Essentials of Real Time PCR

About Real-Time PCR Assays

Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In contrast, an endpoint assay (also called a "plate read assay") measures the amount of accumulated PCR product at the end of the PCR cycle.

About Sequence Detection Chemistries

Overview Applied Biosystems has developed two types of chemistries used to detect PCR products using Sequence Detection Systems (SDS) instruments:

- TaqMan® chemistry (also known as "fluorogenic 5´ nuclease chemistry")
- SYBR® Green I dye chemistry

TaqMan® Chemistry

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles.

Assay Types that Use TaqMan Chemistry

The TaqMan chemistry can be used for the following assay types:

- Quantitation, including:
  - One-step RT-PCR for RNA quantitation
  - Two-step RT-PCR for RNA quantitation
  - DNA/cDNA quantitation
- Allelic Discrimination
- Plus/Minus using an IPC

SYBR Green I Dye Chemistry

The SYBR Green I dye chemistry uses SYBR Green I dye, a highly specific, double-stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles.

The most important difference between the TaqMan and SYBR Green I dye chemistries is that the SYBR Green I dye chemistry will detect all double-stranded DNA, including non-specific reaction products. A well-optimized reaction is therefore essential for accurate results.

Assay Types that Use SYBR Green I Dye Chemistry

The SYBR Green I dye chemistry can be used for the following assay types:

- Quantitation, including:
  - One-step RT-PCR for RNA quantitation
  - Two-step RT-PCR for RNA quantitation
  - DNA/cDNA quantitation

TaqMan Chemistry

Background

Initially, intercalator dyes were used to measure real-time PCR products. The primary disadvantage to these type of probes is that they detect accumulation of both specific and non-specific PCR products.

Development of TaqMan Chemistry

Real-time systems for PCR were improved by the introduction of fluorogenic-labeled probes that use the 5´ nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products. The development of fluorogenic labeled probes also made it possible to eliminate post-PCR processing for the analysis of probe degradation.

How TaqMan Sequence Detection Chemistry Works

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. Here's how it works:

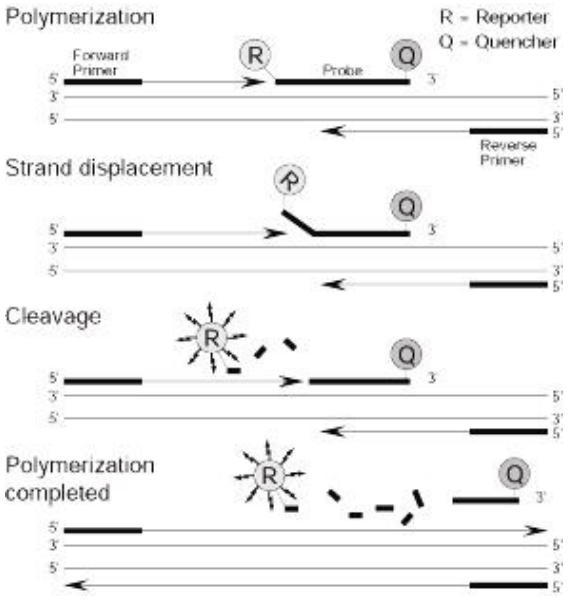
Step Process

1. An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5´ end and a quencher dye on the 3´ end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space.

2. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of Taq DNA polymerase as this primer is extended.

3. This cleavage of the probe:
   - Separates the reporter dye from the quencher dye, increasing the reporter dye signal.
   - Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibitthe overall PCR process.

4. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.



Two Types of TaqMan® Probes

Applied Biosystems offers two types of TaqMan probes:

- TaqMan® probes (with TAMRA™ dye as the quencher dye)
- TaqMan® MGB probes

TaqMan® MGB Probes Recommended for Allelic Discrimination Assays

Applied Biosystems recommends the general use of TaqMan MGB probes for allelic discrimination assays, especially when conventional TaqMan probes exceed 30 nucleotides. The TaqMan MGB probes contain:

- A nonfluorescent quencher at the 3′ end - The SDS instruments can measure the reporter dye contributions more precisely because the quencher does not fluoresce.
- A minor groove binder at the 3′ end - The minor groove binder increases the melting temperature (T_m) of probes, allowing the use of shorter probes.

Consequently, the TaqMan MGB probes exhibit greater differences in T_m values between matched and mismatched probes, which provides more accurate allelic discrimination.

Advantages of TaqMan Chemistry

The advantages of the TaqMan chemistry are as follows:

- Specific hybridization between probe and target is required to generate fluorescent signal
- Probes can be labeled with different, distinguishable reporter dyes, which allows amplification of two distinct sequences in one reaction tube
- Post-PCR processing is eliminated, which reduces assay labor and material costs

Disadvantage of TaqMan Chemistry

The primary disadvantage of the TaqMan chemistry is that the synthesis of different probes is required for different sequences.

SYBR® Green I Dye Chemistry

Background

Small molecules that bind to double-stranded DNA can be divided into two classes:

- Intercalators
- Minor-groove binders

Regardless of the binding method, there are two requirements for a DNA binding dye for real-time detection of PCR:

- Increased fluorescence when bound to double-stranded DNA
- No inhibition of PCR

Applied Biosystems has developed conditions that permit the use of the SYBR Green I dye in PCR without PCR inhibition and increased sensitivity of detection compared to ethidium bromide.

How the SYBR Green I Dye Chemistry Works

The SYBR Green I dye chemistry uses the SYBR Green I dye to detect polymerase chain reaction (PCR) products by binding to double-stranded DNA formed during PCR. Here's how it works:

Step Process

1. When SYBR Green I dye is added to a sample, it immediately binds to all double-stranded DNA present in the sample.
2. During the PCR, AmpliTaq Gold® DNA Polymerase amplifies the target sequence, which creates the PCR products, or "amplicons."
3. The SYBR Green I dye then binds to each new copy of double-stranded DNA.
4. As the PCR progresses, more amplicons are created. Since the SYBR Green I dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportionate to the amount of PCR product produced.

Advantages of SYBR Green I Dye

The advantages of the SYBR Green I dye chemistry are as follows:

- It can be used to monitor the amplification of any double-stranded DNA sequence.
- No probe is required, which reduces assay setup and running costs.

Disadvantage of SYBR Green I Dye

The primary disadvantage of the SYBR Green I dye chemistry is that it may generate false positive signals; i.e., because the SYBR Green I dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences.

Additional Consideration

Another aspect of using DNA binding dyes is that multiple dyes bind to a single amplified molecule. This increases the sensitivity for detecting amplification products. A consequence of multiple dye binding is that the amount of signal is dependent on the mass of double-stranded DNA produced in the reaction. Thus, if the amplification efficiencies are the same, amplification of a longer product will generate more signal than a shorter one. This is in contrast to the use of a fluorogenic probe, in which a single fluorophore is released from quenching for each amplified molecule synthesized, regardless of its length.

About Quantitation Assays

What Is a Quantitation Assay?

A Quantitation Assay is a real-time PCR assay. It measures (quantitates) the amount of a nucleic acid target during each amplification cycle of the PCR. The target may be DNA, cDNA, or RNA. There are three types of Quantitation Assays discussed in this chemistry guide:

- DNA/cDNA quantitation
- RNA quantitation using one-step reverse transcription polymerase chain reaction (RT-PCR)
- RNA quantitation using two-step RT-PCR

Terms Used in Quantitation Analysis

Amplicon A short segment of DNA generated by the PCR process
Amplification plot The plot of fluorescence signal versus cycle number
Baseline The initial cycles of PCR, in which there is little change in fluorescence signal
C_T (threshold cycle) The fractional cycle number at which the fluorescence passes the fixed threshold

NTC (no template control) - A sample that does not contain template. It is used to verify amplification quality.
Nucleic acid target (also called "target template") - DNA or RNA sequence that you wish to amplify
Passive reference A dye that provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for forestallment fluctuations caused by changes in concentration or volume. A passive reference dye is included in all SDS PCR reagent kits.
R_n (normalized reporter) The fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye
R_{n+} The R_n value of a reaction containing all components, including the template
R_{n-} The R_n value of an un-reacted sample. The R_{n-} value can be obtained from:

- The early cycles of a real-time PCR run (those cycles prior to a detectable increase in fluorescence), OR
- A reaction that does not contain any template

ΔR_n (delta R_n) The magnitude of the signal generated by the given set of PCR conditions. The ΔR_n value is determined by the following formula:

$$(R_{n+}) - (R_{n-})$$

Standard A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, you create a standard curve from which you can extrapolate the quantity of an unknown sample.
Threshold The average standard deviation of R_n for the early PCR cycles, multiplied by an adjustable factor. The threshold should be set in the region associated with an exponential growth of PCR product.
Unknown A sample containing an unknown quantity of template. This is the sample whose quantity you want to determine.

How Real-Time PCR Quantitation Assays Work

In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated target. A fixed fluorescence threshold can be set above the baseline. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Absolute vs. Relative Quantitation

Overview

When calculating the results of your quantitation assays, you can use either absolute or relative quantitation.

What is Absolute Quantitation?

The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a standard curve.

Example

Absolute quantitation might be used to correlate viral copy number with a disease state. It is of interest to the researcher to know the exact copy number of the target RNA in a given biological sample in order to monitor the progress of the disease.

Absolute quantitation can be performed with data from all of the SDS instruments, however, the absolute quantities of the standards must first be known by some independent means.

What is Relative Quantitation?

A relative quantitation assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample).

Example

Relative quantitation might be used to measure gene expression in response to a chemical (drug). The level of gene expression of a particular gene of interest in a chemically treated sample would be compared relative to the level of gene expression an untreated sample.

Calculation Methods for Relative Quantitation

Relative quantitation can be performed with data from all of the SDS instruments. The calculation methods used for relative quantitation are:

- Standard curve method
- Comparative C_T method

Determining Which Method to Use

All methods can give equivalent results. When determining which method you want to use, note the following:

- Running the target and endogenous control amplifications in separate tubes and using the standard curve method of analysis requires the least amount of optimization and validation.
- To use the comparative C_T method, a validation experiment must be run to show that the efficiencies of the target and endogenous control amplifications are approximately equal. The advantage of using the comparative C_T method is that the need for a standard curve is eliminated. This increases throughput because wells no longer need to be used for the standard curve samples. It also eliminates the adverse effect of any dilution errors made in creating the standard curve samples.
- To amplify the target and endogenous control in the same tube, limiting primer concentrations must be identified and shown not to affect C_T values. By running the two reactions in the same tube, throughput is increased and the effects of pipetting errors are reduced.

Terms Used

The following terms are used in this discussion of absolute and relative quantitation:

Standard A sample of known concentration used to construct a standard curve.
Reference A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers and probe.
Endogenous control – This is an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.
Exogenous control – This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. Whether or not an active reference is used, it is important to use a passive reference containing the dye ROX in order to normalize for non-PCR-related fluctuations in fluorescence signal.
Normalized amount of target
A unitless number that can be used to compare the relative amount of target in different samples.
Calibrator A sample used as the basis for comparative results.

Standard Curve Method for Relative Quantitation

Overview

It is easy to prepare standard curves for relative quantitation because quantity is expressed relative to some basis sample, such as the calibrator. For all experimental samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1× sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. As an example, in a study of drug effects on expression, the untreated control would be an appropriate calibrator.

Critical Guidelines

The guidelines below are critical for proper use of the standard curve method for relative quantitation:

- It is important that stock RNA or DNA be accurately diluted, but the units used to express this dilution are irrelevant. If two-fold dilutions of a total RNA preparation from a control cell line are used to construct a standard curve, the units could be the dilution values 1, 0.5, 0.25, 0.125, and so on. By using the same stock RNA or DNA to prepare standard curves for multiple plates, the relative quantities determined can be compared across the plates.
- It is possible to use a DNA standard curve for relative quantitation of RNA. Doing this requires the assumption that the reverse transcription efficiency of the target is the same in all samples, but the exact value of this efficiency need not be known.
- For quantitation normalized to an endogenous control, standard curves are prepared for both the target and the endogenous reference. For each experimental sample, the amount of target and endogenous reference is determined from the appropriate standard curve. Then, the target amount is divided by the endogenous reference amount to obtain a normalized target value. Again, one of the experimental samples is the calibrator, or 1× sample. Each of the normalized target values is divided by the calibrator normalized target value to generate the relative expression levels.

Endogenous Control

Amplification of an endogenous control may be performed to standardize the amount of sample RNA or DNA added to a reaction. For the quantitation of gene expression, researchers have used ß-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA (rRNA), or other RNAs as an endogenous control.

Standards

Because the sample quantity is divided by the calibrator quantity, the unit from the standard curve drops out. Thus, all that is required of the standards is that their relative dilutions be known. For relative quantitation, this means any stock RNA or DNA containing the appropriate target can be used to prepare standards.

Comparative C_T method for Relative Quantitation

The comparative C_T method is simlar to that standard curve method, except it uses the arithmetic formula, $\mathbf{2}^{-\Delta\Delta C}_{\mathbf{T}}$ to achieve the same result for relative quantitation.

Arithmetic Formulas:

For the comparative C_T method to be valid, the efficiency of the target amplification (your gene of interest) and the efficiency of the reference amplification (your endogenous control) must be approximately equal.

For more information on using the comparative C_T method for relative quantitation, please refer to User Bulletin #2: Relative Quantitation of Gene Expression (PN 4303859).

Standard Curve Method for Absolute Quantitation

Overview

The standard curve method for absolute quantitation is similar to the standard curve method for relative quantitation, except the absolute quantities of the standards must first be known by some independent means.

Critical Guidelines

The guidelines below are critical for proper use of the standard curve method for absolute quantitation:

- It is important that the DNA or RNA be a single, pure species. For example, plasmid DNA prepared from E. coli often is contaminated with RNA, which increases the A_{260} measurement and inflates the copy number determined for the plasmid.
- Accurate pipetting is required because the standards must be diluted over several orders of magnitude. Plasmid DNA or in vitro transcribed RNA must be concentrated in order to measure an accurate A_{260} value. This concentrated DNA or RNA must then be diluted 10^6–10^{12}-fold to be at a concentration similar to the target in biological samples.
- The stability of the diluted standards must be considered, especially for RNA. Divide diluted standards into small aliquots, store at –80 °C, and thaw only once before use.
- It is generally not possible to use DNA as a standard for absolute quantitation of RNA because there is no control for the efficiency of the reverse transcription step.

Standards

The absolute quantities of the standards must first be known by some independent means. Plasmid DNA and in vitro transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A_{260} and converted to the number of copies using the molecular weight of the DNA or RNA.