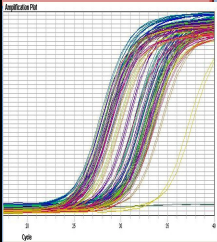
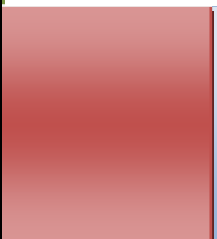


NanoCinna

Pharmacogenomics Center

Introduction to

Real-Time PCR



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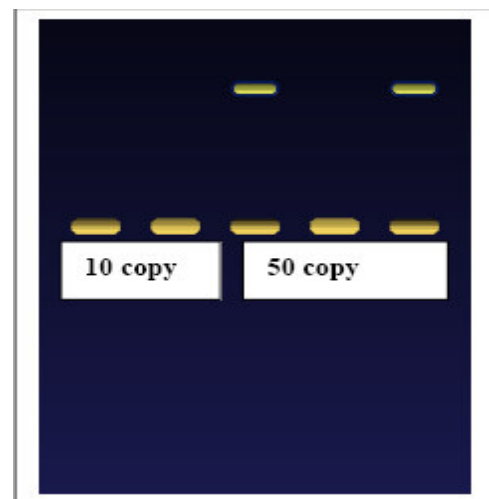
Introduction to Real-Time PCR:

Cells in all organisms regulate gene expression and turnover of gene transcripts (messenger RNA, abbreviated to mRNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation. Numerous techniques have been developed to measure gene expression in tissues and cells. These include Northern blots, RNase protection assays, *in situ* hybridization, dot blots, S1 nuclease assays and coupled reverse transcription and PCR amplification (RT-PCR). Of these methods, RT-PCR is the most sensitive and versatile. The technique can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library. Real-time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles. Quantitative real-time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample.

Real-Time Vs Traditional PCR

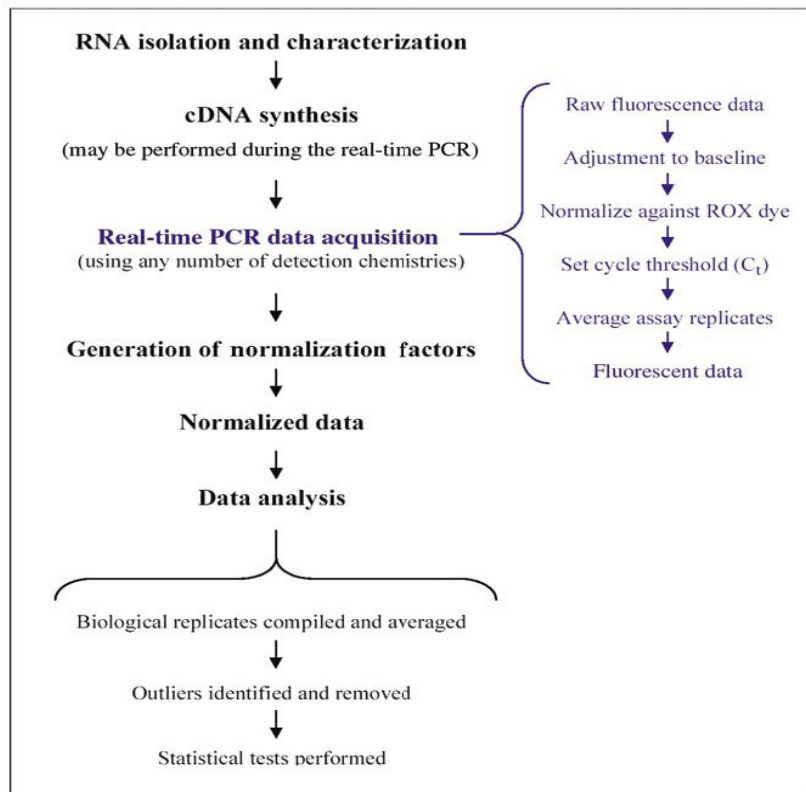
Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. Agarose gel results are obtained from the end point of the reaction. Endpoint detection is very time consuming, results may not be obtained for days and they are based on size discrimination, which may not be very precise. The end point is variable from sample to sample. While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect these changes. Agarose Gel resolution is very poor, about 10 fold. Real-Time PCR can detect as little as a two-fold change!

Figure 1: As you can see from the figure, the samples in the gel contain 10 copies and 50 copies, respectively. It is hard to differentiate between the 5-fold changes on the Agarose gel. Real-Time PCR is able detect a two-fold change (i.e. 10 vs. 20 copies).



One-Step Vs Two-Step Real-Time

There are two primary ways that real-time RT-PCR can be carried out. One method involves including the RT step into the same tube as the PCR reaction (one-step). The other method involves creating cDNA first by means of a separate reverse transcription reaction and then adding the cDNA to the PCR reaction (two-step). There are advantages and disadvantages to both systems. There are times when the use of one or the other assay system makes sense and it is up to the investigator to make that decision.



1. **Figure 2. Steps performed when measuring gene expression using real-time PCR.** RNA is first isolated and characterized for quantity and integrity. If performing a one-step reaction, RNA is used as a template for the real-time PCR assay, and reverse transcription occurs during the assay. During a two-step reaction, cDNA is first synthesized and then used as a PCR template. The steps performed on the real-time PCR machine are shown in blue, the time during which raw fluorescence data are collected, adjusted, and manipulated to generate the output data used for analysis. For normalizing results with multiple housekeeping genes, a normalization factor must be calculated for each individual sample. Dividing the fluorescent data by its normalization factor produces the normalized data, which is followed by statistical analysis.

cDNA synthesis:

RNA quantification begins with the making of cDNA (complementary DNA) by reverse transcriptase. There are two kinds of RT enzymes readily available on the

market, AMV and MMLV. AMV is a dimeric protein from the avian myeloblastosis virus and MMLV is derived from the Moloney murine leukemia virus and is a monomeric protein.

There are three ways to prime a reverse transcriptase reaction: **oligo-dT**, **random primers** or **assay-specific primers**. There are pros and cons for each type and in the end; Oligo-dT has been used extensively because it will prime primarily mRNAs. Furthermore it is possible that priming will be initiated within the PCR amplicon (the sequence amplified during PCR) by one of the random primers, eliminating that molecule from possible detection. A third alternative is the use of assay-specific primers. In this case, the reverse primer for the following PCR is used to prime cDNA synthesis in the reverse transcriptase reaction. This ensures, in theory, that every potential transcript targeted by the PCR assay will have a corresponding cDNA synthesized.

The amplification of any template is defined by four phases: 1 – baseline; 2 – exponential; 3 – linear and 4 – plateau. The baseline phase contains all the amplification that is below the level of detection of the real-time instrument. The exponential phase is comprised of the earliest detectible signal from a polymerase chain reaction where the amplification is proceeding at its maximal exponential rate. The linear phase is also a straight line but amplification is no longer 2 products from every 1 template molecule in each cycle

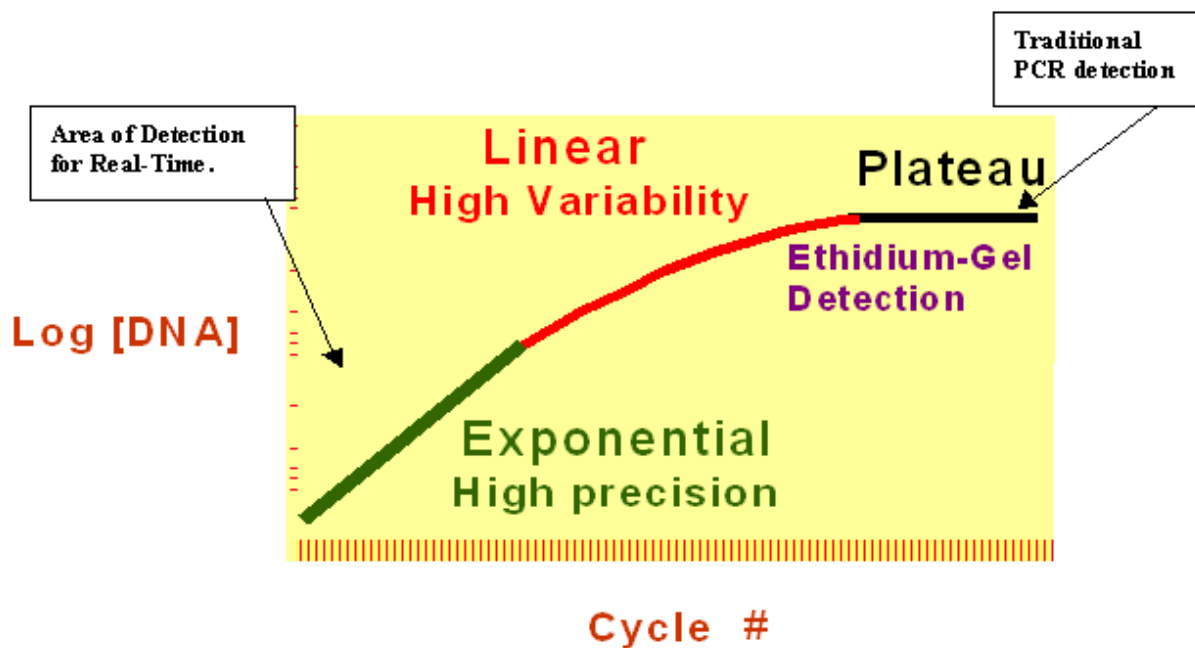


Figure 2: PCR phases in Log view

Detection chemistries:

There are two types model for detection of quantitative Real-Time PCR: Non specific form and specific form.

DNA binding dyes:

DNA binding dyes such as SYBR Green emit fluorescence when bound to dsDNA (Figure 3). As the double-stranded PCR product accumulates during cycling, more dye can bind and emit fluorescence. Thus, the fluorescence intensity increases proportionally to dsDNA concentration.

SYBR® Green I Detection Chemistry

High Flexibility and Low Cost

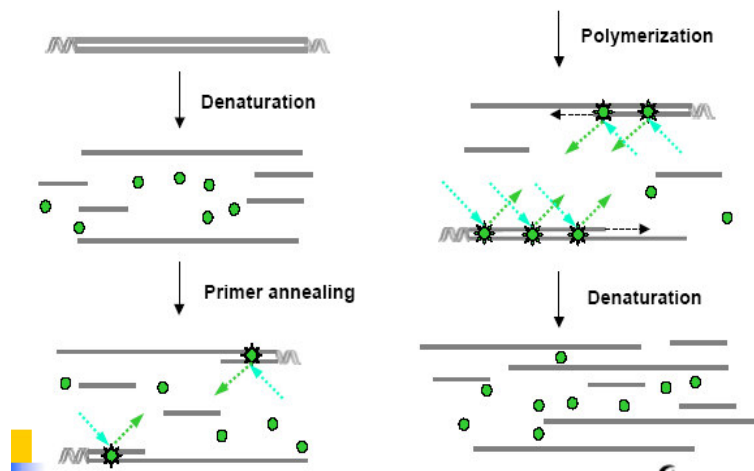
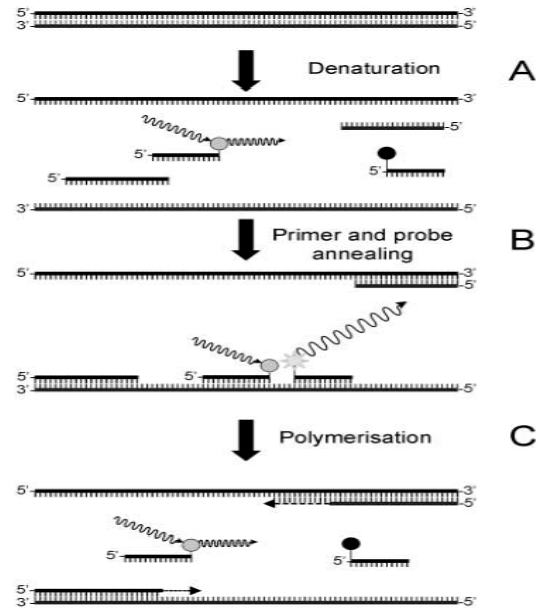


Figure 3: Graphical representation of the incorporation of SYBR® Green I dye resulting in an increase in fluorescent signal during the PCR.

Hybridisation probs:

In this technique one probe is labelled with a donor fluorochrome at the 3' end and a second –adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

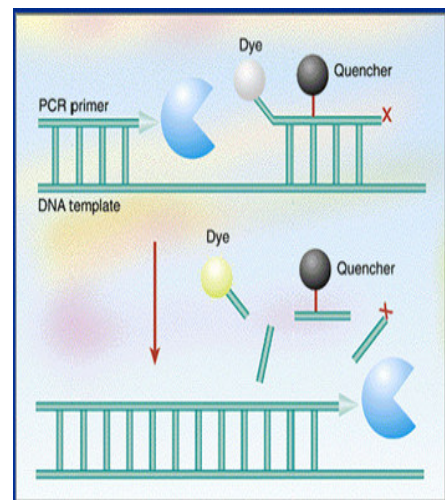
Figure 4: During the denaturation step, both hybridisation probes remain in solution and separate. Any emission from fluorescein is at 530 nm, and is disregarded by the detector. (B) During the annealing step, the probes hybridise in a head-to-tail arrangement, the two dyes come in close proximity and the emitted energy excites the second dye, which emits red fluorescent light at a longer wavelength. (C) At the polymerisation temperature, both probes return into solution and any emissions from fluorescein are ignored.



Hydrolysis Probes:

Hydrolysis probes, exemplified by the **TaqMan** chemistry, also known as 5' nuclease assay, fluoresce upon probe hydrolysis to detect PCR product accumulation (Figure 5). The sequence-specific probe is labeled with a reporter dye on the 5' end and a quencher dye on the 3' end, which allows the quencher to reduce the reporter fluorescence intensity by FRET when the probe is intact. While both hydrolysis and hybridization probes rely on FRET to alter the intensity of fluorescence emission, the energy transfer works in opposite manners in these two chemistries. When annealed to the target sequence, the bound and quenched probe will be degraded by the DNA polymerase's 5' nuclease ability during the extension step of the PCR. Probe degradation allows for separation of the reporter from the quencher dye, resulting in increased fluorescence emission.

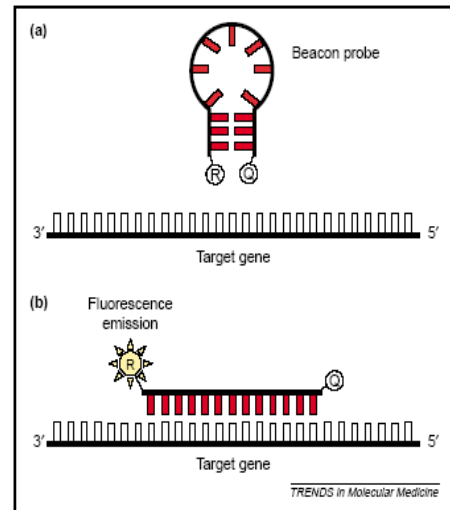
Figure 5: Upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable.



Hairpin Probes:

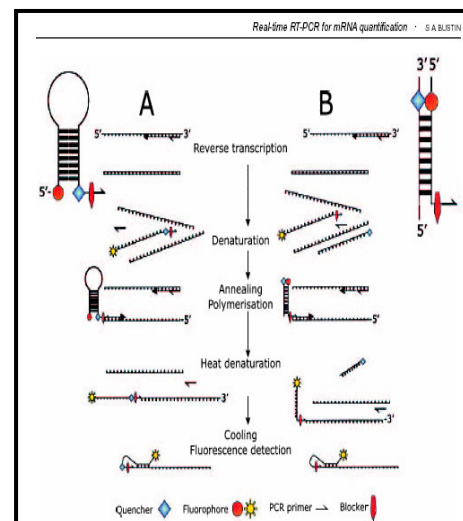
Molecular beacons. Consisting of a sequence-specific region (loop region) flanked by two inverted repeats, molecular beacons are the simplest hairpin probe (Figure 3D) (53). Reporter and quencher dyes are attached to each end of the molecule, causing a reduction in fluorescence emission via contact quenching (FRET) when the beacon is in hairpin formation (free in solution). When bound to the target, the beacon is in hairpin formation (free in solution). When bound to the target, the quencher and reporter are separated, allowing reporter emission.

Figure 6: Following the melting of the template and probe structures, probe molecules anneal to complementary sequences within the template as the temperature is lowered to the annealing set point. At this time, the molecular beacon is completely linear on the template, fully separating the reporter and quencher dyes and resulting in full reporter signal due to the loss of FRET.



Scorpions: Scorpions combine the detection probe with the upstream PCR primer and consist of a fluorophore on the 5' end, followed by a complementary stem-loop structure (also containing the specific probe sequence), quencher dye, DNA polymerase blocker (a nonamplifiable monomer that prevents DNA polymerase extension), and finally a PCR primer on the 3' end. The probe sequence contained within the hairpin allows the scorpion to anneal to the template strand, which separates the quencher for the fluorophore and results in increased fluorescence.

Figure 7: Real-Time PCR with Scorpions



Types of Real-Time quantification:

Absolute Quantitation:

Absolute quantitation uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values. This method assumes all standards and samples have approximately equal amplification efficiencies. In addition, the concentration of serial dilutions should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay. The PCR standard is a fragment of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or cRNA bearing the target sequence.

Relative Quantitation:

During relative quantitation, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator or **housekeeping gene**. Traditionally, genes thought to have stable expression have been employed as controls in gene expression assays. Many of the well-known housekeeping genes such as **GAPDH**, **β -actin**, **cyclophilin** and **28 S** or **18 S rRNA** have been shown to be affected by different treatments, biological processes, and even different tissues or cell types. When using a calibrator, the results are expressed as a target/reference ratio. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays. Depending on the method employed, these can yield different results and thus discrepant measures of standard error.

Comparative Ct ($2^{-\Delta\Delta Ct}$) method:

The comparative Ct method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample. While this method includes a correction for nonideal amplification efficiencies, the amplification kinetics of the target gene and reference gene assays must be approximately equal because different efficiencies will generate errors when using this method. Consequently, a validation assay must be performed where serial dilutions are assayed for the target and reference gene and the results plotted with the log input concentration for each dilution on the x-axis, and the difference in Ct (target-reference) for each dilution on the y-axis. Because the comparative Ct method does not require a standard curve, it is useful when assaying a large number of samples since all reaction wells are filled with sample reactions rather than standards.

Pfaffl model:

The Pfaffl model combines gene quantification and normalization into a single calculation. This model incorporates the amplification efficiencies of the target and reference (normalization) genes to correct for differences between the two assays. The relative expression software tool (REST[®]), which runs in Microsoft[®] Excel, automates data analysis using this model. REST uses the Pairwise Fixed Reallocation Randomization Test[®] to calculate result significance and will indicate if the reference gene used is suitable for normalization.

$$\text{Ratio} = \frac{2^{\Delta Ct (B_1 - B_2)}}{2^{\Delta Ct (C_1 - C_2)}}$$

B1: Target gene before treatment

B2: Target gene after treatment

C1: Reference gene before treatment

C2: Reference gene after treatment

Material description:

This kit has designed for expression analysis of 20 genes in order to evaluation of trastuzumab resistance in breast cancer patients using Real-Time PCR. Each tube contains RT Array™ SYBR Green mix (2X) and specific forward/reverse primers for related gene.

RT Array™ SYBR Green/ROX qPCR Master Mix (2X) is a ready-to-use solution optimized for quantitative real-time PCR and two-step RT-PCR. The master mix includes a Hot Start *Taq* DNA polymerase and dNTPs in an optimized PCR buffer. It contains SYBR Green dye and is supplemented with ROX passive reference dye. For each tube only template and dionized water need to be added.

The RT Array™ Hot Start *Taq* DNA polymerase in combination with an optimized buffer ensures PCR specificity and sensitivity. dUTP is included in the mix for optional carryover contamination control using uracil DNA glycosylase (UDG). The SYBR Green intercalating dye allows DNA detection and analysis without using sequence-specific probes. ROX serves as a passive reference dye. The use of RT Array™ SYBR Green/ROX qPCR Master Mix (2X) in real time PCR ensures reproducible, sensitive and specific quantification of genomic, plasmid, viral and cDNA templates. The master mix can be used with most real-time thermal cyclers, including instruments from Applied Biosystems, Eppendorf, Corbett, Bio-Rad and Roche.

Features:

- **Specificity** – RT Array™ Hot Start *Taq* DNA Polymerase and the optimized buffer eliminate non-specific amplification and formation of primer dimers.
- **Sensitivity** – detects low copy number targets.
- **ROX** – supplemented with ROX passive reference dye.
- **Wide linear range** – accurate quantification across 9 orders of magnitude.
- **Universal** – can be used on most real-time thermal cyclers.
- **Reproducibility and convenience** – ready-to-use 2X master mix.

PROCEDURES

1. RNA Extraction
2. cDNA synthesis
3. Real-Time PCR

1. RNA Extraction (strongly recommended using the Qiagene RNeasy kit, CinnaPure RNA extraction or Nucleu.exe RNA/DNA purification kit).

Optional: After RNA extraction, it is recommended to use DNase I, RNase free, to remove tract amounts of DNA from RNA preparations.

Note: RNA purity and integrity is essential for synthesis of full-length cDNA, which results in high quality RT-PCR products. Thus template RNA for RT-PCR has to be free of DNA contamination.

Removal of genomic DNA from RNA preparation

a. Add the following components to an RNase-free tube:

Component	Amount
RNA	0.1-5µg
10X reaction buffer with MgCl ₂	1 ul
DNase I, RNase-free	1 ul (1 u)
DEPC_treated water	To 10 ul
Total volume	10 ul

b. Incubate 30 min at 37 °C.

c. Add 1 ul of 25 mM EDTA and incubate 10 min at 70 °C. RNA hydrolyzes if heated in the absence of a chelating agent. Template RNA is ready for the first strand cDNA synthesis.

2. Synthesis of First Strand cDNA Suitable for PCR Amplification

2a. Prepare the following reaction mixture in a tube on ice:

template RNA:

total RNA	0.1-5µg
or poly(A)+ RNA	10ng-0.5µg
or specific RNA	0.01pg - 0.5µg

primer:

oligo(dT)18 primer (0.5µg/µl)	1µl
or random hexamer primer (0.2µg/µl)	1µl
or sequence-specific primer	15-20pmol

DEPC-treated water

to 12µl

Mix gently and spin down for 3-5sec. in a microcentrifuge.

2b. Incubate the mixture at 70°C for 5min, chill on ice and collect drops by brief centrifugation.

3b. Place the tube on ice and add the following components in the indicated order:

5x reaction buffer	4µl
RiboLock™ Ribonuclease Inhibitor (20u/µl)	1µl
10mM dNTP mix	2µl

Mix gently and collect drops by brief centrifugation.

4b. Incubate at 37°C for 5min (at 25°C for 5min if random hexamer primer is used).

5b. Add RevertAid™ M-MuLV Reverse Transcriptase (200u/µl) 1µl

Final volume 20µl

5b. Incubate the mixture at 42°C for 60min (incubate at 25°C for 10min and finally at 42°C for 60min if the random hexamer primer is used).

6b. Stop the reaction by heating at 70°C for 10min. Chill on ice. The first strand cDNA synthesized can be used directly for amplification by PCR.

The reverse transcription can be used immediately in second strand cDNA synthesis reactions or stored in - 20 °C for less than a week. For longer storage, -70 °C is recommended.

3. SYBR Green based Real-Time RT-PCR:

Real-time PCR with SYBR Green detection is just as specific and sensitive as probe-based detection, and also saves time and costs as there is no need to synthesize target-specific probes.

Follow the instructions to assemble RT-PCRs:

1. Prepare RT Array™ Mix on ice.

- Prepare 5–10% extra master mix.

- Negative controls: Include duplicate no-template controls using nuclease-free water in place of sample.

2. Add cDNA to each tube.

3. Distribute RT Array™ Mix to a PCR plate or to tubes.

No	Component	Amount	
1	RT Array™ Mix (2X)	12 μ l	
2	cDNA	0.5-1 μ l	
3	Nuclease-free water	To 20 μ l	
4	Total volume per reaction	20 μ l	

4. Perform thermal cycling and analyze the data.

Run the thermal cycle and analyze RT-PCR data according to the PCR instrument manufacturer's instructions.

Optional: melting curve analysis may be performed to verify the specificity and identity of the PCR product.

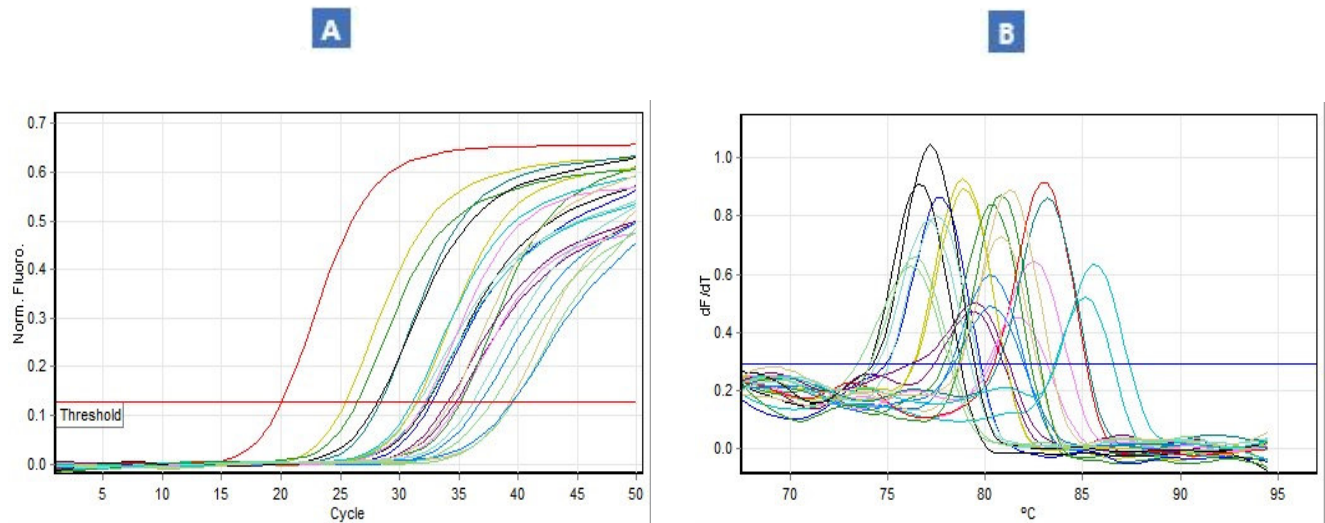


Figure 8: Quantification data for cycling A. Green and melting curve analysis

Troubleshooting

Problem	Possible cause	Solution
No amplification	You may have problems setting up the reaction conditions on your real-time PCR instrument.	Follow the specific reagent protocol. Be sure to use the appropriate hot-start temperature to activate the DNA polymerase. Use the appropriate time and/or temperature for amplification.
	Degradation of primers.	Check PCR primers for possible degradation on polyacrylamide gel.
	Pipetting error or missing reagent.	Repeat the PCR reaction; check the concentrations of template and primers; ensure proper storage conditions of all reagents. Make new serial dilutions of template DNA or RNA.
	Primer design is suboptimal.	Verify your primer design, use reputable primer design programs or validated pre-designed primers.
	PCR inhibitors presented in the reaction mixture.	Re-purify your template RNA.
	RT-qPCR: inhibition by excess volume of the RT reaction.	Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.
Late amplification	Annealing temperature is not optimal.	Optimize the Annealing temperature in 3 °C increments.
	RNA sample may be of poor quality and containing PCR inhibitors.	Perform RNA purification on a sample using a new purification method; Select the extraction method that can assure a high yield and purity of nucleic acid and a complete removal of PCR inhibitors. Further purify your samples- RNA with a significantly lower A_{260}/A_{280} ratio should be further purified by

Late amplification

Reverse transcription may not be optimal and the input amount of RNA may not be optimal for the size of RT reaction

Template sequence may not be enough.

Samples may be containing degraded RNA. Check RNA integrity in denaturing Agarose gel electrophoresis or Agilent 2100 bioanalyzer. Sharp 18S and 28S RNA bands should be visible after denaturing agarose gel electrophoresis of total eukaryotic RNA.

phenol-chloroform extraction, LiCl precipitation, or washing to remove residual salt.

Test your sample using a lower template concentration at which it is known that PCR inhibition does not affect the real-time PCR results.

Check type of reverse transcriptase and priming, quality of starting template, amount and quantification of starting RNA. It is fundamental to accurately quantities the starting template (RNA) to be reverse transcribed.

Gene of interest may not be expressed at a high level in sample type, so that increase the amount of template you use in the real-time PCR reaction.

Select an RNA isolation kit based on your sample type to maximize yield.

Pre-amplify your target sequences.

Maintain your laboratory workspace free of RNase contamination.

Store samples carefully prior to RNA isolation.

Disrupt samples completely to prevent RNA degradation and to increase RNA yield.

Choose an appropriate RNA isolation kit.

Store RNA in RNase-free solution.

Accurately quantities RNA and confirm that RNA is high integrity

<p>Late amplification</p>	<p>Assay design may not detect all of the splice variants for the gene of interest.</p>	<p>and purity. When you use TaqMan[®] Gene Expression Assay, check the alignment of the assay with target sequence.</p> <p>When you use custom TaqMan[®] Gene Expression Assay, submit a template sequence that is common to all of the alternative splicing variants.</p> <p>When assay designed by yourself, design the primer and probe on a template sequence that is common for all of the alternative splicing variants.</p>
<p>RT-PCR product longer than expected</p>	<p>RNA template is contaminated with DNA.</p>	<p>Perform DNase I digestion prior reverse transcription. To avoid amplification of genomic DNA, design PCR primers on exon-intron boundaries.</p>
<p>Amplification signal in non-template control</p>	<p>DNA contamination of reagents.</p> <p>RT-qPCR: RNA contaminated with genomic DNA.</p> <p>Primer-dimers.</p>	<p>Follow general guidelines to avoid carry over contamination</p> <p>Discard reagents and repeat with new reagents.</p> <p>Design primers on intron/exon boundaries, treat RNA sample with DNaseI, RNA free prior to reverse transcription.</p> <p>Use melting curve analysis to identify primer-dimers by the lower melting temperature compared to amplicon. If presence of dimers is confirmed:</p> <ul style="list-style-type: none"> • Redesign your primers according to recommendations or use validated pre-designed primers. • Optimize annealing temperature by increasing in 3°C increments.

Poor standard curve	Excessive amount of template.	Do not exceed maximum recommended amounts of template DNA (500 ng DNA for 25 μ l reaction).
	Suboptimal amount of template. RT-qPCR: inhibition by excess volume of the RT reaction.	Increase the amount of template, if possible. Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.
Non-uniform fluorescence intensity	Contamination of the thermal cycler.	Perform decontamination of your real-time cycler according to the supplier's instructions.
	Poor calibration of the thermal cycler.	Perform calibration of the real-time cycler according to the supplier's instructions.

References

1. <http://www.appliedbiosystems.com/absite/us/en/home/support/tutorials/realtime-pcr-trouble-shooting-guide.html>.
2. www.fermentas.com

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