension:** At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

SYBR Green dye

The most commonly used DNA-binding dye for real-time PCR is Sybr Green which binds nonspecifically to double-stranded DNA (dsDNA). Sybr Green exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1000 fold when it binds dsDNA. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified.

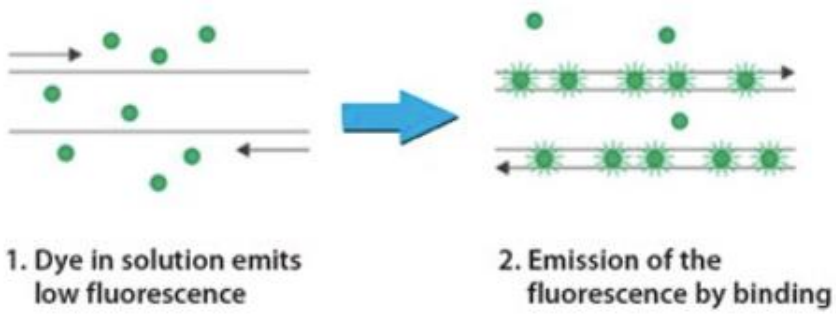


Figure 1. Technology Overview: SYBR Green qPCR

EQUIPMENT

Fixed Equipment

- DNA Engine Opticon 2 Continuos Fluorescence Detection System (MJ Research)
- Centrifuge
- Pipettes suitable for measuring 10, 200 and 1000 µl, respectively

Consumables

- Strip 8x0.1 mL
- Optical strip caps
- Filter pipette tips 10, 200, 1000 µl

REAGENTS

- cDNAs from step 1
- 2X SYBR Green Mix
- Mix Trpv1 Forward + Reverse Primer 10 µM
- Mix Beta-Actin Forward + Reverse Primer 10 µM
- H_2O nuclease free

2.1 Real-Time Mix

2 genes:
- Housekeeping: B-Actin
- Gene of interest: TRPV1

Each sample must be runed in duplicate for each gene of interest.

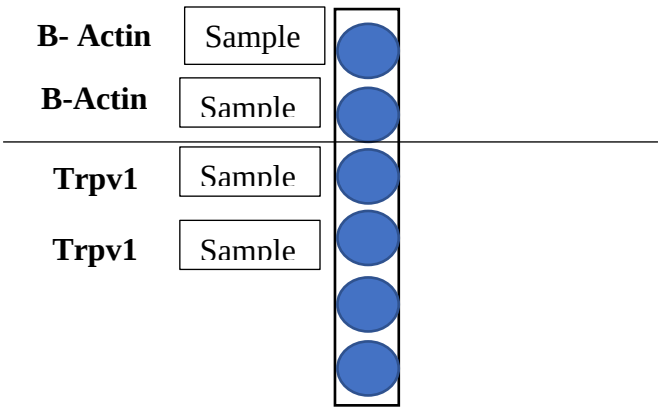
Thus, each group has to prepare **2 reaction mix**, one for gene (one for B-Actin and one for Trpv1). Each reaction mix must be for 3 samples (one sample in duplicate + 1 in excess)

- After thawing, mix and briefly centrifuge the components of the reaction. Store in ice.
- Add the following reagents into a sterile tube, previously signed with an indelible marker:

Reagents	**Final Concentration**	**Volume**
2X SYBR Green Mix	1X	?
Mix F +R 10 µM	1 µM	?
H_2O nuclease free		Up to 30 µl

Final Volume (for 1 gene, 3 samples)		**30 µl**

- Load samples in duplicate in each strip: 1 ul of sample for well . Then add 9 ul of reaction mix following this plan:



- Close the strips with the caps and spin down the strips.

2.2 Real Time Run

- Load the strips in the Real-Time PCR system with the following program:

95°C 5 minutes
95°C 10 seconds
60°C 20 seconds

Plate read
Go to line 2 for 45 times
Melting curve from 65°C to 95°C, read any 0.5°C, hold 00:00:02
Incubate at 4°C for ever

2.3 Real Time Data Analysis

In this plot, the PCR cycle number is shown on the x-axis and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, on the y-axis.

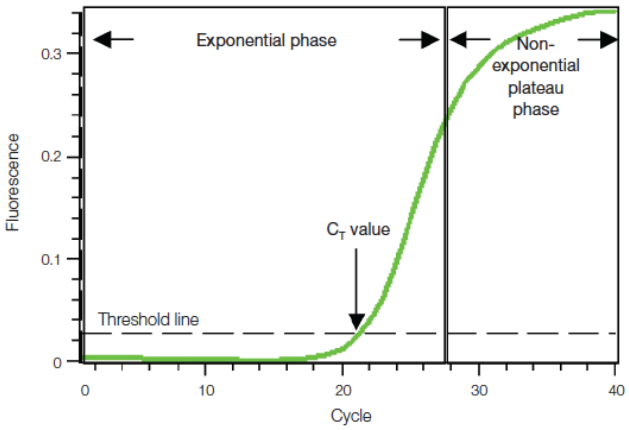


Fig. 1.1. Amplification plot. Baseline-subtracted fluorescence is shown.

The amplification plot shows two phases, an exponential phase followed by a nonexponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28-40 in Figure).

Initially fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1-18 in Figure) even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which occurs is called the **threshold cycle** or **Ct.** Since the Ct is value is measured in the exponential phase when reagents are not limited, real time PCR can be used to reliably and accurately calculate the initial amount of template present in the reaction.

The Ct of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early, Ct. In contrast, If a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, Ct.

Comparative quantification

In this technique, which applies to most gene expression studies, the expression level of a gene of interest is assayed for up- or down-regulation in a calibrator (control) sample and one or more experimental samples. Precise copy number determination is not necessary with this technique, which instead focuses on fold change compared to the calibrator sample.

Comparative quantification algorithms-ΔΔCt

The ΔΔCt method is a very popular technique that compares results from experimental samples with both a calibrator (e.g., untreated or wild-type sample) and a normalizer (e.g., housekeeping gene expression). With this method, Cts for the gene of interest (GOI) in both the test sample(s) and calibrator sample are now adjusted in relation to a normalizer (norm) gene Ct from the same two samples. The resulting ΔΔCt value is incorporated to determine the fold difference in expression.

$\Delta Ct_{sample} - \Delta Ct_{calibrator} = \Delta\Delta Ct$

$Ct_{GOI}^{s} - Ct_{norm}^{s} = \Delta Ct_{sample}$

$Ct_{GOI}^{c} - Ct_{norm}^{c} = \Delta Ct\ calibrator$

$Fold\ difference = 2^{-\Delta\Delta Ct}$

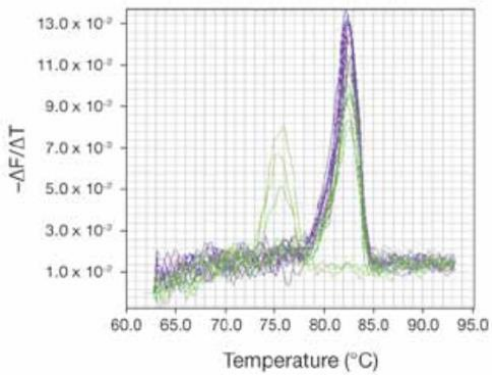
The result obtained is the fold increase (or decrease) of the target gene in the sample relative to the calibrator and is normalized to the expression of a reference gene. Normalizing the expression of the gene of interest to that of the reference gene compensates for any difference in the amount of sample tissue.

NB: This method assumes that both target and reference genes are amplified with efficiencies near 100%.

Melting Curve Analyses

The specificity of a real-time PCR assay is determined by the primers and reaction conditions used. However, there is always the possibility that even well designed primers may form primer-dimers or amplify a nonspecific product. There is also the possibility when performing qRT-PCR that the RNA sample contains genomic DNA, which may also be amplified. The specificity of the real-time PCR reaction can be confirmed using melting curve analysis.
Primer-dimers occur when two PCR primers (either same sense primers or sense and antisense primers) bind to each other instead of to the target. Melting curve analysis can identify the presence of primer-dimers because they exhibit a lower melting temperature than the amplicon.

The presence of primer-dimers is not desirable in samples that contain template, as it decreases PCR efficiency and obscures analysis.



To perform melting curve analysis, the real-time PCR instrument can be programmed to include a melting profile immediately following the thermal cycling protocol. After amplification is complete, the instrument will reheat your amplified products to give complete melting curve data.