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q RT PCR

Real-time PCR is increasingly being adopted by diagnostic laboratories, both in the human and veterinary medical fields. Since the first description of real-time PCR in 1992, the field has expanded rapidly, with significant improvements in chemistry, analysis of data, and availability and affordability of real-time PCR platforms. In Real Time PCR, the thermocycler is equipped with a fluorescence detector to monitor the buildup of product.

In RT-PCR, the RNA template is first **converted** into a complementary DNA (**cDNA**) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR.

rTth DNA polymerase

Tth DNA polymerase is a thermostable DNA polymerase derived from the thermophilic bacteria ***Thermus thermophilus* HB8**. The enzyme has a reverse transcriptase activity in addition to a 5'- 3' polymerase activity and a double strand specific 5'- 3' exonuclease activity in the presence of Mn^{++} ions. Therefore, this enzyme enables "one-step RT-PCR" including the reverse transcription and PCR steps. The features of rTth polymerase include:

1. Exhibits reverse transcriptase activity in the presence of Mn^{++} ions.
2. Effective for the amplification of GC-rich targets and crude samples.
3. Effective for reverse transcription of RNA with complicated secondary structure due to the reaction occurring at high temperature (i.e., $60^{o}C$).

Nucleic Acid Sample Preparation

DNA

There are many methods used to purify genomic and plasmid DNA, depending on sample type, sample amount, and budget. Pure, intact DNA is highly recommended for QPCR analysis. DNA purity can be measured by spectrophotometer. Intact DNA can be verified using gel electrophoresis and visual analysis of the gel image for lack of degradation.

RNA

Purification of undegraded RNA from biological sources is central to any investigation of gene expression and regulation studies, including those based on qRT-PCR. Traces of extraction components, **DNA and protein contamination, and RNA degradation** can dramatically affect the quality of qRT-PCR data. Therefore, its highly recommend starting with high quality, intact, DNA- free RNA.

Because **ribonucleases** are found in virtually all tissues and are often the leading cause of RNA degradation during RNA isolation, they represent a major challenge in isolating high- quality RNA. To avoid degradation caused by ribonucleases, RNA isolation protocols should involve cellular lysis tissue disruption with **guanidine isothiocyanate**, a strong protein denaturant. Complete **lysis and homogenization** is important in order to ensure that ribonuclease activity is eliminated. Additionally, proper handling of tissue prior to homogenization and RNA isolation, as well as using sterile RNase- free reagents and plastic ware, will ensure recovery of full length, intact RNA.

Total RNA vs. mRNA

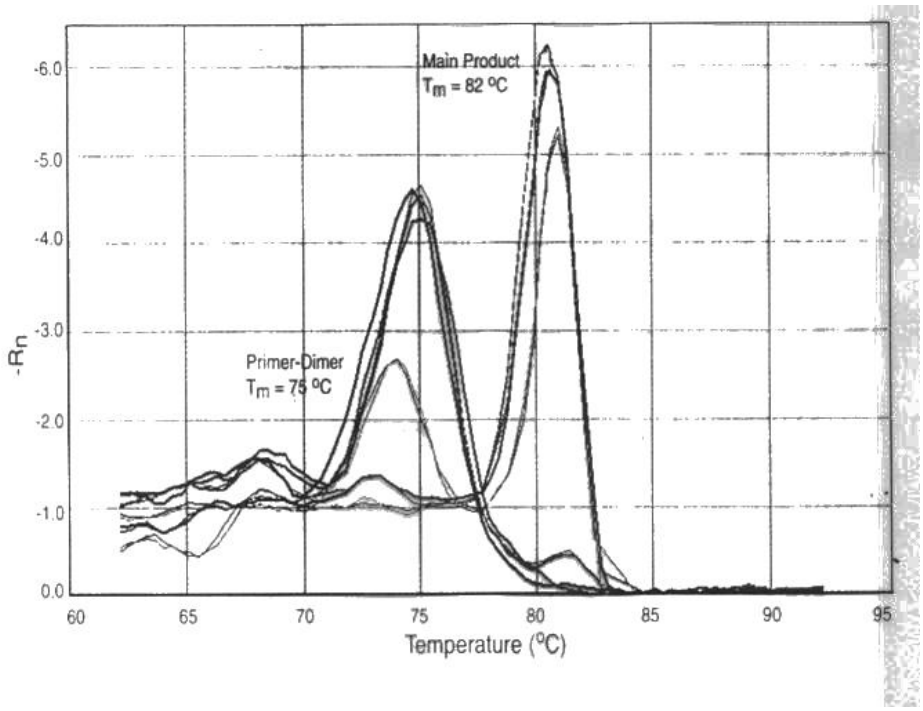
Total RNA includes the full complement of RNA: mRNA, miRNA, tRNA, and rRNA. Messenger RNA (mRNA) constitutes 1–5% of the total RNA depending on a variety of factors (e.g., tissue type, disease state, etc.). Whether to use total RNA or mRNA is typically dependent on the **preferences** of the researcher. Several publications and a majority of microarray researchers support the use of total RNA to achieve quality gene expression data.

mRNA can be **difficult** to isolate, depending on tissue type, and mRNA isolation techniques produce, very low yields. However, using mRNA can increase detection sensitivity.

Detection methods

SYBR Green

A method that is straight forward, but **lacks specificity** for the target sequence, is to incorporating a fluorescent dye that detects double stranded DNA into the PCR reaction. Although some initial real time experiments employed ethidium bromide for detection, now a more sensitive fluorophore named SYBR Green is used for this purpose, the dye fluoresces after associating with double stranded DNA, so it directly detects the buildup of product. Since this detection method is not sequence-specific, one is vulnerable to being mislead by amplification of spurious products. The instrument can be programmed to do a denaturation curve on the sample after amplification, which may provide a warning that the amplified product is very short as might occur for **a primer dimer**.

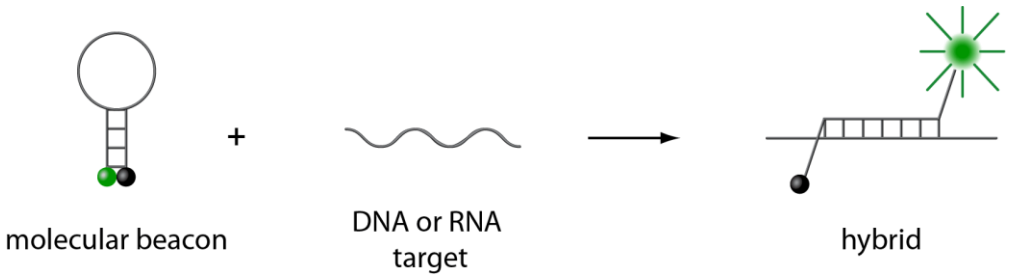


Denaturation curves: The Y axis is actually - d (Rn)/dT, where Rn is the ratio of the SYBR green signal to a reference dye that does not change fluorescence during the reaction. The peak therefore appears at the midpoint of the melting transition (the point of maximum slope).

Sequence-specific detection

The available sequence specific detection methods involve an **oligo probe** with a **fluorophore** on one end and a **quencher** on the other. These probes are added in addition to the two PCR primers, and are designed to fluoresce if they can hybridize to the accumulating PCR product. In order to **prevent** the probe from acting as a primer, they are phosphorylated at the 3' end. There are several variations on two general formats:

One type of probe fluoresces directly upon hybridization. The **molecular beacon** web site has extensive documentation: (http://www.molecular-beacons.org/).



The oligos with a fluorophore on one end and a quencher on the other has the ends in a hairpin so that usually the quencher is brought close to the fluorophore and quenches it. If the oligo is hybridized to another nucleic acid, the quencher is separated from the fluorophore and fluorescence increases. Hybridization is therefore detected directly by fluorescence without having to remove the unhybridized oligo. This kind of probe can also be used for quantitative in-solution hybridization or as a substrate on a microchip or microarray.

Another type of probe is called a **hydrolysis probe (TaqMan)**. These are also called "**fluorogenic probes**". The fluorophore is at the 5' end and the quencher at the 3' end. In this case the probe is fully complementary to the intended product and is short enough that fluorescence is quenched as long as the probe is intact. This probe **hybridizes** to the template in the path of the polymerase. Upon colliding with the probe, the polymerase hydrolyzes the 5' nucleotides **releasing** the fluorophore which is now unquenched. When designing hydrolysis probes, one is advised to give it an extra 5°C over the Tm of the primers to account for any destabilizing effect of the dyes. Some of the available fluorophores are quenched by guanine, so one is advised to not put a G at the 3' end of the probe. Also one is advised to choose the strand that puts more C's than G's in the probe. A common strategy to avoid detection of genomic DNA during a reverse-transcription PCR assay is to make the probe cross an intron exon boundary. Finally, more than one probe with different fluorophores may be used to detect the same amplicon.