Real-time PCR

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences within a DNA or cDNA template can be copied, or "amplified", many thousand- to a million-fold using sequence specific oligonucleotides, heat stable DNA polymerase, and thermal cycling. In traditional (endpoint) PCR, detection and quantification of the amplified sequence are performed at the end of the reaction after the last PCR cycle, and involve post-PCR analysis such as gel electrophoresis and image analysis. In real-time quantitative PCR, PCR product is measured at each cycle. By monitoring reactions during the exponential amplification phase of the reaction, users can determine the initial quantity of target with great precision. PCR theoretically amplifies DNA exponentially, doubling the number of target molecules with each amplification cycle. When it was first developed, scientists reasoned that the number of cycles and the amount of PCR end-product could be used to calculate the initial quantity of genetic material by comparison with a known standard. To address the need for robust quantification, the technique of real-time quantitative PCR was developed and end-point PCR is used mostly to amplify specific DNA for sequencing, cloning, and use in other molecular biology techniques. 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

The advantages of real-time PCR include:

• Ability to monitor the progress of the PCR reaction as it occurs in real time

• Ability to precisely measure the amount of amplicon at each cycle, which allows highly accurate quantification of the amount of starting material in samples

• An increased dynamic range of detection

• Amplification and detection occurs in a single tube, eliminating post-PCR manipulations

Over the past several years, real-time PCR has become the leading tool for the detection and quantification of DNA or RNA. Using these techniques, you can achieve precise detection that is accurate within a two-fold range, with a dynamic range of input material covering 6 to 8 orders of magnitude.

Overview of real-time PCR

This section provides an overview of the steps involved in performing real-time PCR. Real-time PCR is a variation of the standard PCR technique that is commonly used to quantify DNA or RNA in a sample. Using sequence-specific primers, the number of copies of a particular DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles; if the sequence is scarce, amplification is observed in later cycles. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing the thermal cycling needed for the 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

Two-step qRT-PCR

Two-step quantitative reverse transcriptase PCR (qRT-PCR) starts with the reverse transcription of either total RNA or poly(A)+ RNA into cDNA using a reverse transcriptase (RT). This first-strand cDNA

synthesis reaction can be primed using random primers, oligo(dT), or gene-specific primers (GSPs). To give an equal representation of all targets in real-time PCR applications and to avoid the 3_ bias of oligo (dT) primers, many researchers use random primers or a mixture of oligo(dT) and random primers. The temperature used for cDNA synthesis depends on the RT enzyme chosen. Next, approximately 10% of the cDNA is transferred to a separate tube for the real-time PCR reaction.

One-step qRT-PCR

One-step qRT-PCR combines the first-strand cDNA synthesis reaction and real-time PCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination. Gene-specific primers (GSP) are required. This is because using oligo(dT) or random primers will generate nonspecific products in the one-step procedure and reduce the amount of product of interest.

Real-time PCR components

1- DNA polymerase

2-Reverse transcriptase

The reverse transcriptase (RT) is as critical to the success of qRT-PCR as the DNA polymerase. It is important to choose an RT that not only provides high yields of full-length cDNA, but also has good activity at high temperatures. High-temperature performance is also very important for denaturation of RNA with secondary structure. In one-step qRT-PCR, an RT that retains its activity at higher temperatures allows you to use a GSP with a high melting temperature (Tm), increasing specificity and reducing background.

3- dNTPs

4- Magnesium

5- Template

Use 10 to 1,000 copies of template nucleic acid for each real-time PCR reaction. This is equivalent to approximately 100 pg to 1 μg of genomic DNA, or cDNA generated from 1 pg to 100 ng of total RNA. Excess template may bring with it higher contaminant levels that can greatly reduce PCR efficiency. Depending on the specificity of the PCR primers for cDNA rather than genomic DNA, it may be important to treat RNA templates to reduce the chance that they contain genomic DNA contamination. One option is to treat the template with DNase I.

Ultrapure, intact RNA is essential for full-length, high-quality cDNA synthesis and may be important for accurate mRNA quantification. RNA should be devoid of any RNase contamination, and aseptic conditions should be maintained. Total RNA typically works well in qRT-PCR; isolation of mRNA is typically not necessary, although it may improve the yield of specific cDNAs.

Good experimental technique

Do not underestimate the importance of good laboratory technique. It is best to use dedicated equipment and solutions for each stage of the reactions, from preparation of the template to post-PCR analysis. The use of aerosol-barrier tips and screwcap tubes can help decrease cross-contamination problems. To obtain tight data from replicates (ideally, triplicates), prepare a master mix that contains all the reaction components except sample. The use of a master mix reduces the number of pipetting steps and, consequently, reduces the chances of cross-well contamination and other pipetting errors.

Real-time PCR fluorescence detection systems

Real-time fluorescent PCR chemistries

Many real-time fluorescent PCR chemistries exist, but the most widely used are

1- The **5'nuclease assay;** the most well-known of which is the TaqManR Assay **and**

2-SYBRR Green dye-based assays.

1- The 5'nuclease assay is named for the 5'nuclease activity associated with Taq DNA polymerase. The 5'nuclease domain has the ability to degrade DNA bound to the template, downstream of DNA synthesis. A second key element in the 5'nuclease assay is a phenomenon called FRET: fluorescent resonance energy transfer. In FRET, the emissions of a fluorescent dye can be strongly reduced by the presence of another dye, often called the quencher, in close proximity FRET can be illustrated by two fluorescent dyes: green and red. The green fluorescent dye has a higher energy of emission compared to the red, because green light has a shorter wavelength compared to red. If the red dye is in close proximity to the green dye, excitation of the green dye will cause the green emission energy to be transferred to the red dye. In other words, energy is being transferred from a higher to a lower level. Consequently, the signal from the green dye will be suppressed or "quenched." However, if the two dyes are not in close proximity, FRET cannot occur, allowing the green dye to emit its full signal. A 5' nuclease assay for target detection or quantification typically consists of two PCR primers and a TaqManR probe. Before PCR begins, the TaqManR probe is intact and has a degree of flexibility. While the probe is intact, the

reporter and quencher have a natural affinity for each other, allowing FRET to occur. The reporter signal is quenched prior to PCR.

5' nuclease assay specificity

Assay specificity is the degree that the assay include signal from the target and excludes signal from non-target in the results. Specificity is arguably the most important aspect of any assay. The greatest threat to assay specificity for 5' nuclease assays is homologs. Homologs are genes similar in sequence to that of the target, but they are not the intended target of the assay. Homologs are extremely common within species and across related species. 5' nuclease assays offer two tools for specificity: primers and probes. For maximal impact on specificity by primers, a mismatch between the target and homolog must be positioned at the 3' most base of the primer. A mismatch further away from the 3' end may have little to no impact on specificity. In contrast, mismatches across most of the length of a MGB probe, which is shorter than a TaqManR probe, can have a strong impact on specificity' TaqManR MGB probes are stronger tools for specificity than primers. For example, a one- or two-base random mismatch in the primer binding site will very likely allow the DNA polymerase to extend the primer bound to the homolog with high efficiency. A one or two base extension by DNA polymerase will stabilize the primer bound to the homolog, so it is just as stably bound as primer bound to the intended, fully complementary target. At that point, there is nothing to prevent the DNA polymerase from continuing synthesis to produce a copy of the homolog. In contrast, mismatches on the 5' end of the TaqManR probe binding site cannot be stabilized by the DNA polymerase due to the quencher block on the 3'end. Mismatches in a TaqManR MGB probe binding site will reduce how tightly the probe is bound, so that instead of cleavage, the intact probe is displaced. The intact probe returns to its quenched configuration, so that when data is collected at the end of the PCR cycle, signal is produced from the target, but not the homolog, even though the homolog is being amplified. In addition to homologs, PCR may also amplify non-specific products, produced by primers binding to seemingly random locations in the sample DNA or sometimes to themselves in so-called "primer-dimers". Since non-specific products are unrelated to the target, they do not have TaqManR probe binding sites, and thus are not seen in the real-time PCR data.

TaqMan® probe types

TaqManR probes may be divided into two types: MGB and non-MGB. The first TaqManR probes could be classified as "non-MGB." They used a dye called TAMRA™ dye as the quencher. Early in the development of real-time PCR, extensive testing revealed that TaqManR probes required an annealing temperature significantly higher than that of PCR primers to allow cleavage to take place. TaqManR

probes were therefore longer than primers. A one-base mismatch in such long probes had a relatively mild effect on probe binding, allowing cleavage to take place. However, for many applications involving high genetic complexity, such as eukaryotic gene expression and SNPs, a higher degree of specificity was desirable. TaqManR MGB probes were a later refinement of the TaqManR probe technology. TaqManR MGB probes possess a minor-groove binding (MGB) molecule on the 3_end. Where the probe binds to the target, a short minor groove is formed in the DNA, allowing the MGB molecule to bind and increase the melting temperature; thus strengthening probe binding. Consequently, TaqManR MGB probes can be much shorter than PCR primers. Because of the MGB moiety, these probes can be shorter than TaqManR probes and still achieve a high melting temperature. This enables TaqManR MGB probes to bind to the target more specifically than primers at higher temperatures. With the shorter probe size, a one-base mismatch has a much greater impact on TaqManR MGB probe binding. And because of this higher level of specificity, TaqManR MGB probes are recommended for most genetic complexity applications.

2- SYBR® Green dye

SYBRR Green I dye is a fluorescent DNA binding dye, binding to the minor groove of any double-stranded DNA. Excitation of DNA-bound SYBRR Green dye produces a much stronger fluorescent signal compared to unbound dye. A SYBRR Green dye based assay typically consists of two PCR primers. Under ideal conditions, a SYBRR Green assay follows a similar amplification pattern as a TaqManR probe-based assay. In the early PCR cycles, a horizontal baseline is observed. If the target was present in the sample, sufficient accumulated PCR product will be produced at some point so that amplification signal becomes visible.

SYBR® Green assay specificity

Assay specificity testing is important for all assays, but especially for those most vulnerable to specificity problems. SYBRR Green assays do not benefit from the specificity of a TaqManR probe, making them more vulnerable to specificity problems. SYBRR Green dye will bind to any amplified product, target or non-target, and all such signals are summed, producing a single amplification plot. SYBRR Green amplification plot shape cannot be used to assess specificity. Plots usually have the same appearance, whether the amplification consists of target, non-target, or a mixture. The fact that a SYBRR Green assay produced amplification should not be automatically taken to mean the majority of any of the signal is derived from target. Since amplification of non-target can vary sample-to sample, at least one type of specificity assessment should be performed for every SYBRR Green reaction. Most commonly, this ongoing assessment is the dissociation analysis.

Real-time PCR instrumentation

Many different models of real-time PCR instruments are available. Each model must have an excitation source, which excites the fluorescent dyes, and a detector to detect the fluorescent emissions. In addition, each model must have a thermal cycler. The thermal block may be either fixed, as in the StepOnePlus ^{R} system or user interchangeable, as in the ViiA™ 7 system and QuantStudio™ 12K Flex system. Blocks are available to accept a variety of PCR reaction vessels: 48-well plates, 96-well plates, 384-well plates,384-microwell cards, 3072-through-hole plates, etc. All real-time PCR instruments also come with software for data collection and analysis.

Dye differentiation

Most real-time PCR reactions contain multiple dyes, e.g., one or more reporter dyes, in some cases a quencher dye, and, very often, a passive reference dye. Multiple dyes in the same well can be measured independently, either through optimized combinations of excitation and emission filters or through a process called multicomponenting. Multicomponenting is a mathematical method to measure dye intensity for each dye in the reaction. Multicomponenting offers the benefits of easy correction for dye designation errors, refreshing optical performance to factory standard without hardware adjustment, and provides a source of troubleshooting information.