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Introduction to Gene Expression

Getting Started Guide

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Introduction to Gene Expression Getting Started Guide

What is gene expression?

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is a structural or housekeeping RNA. In addition, small non-coding RNAs (miRNAs, piRNA) and various classes of long non-coding RNAs are involved in a variety of regulatory functions (Taft, R.; Pang, K.C.; Mercer, T.R.; Dinger, M.; and Mattick, J.S. 2010).

When studying gene expression with real-time polymerase chain reaction (PCR), scientists usually investigate changes – increases or decreases – in the expression of a particular gene or set of genes by measuring the abundance of the gene-specific transcript. The investigation monitors the response of a gene to treatment with a compound or drug of interest, under a defined set of conditions. Gene expression studies can also involve looking at profiles or patterns of expression of several genes. Whether quantitating changes in expression levels or looking at overall patterns of expression, real-time PCR is used by most scientists performing gene expression.

Real-Time PCR concepts

Real-time PCR —also known as quantitative reverse transcription PCR (RT-qPCR), and quantitative PCR (qPCR)—is one of the most powerful and sensitive gene analysis techniques available. It is used for a broad range of applications including quantitative gene expression analysis, genotyping, copy number, drug target validation, biomarker discovery, pathogen detection, and measuring RNA interference.

Real-time PCR measures PCR amplification as it occurs, so that it is possible to determine the starting concentration of nucleic acid. In traditional PCR, which is based on end-point detection, results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid.

Every real-time PCR contains a fluorescent reporter molecule—a TaqMan® probe or SYBR® Green dye, for example—to monitor the accumulation of PCR product. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore.

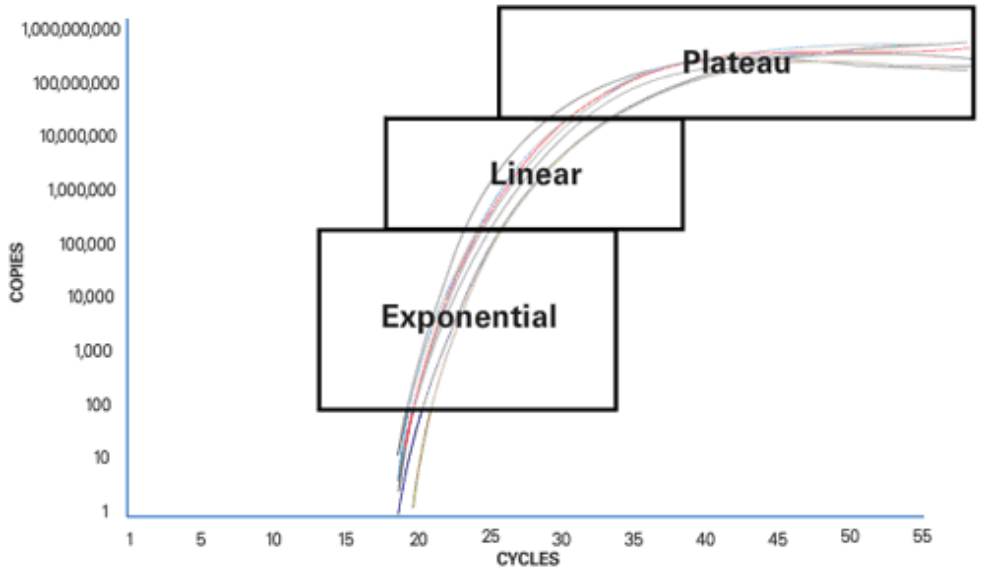
Advantages of real-time PCR include:

- Generation of accurate quantitative data
- Increased dynamic range of detection
- Elimination of post-PCR processing
- Detection down to one copy
- Increased precision to detect smaller fold changes
- Increased throughput

End-point phase measurement in traditional PCR

There are three phases in a basic PCR run:

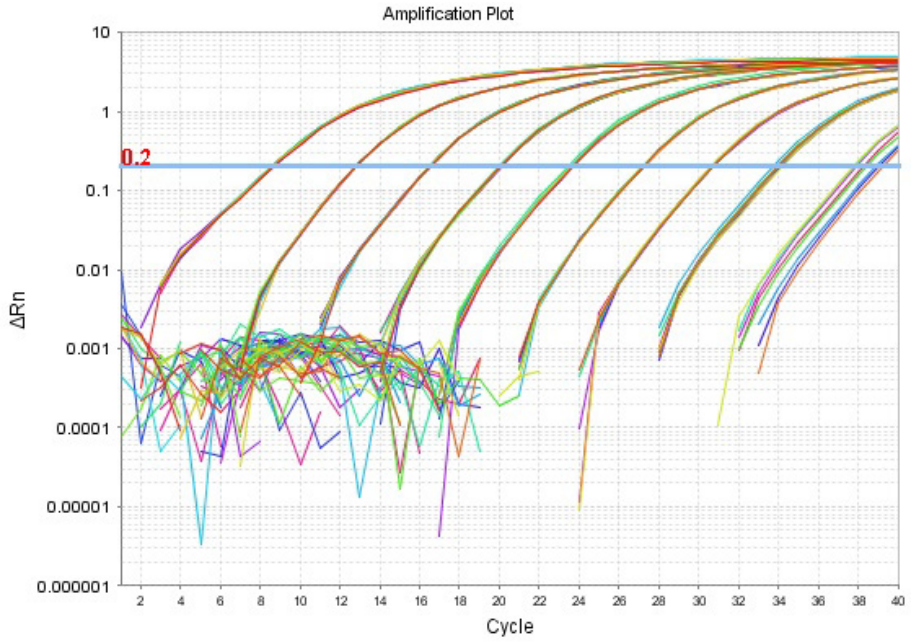
- **Exponential** – Exact doubling of product occurs at every cycle (assuming 100% reaction efficiency). Exponential amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon.
- **Linear (High Variability)** – As the reaction progresses, some of the reagents are consumed as a result of amplification. The reactions start to slow down and the PCR product is no longer doubled at each cycle.
- **Plateau (End-Point: Gel detection for traditional methods) –** The reaction has stopped, no more products are made, and if left long enough, the PCR products begin to degrade. Each tube or reaction plateaus at a different point, due to the different reaction kinetics for each sample. These differences can be seen in the plateau phase. The plateau phase is the end point, where traditional PCR takes its measurement.



Exponential phase measurement in real-Time PCR

Real-Time PCR focuses on the exponential phase, which provides the most precise and accurate data for quantitation. During the exponential phase, the real-time PCR instrument calculates two values:

- **Threshold** – The level of detection at which a reaction reaches a fluorescent intensity above background.
- **C_T**– The PCR cycle at which the sample reaches the threshold. The C_T value is used in absolute or relative quantitation.



Gene expression using real-time PCR

Before you set up an experiment:

- Decide the type of real-time chemistry to use (TaqMan® or SYBR®).
- Select a reverse transcription method.
- Select or design assays.
- Select a quantitation method.

The workflow for performing gene expression using real-time PCR is shown:

Selecting the detection chemistry page 8 → **Selecting the reverse transcription method** page 12 → **Selecting or designing assays** page 13 → **Selecting the quantitation method** page 15

The following topic, "Selecting the detection chemistry", provides a brief overview of the two real-time PCR chemistries, TaqMan probe and SYBR Green I dye, and guidelines for selecting a chemistry.

Selecting the detection chemistry

The two types of chemistries that have been developed for gene expression studies using real-time PCR are:

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

About TaqMan chemistry

Real-time PCR systems 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.

About TaqMan probes

TaqMan probes are dual labeled, hydrolysis probes that increase the specificity of real-time PCR assays. TaqMan probes contain:

- A reporter dye (for example, FAM™ dye) linked to the 5′ end of the probe
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe
- MGB moiety attached to the NFQ

TaqMan MGB probes also contain a minor groove binder (MGB) at the 3′ end of the probe. MGBs increase the melting temperature (T_m) without increasing probe length ; allowing for the design of shorter probes (Afonina et al., 1997; Kutyavin et al., 1997).

How TaqMan real-time chemistry works

Here is how TaqMan real-time chemistry works:

1. An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5′ end and a quencher on the 3′ end.

   While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer through space (Förster, V. T. 1948).

2. If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5′ nuclease activity of the taq DNA polymerase during extension. This cleavage of the probe:
   - Separates the reporter dye from the quencher, 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 inhibit the overall PCR process.

3. 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. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

About SYBR Green I dye chemistry

About SYBR Green I dye

SYBR Green I dye chemistry uses SYBR Green I dye, which binds to double-stranded DNA, to detect PCR products as they accumulate during PCR cycles.

An important difference between the TaqMan probes and SYBR Green I dye chemistries is that the SYBR Green I dye chemistry binds all double-stranded DNA, including nonspecific reaction products. A well-optimized reaction is therefore essential for accurate results.

How SYBR Green I dye chemistry works

The SYBR Green I dye chemistry uses the SYBR Green I dye to detect PCR products by binding to the double-stranded DNA formed during PCR. Here is how this chemistry works:

1. SYBR Green I dye fluoresces when bound to double-stranded DNA.
2. During the PCR, taq DNA Polymerase amplifies the target sequence, which creates the PCR product, or "amplicon."
3. As the PCR progresses, more amplicons are created. Since SYBR Green binds to all double-stranded DNA, the result is an increase in fluorescent intensity proportional to the amount of PCR product produced.

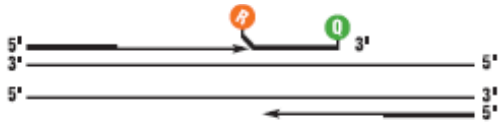
Comparison of TaqMan and SYBR Green I Dye chemistries

The illustration on page 10 shows a comparison of TaqMan-based and SYBR Green-based detection workflows.

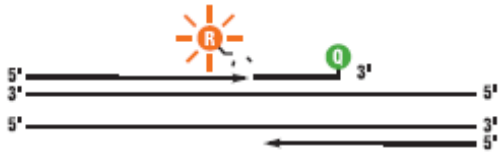
TAQMAN® PROBE-BASED ASSAY CHEMISTRY

1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5′ and 3′ ends of a TaqMan probe respectively.

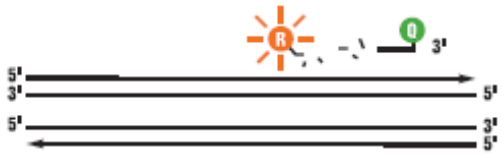
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



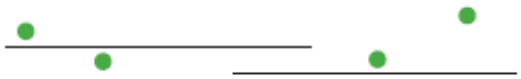
4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



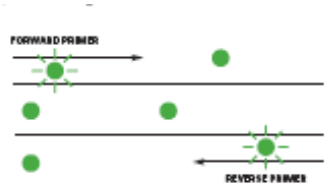
SYBR® GREEN I DYE ASSAY CHEMISTRY

1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

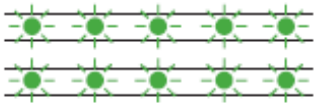
2. **Denaturation:** When the DNA is denatured, the SYBR Green I Dye is released and the fluorescence is drastically reduced.



3. **Polymerization:** During extension, primers anneal and the PCR product is generated.



4. **Polymerization completed:** When polymerization is complete, SYBR Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence.



Comparison of TaqMan and SYBR Green chemistries

TaqMan probe-based chemistry and SYBR Green I dye can be used for the assay types listed below.

Chemistry	Assay type	
	Quantification	Multiplex
TaqMan probes	Yes	Yes
SYBR Green I Dye	Yes	No

Each chemistry has its advantages and limitations. For example, TaqMan chemistry enables you to perform multiplex PCR. If high sensitivity is your priority, SYBR Green chemistry offers that advantage. Consider the following aspects of each chemistry type when choosing between TaqMan probe-based and SYBR Green chemistry for your assays:

Detection	Description	Advantages	Limitations
TaqMan	Uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. Detects specific amplification products only.	• Specific hybridization between probe and target is required to generate fluorescent signal, significantly reducing background and false positives. • Two or more specific targets may be detected in the same reaction when the probes are labeled with different dyes. Multiplex PCR can reduce cost and improve precision. • Post-PCR processing is eliminated, saving time.	A different probe has to be synthesized for each unique target sequence.
SYBR Green I dye	Uses SYBR Green I dye, a double-stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles. Detects all double-stranded DNA, including both specific and non-specific reaction products.	• Enables you to monitor the amplification of any double-stranded DNA sequence. • Does not require probes, so your assay setup and running costs are reduced. • Multiple dye molecules can bind to a single amplified target sequence, increasing sensitivity for detecting amplification products.	• Because SYBR Green I dye binds to any double-stranded DNA—including nonspecific double-stranded DNA sequences—it may generate false positive signals. • Primer optimization is sometimes necessary to improve the performance of SYBR Green assays. • Multiplex PCR cannot be done when using SYBR Green. • A dissociation or "melt" of the PCR products is highly recommended for SYBR Green assays, which lengthens the protocol, and requires visual analysis of the peaks.

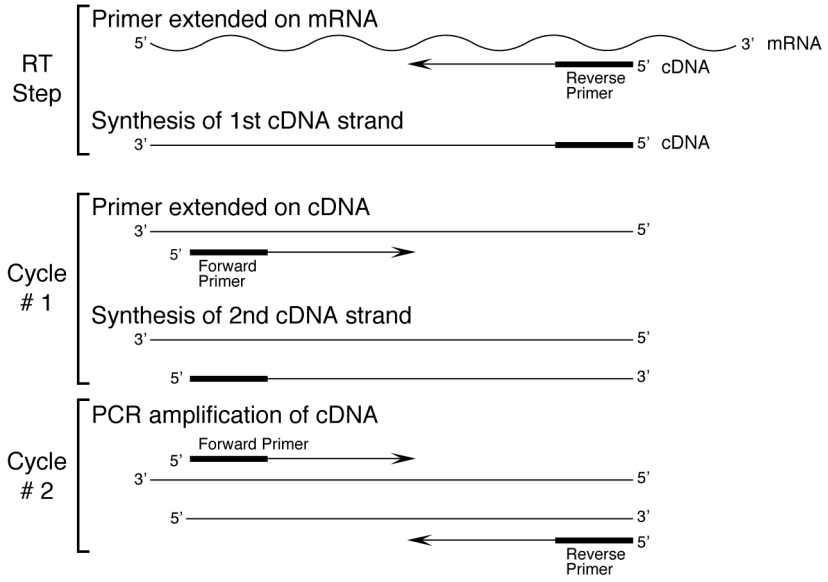
Selecting the reverse transcription method

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is used to quantify RNA. RT-qPCR can be performed as a one-step or two-step procedure.

The most common method for looking at gene expression is two-step RT-qPCR.

About one-step RT-qPCR

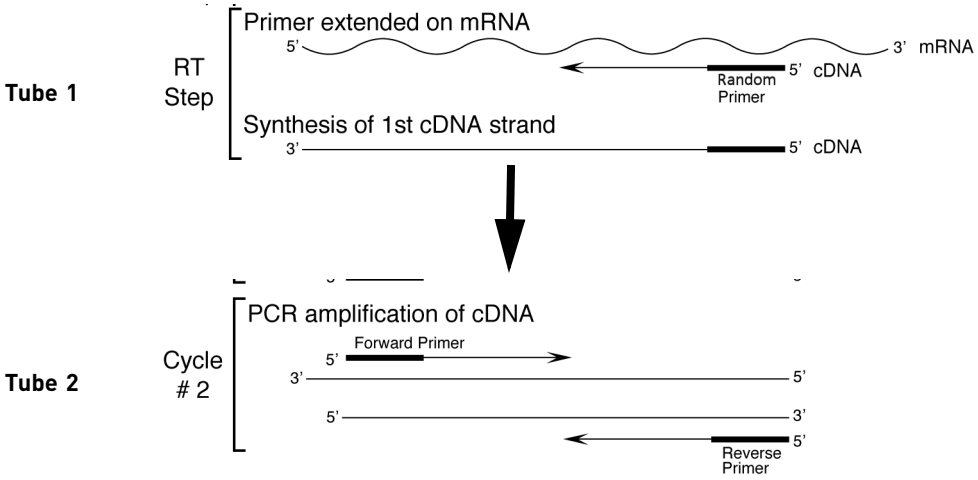
With one-step RT-qPCR, the reverse transcription and PCR amplification steps are performed in a single buffer system:



The reaction proceeds without the addition of reagents between the RT and PCR steps. One-step RT-qPCR offers the convenience of a single-tube preparation for RT and PCR amplification. This method is target- or gene-specific. Only your specific target is transcribed because you use one of your PCR primers to prime the reverse transcription. This approach is useful when studying a single gene in many samples.

About two-step RT-PCR

With two-step RT-qPCR, the reverse transcription and PCR amplification steps are performed in two separate reactions:



Two-step RT-qPCR is useful when detecting multiple transcripts from a single sample, or when storing a portion of the cDNA for later use. In a two-step approach, the reverse transcription is usually primed with either oligo $d(T)_{16}$ or random primers. Oligo $d(T)_{16}$ binds to the poly-A tail of mRNA, and random primers bind across the length of the RNA being transcribed.

Guidelines for selecting the reverse transcription and amplification reagents

For guidelines on selecting the reverse transcription and amplification reagents, see the *Applied Biosystems Gene Expression Assays Protocol* (PN 4333458) or the Real-Time PCR Decision Tree at **www.ambion.com/techlib/trees/qpcr/**.

Preamplification

If you have difficult samples, use TaqMan PreAmp Master Mix, which preamplifies small amounts of cDNA without introducing amplification bias to the sample. Preamplification enables you to stretch your limited sample into many more real-time PCR reactions. For more information, see the Applied Biosystems *TaqMan PreAmp Master Mix Kit Protocol* (PN 4384557A) and the Product Description on **www.appliedbiosystems.com**.

Selecting or designing assays

When you are deciding whether to select a predesigned assay or design a custom assay, think about your goals for the assay. These considerations should be taken into account whether you purchase commercially available, preformulated primer or probe sets or you design your own assays.

Considerations for optimal assay performance

Target of interest

Identify the gene(s) or pathway of interest.

Specificity

Depending on the level of specificity you require, you can select or design an assay to:

- Detect all known transcripts of your gene of interest (gene-specific detection).
- Detect a unique splice variant (transcript-specific detection).
- Discriminate between closely related members of a gene family (homologs and potentially orthologs).

Ensure specificity by checking against known sequences databases such as NCBI and Ensembl.

Efficiency

Ensure that your assay has an amplification efficiency close to 100%. Less efficient assays may result in reduced sensitivity and linear dynamic range, thereby limiting your ability to detect low abundance transcripts.

Reproducibility

You should be able to repeat your experiment and produce the same results. Factors that could affect reproducibility are oligo manufacturing and assay formulation, and primer dimer formation.

Preformulated assays and PCR arrays

Whether you are studying single genes or whole pathways, you now have many choices of preformulated assays and PCR arrays from multiple vendors:

- Preformulated assays in tubes are appropriate when you are a studying small number of genes or when you need maximum flexibility.
- PCR arrays are 96 or 384 well plates or microfluidic cards loaded with assays corresponding to pathways or other common gene sets. This format is appropriate when you are studying a large number of genes or when you are trying to narrow down the number of genes you want to focus on in your experiment.

If your gene targets are available as commercial primer or probe sets, you could use either of the following tools to design customized assays:

- A public primer or probe design tool, or
- A commercial assay design tool or service, such as the Applied Biosystems Custom TaqMan Assay Design Tool (**www.appliedbiosystems.com/tools/cadt/**)

Endogenous controls

In any gene expression study, selecting a valid normalization or endogenous control to correct for differences in RNA sampling is critical to avoid misinterpretation of results. TaqMan Endogenous Controls consist of the most commonly used housekeeping genes in human, mouse, and rat, and these controls are provided as a preformulated set of predesigned probe and amplification primers.

For more information on selecting an endogenous control, refer to *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies* (Publication 127AP08-01), available at **www.appliedbiosystems.com**.

Singleplex PCR vs. duplex PCR

Duplex PCR is the simultaneous amplification of two target sequences in a single reaction.

Duplex real-time PCR is possible using TaqMan probe–based assays, in which each assay has a specific probe labeled with a unique fluorescent dye, resulting in different observed colors for each assay. Real-time PCR instruments can discriminate between the different dyes. The signal from each dye is used to separately quantitate the amount of each target.

Typically one probe is used to detect the target gene; another probe is used to detect an endogenous control (reference gene). Running both assays in a single tube reduces both the running costs and the dependence on accurate pipetting when splitting a sample into two separate tubes. Duplex PCR is not possible when using SYBR Green chemistry.

Consider the following advantages and limitations when choosing between duplex and singleplex PCR:

PCR	Description	Advantage	Limitation
Singleplex	A reaction in which a single target is amplified in the reaction tube or well.	• No optimization is required for TaqMan assays. • Flexibility to use TaqMan or SYBR Green reagents.	Requires separate reactions for the target and the endogenous control assay.
Duplex	A reaction in which two targets are amplified in the same reaction tube or well.	Reduces the: • Running costs. • Dependence on accurate pipetting.	Requires validation and optimization.

Note: Applied Biosystems has predesigned assays to select a VIC reporter dye.

Selecting the quantitation method

Methods for relative quantitation of gene expression enable you to quantify differences in the expression level of a specific target (gene) between different samples. The data output is expressed as a fold-change or a fold-difference of expression levels. For example, you might want to look at the change in expression of a particular gene over a given time period in treated versus untreated samples.

When you are designing your Real-Time PCR gene expression experiment, select the method to use to quantify the target sequence:

- Comparative C_T ($\Delta\Delta C_T$) method (relative quantitation)
- Relative standard curve method (relative quantitation)
- Standard curve method (absolute quantitation)

Comparative C_T method

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

Uses

Comparative C_T experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in treated versus untreated samples.
- Compare expression levels of genes in samples treated with a compound under different experimental conditions, over a time-course-study-defined period of time.

For more information, read *Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ C_T method* by Kenneth J. Livak and Thomas D. Schmittgen.

Experimental validation

To use the comparative C_T method, run a validation experiment to show that the efficiencies of the target and endogenous control amplifications are approximately equal (Livak, K.J. and Schmittgen, T.D. 2001).

Relative standard curve method

Similar to the comparative C_T method, the relative standard curve method can be used to determine fold changes in gene expression. Generally, use the relative standard curve method when you use two assays for quantitation (an assay for the target gene and an assay for endogenous control) that did not have equivalent amplification efficiency. A dilution series is created from a common sample and run with both the target and the endogenous control gene. For all experimental samples, a quantity is determined from this dilution series, and a fold change in expression can be calculated from this data.

For more information, read the Applied Biosystems User Bulletin for the ABI PRISM 7700 Sequence Detection System: *Relative Quantitation of Gene Expression* (PN 4303859).

Standard curve method

Use the standard curve method to determine the absolute target quantity in samples. With the standard curve method, the real-time PCR system software measures amplification of the target in samples and in a standard dilution series of known copy number. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples. The standard curve method is probably the least common method for quantitation of gene expression.

Guidelines for selecting the quantitation method

Consider the following advantages and limitations when selecting the quantitation method:

Experiment type	Advantage	Limitation
Comparative C_T ($\Delta\Delta C_T$)	• Relative levels of target in samples can be determined without the use of a standard curve or dilution series. • Requires reduced reagent usage. • More space is available in the reaction plate. • Because a standard curve is not needed, throughput can increase. • Dilution errors made in creating the standard curve samples are eliminated. • The target and endogenous control can be amplified in the same tube, increasing throughput and reducing pipetting errors.	• Suboptimal (low PCR efficiency) assays may produce inaccurate results. • Before you can use the comparative C_T method, the PCR efficiencies for the target assay and the endogenous control assay must be approximately equal.
Relative standard curve	Requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not need to be equivalent.	A dilution series must be run for each target; a series requires more reagents and more space in the reaction plate.
Absolute quantitation (standard curve)	Absolute, rather than relative, quantities of transcripts are calculated.	The required standard curve for each target requires more reagents and more space in the reaction plate.

Analyzing data

Analyzing the data requires you to:

- View the amplification plots for the entire plate.
- Set the baseline and threshold values.
- Use the relative standard curve or the comparative C_T method to analyze your data.

Resources for data analysis

The details of data analysis depend on the real-time PCR instrument that you use; refer to the appropriate user guide for instructions on how to analyze your data.

Real-time PCR system	Document	Part number
7900HT Fast system	*Relative Quantitation Using Comparative C_T Getting Started Guide*	4364016
	Performing Fast Gene Quantification: Quick Reference Card	4351892
	Performing Fast Gene Quantitation with 384-Well Plates: User Bulletin	4369584
7300/7500/ 7500 Fast system	*Relative Quantification: Getting Started Guide*	4347824
	Relative Standard Curve and Comparative C_T Experiments Getting Started Guide	4387783
StepOne™/ StepOnePlus™ system	*Comparative C_T/Relative Standard Curve and Comparative C_T Experiments Getting Started Guide*	4376785
All	*Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems Chemistry Guide*	4348358

Tools for data analysis

Applied Biosystems recommends the following software for analyzing data generated using TaqMan Gene Expression Assays.

DataAssist™ Software

DataAssist™ Software is a simple, yet powerful data analysis tool for sample comparison when using the comparative C_T ($\Delta\Delta C_T$) method for calculating relative quantitation of gene expression. The software is compatible with all Applied Biosystems instruments. It contains a filtering procedure for outlier removal, and various normalization methods based on single or multiple genes, and it provides relative quantification analysis of gene expression through a combination of statistical analysis and interactive visualization.

DataAssist™ Software is free and can be downloaded from:

www.appliedbiosystems.com/dataassist

For more information about comparative C_T, read *Analyzing real-time PCR data by the comparative C_T method* by Kenneth J. Livak and Thomas D. Schmittgen.

Real-Time StatMiner® Software

Real-Time StatMiner® Software from Integromics is a software analysis package for qPCR experiments that is compatible with all Applied Biosystems instruments. Real-Time StatMiner® Software uses a step-by-step analysis workflow guide that includes parametric, non-parametric, and paired tests for relative quantification of gene expression, as well as two-way ANOVA for two-factor differential expression analysis.

For more information, visit:

www.integromics.com/StatMiner

Ordering Information

This appendix covers:

Reverse transcription and PCR master mix reagents

Reagent	Description and part number
High Capacity RNA-to-cDNA™ Kit	50 reactions (PN 4387406)
High Capacity cDNA Reverse Transcription Kit	• 200 reactions (PN 4368814) • 200 reactions with RNase Inhibitor (PN 4374966) • 1000 reactions (PN 4368813) • 1000 reactions with RNase Inhibitor (PN 4374967)
TaqMan® RNA-to-C_T™ *1-Step* Kit	• 40 × 50-µL reactions (PN 4392653) • 200 × 50-µL reactions (PN 4392938) • 2000 × 50-µL reactions (PN 4392656)
TaqMan® Gene Expression Master Mix (2X)	• One 1-mL tube (PN 4370048) • One 5-mL bottle (PN 4369016) • One 6-mL bottle (PN 4393469) • Two 5-mL bottles (PN 4369514) • Five 5-mL bottles (PN 4369510) • Ten 5-mL bottles (PN 4369542) • One 50-mL bottle (PN 4370074)
TaqMan® Universal Master Mix II, no UNG	• One 1-mL tube (PN 4440043) • One 5-mL bottle (PN 4440040) • Two 5-mL bottles (PN 4440047) • Five 5-mL bottles (PN 4440048) • Ten 5-mL bottles (PN 4440049) • One 50-mL bottle (PN 4440041)

Reagent	Description and part number
TaqMan® Universal Master Mix II, with UNG	• One 1-mL tube (PN 4440042) • One 5-mL bottle (PN 4440038) • Two 5-mL bottles (PN 4440044) • Five 5-mL bottles (PN 4440045) • Ten 5-mL bottles (PN 4440046) • One 50-mL bottle (PN 4440039)
TaqMan® Fast Universal Master Mix (2X) No AmpErase® UNG	• 250 × 20-µL reactions (PN 4352042) • 500 × 20-µL reactions (PN 4366072) • 1250 × 20-µL reactions (PN 4366073) • 2500 × 20-µL reactions (PN 4364103) • 5000 × 20-µL reactions (PN 4367846)
TaqMan® PreAmp Master Mix	40 reactions (PN 4381128)
TaqMan® PreAmp Master Mix Kit	40 reactions (PN 4384267)

TaqMan® Gene Expression Assays and Arrays

Applied Biosystems offers more than 1,000,000 TaqMan Gene Expression Assays, the most comprehensive set of predesigned Real-Time PCR assays available.

All TaqMan® Gene Expression Assays have been designed through our validated bioinformatics pipeline, and run with the same PCR conditions, eliminating the need to design primers or optimize PCR conditions.

There are more than 1,200,000 TaqMan Gene Expression Assays available from 19 species.

Species	Number of Assays
H. sapiens	209,008
M. musculus	181,951
R. norvegicus	150,510
C. familiaris (dog)	54,864
M. mulatta (Rhesus Macaque)	64,851
A. thaliana	97,879
B. taurus (Cow)	67.083
G.gallus (chicken)	48,432
O. cuniculus (rabbit)	3,587
S. scrofa (pig)	7,240
C. elegans	92,687
D. melanogaster	41,607
D. rerio (zebrafish)	63,712

Species	Number of Assays
E. caballus (horse)	2,967
O. sativa (rice)	99,822
S. pombe (yeast)	6,538
X. tropicalis (frog)	56,764
G. max (soybean)	3,456
C. porcellus (guinea pig)	962
Total number of assays	**1,253,920**

Order Custom TaqMan Assays

Use the online Custom TaqMan Assay Design Tool to order Custom TaqMan Assays. To order an assay:

- Go to the Custom TaqMan Assay Design Tool web site:
  www5.appliedbiosystems.com/tools/cadt/

Submit your target sequences of interest or custom primer and probe sequences from any organism. If a predesigned assay is not available, your sequence information will be submitted to the assay design pipeline, which will design a TaqMan assay to your submitted sequence.

Refer to the Applied Biosystems Design and Ordering Guide, *Custom TaqMan Assays: For New SNP Genotyping and Gene Expression Assays* (PN 4367671) for instructions on how to use the Custom TaqMan Assay Design Tool.

Additional Applied Biosystems publications that contain ordering instructions are listed in "Documentation" on page 31.

TaqMan Arrays (384-Well micro fluidic cards)

TaqMan Array micro fluidic cards are 384-well cards preloaded with TaqMan Gene Expression Assays. The TaqMan Array cards allow you to measure gene expression using the comparative C_T ($\Delta\Delta C_T$) method of relative quantitation. You can run 1 to 8 samples per card, against 12 to 384 TaqMan Gene Expression Assay targets (including controls). The key features of TaqMan Arrays are:

- Provides small-volume design, minimizing sample and reagent consumption.
- Provides streamlined reaction setup, saving time and reducing labor-intensive steps.
- Provides access to high-throughput, 384-Well format without liquid-handling robotics.
- Detects two-fold discrimination at the 99.7% confidence level.
- Provides standardization across multiple samples in multiple laboratories.

Four types of TaqMan Array cards are available from Applied Biosystems:

- **TaqMan Custom Array** – Customizable card. You choose the assays to include in your card. Select TaqMan Gene Expression Assays from the Applied Biosystems collection of inventoried assays. The TaqMan Custom Array micro fluidic cards are manufactured when ordered.
- **TaqMan Gene Sets** – Customizable card. You choose the assays to include in your card. The TaqMan Gene Sets are a collection of TaqMan Gene Expression Assays that define specific target classes, pathways, or diseases (for example, the TaqMan Array Human Apoptosis micro fluidic card). Select assays from the TaqMan Gene Sets, then customize the card according to your research needs. TaqMan Array cards with TaqMan Gene Sets are manufactured when ordered.

  Note: You can substitute assays within a TaqMan Gene Set with TaqMan Gene Expression Assays from Applied Biosystems collection of Inventoried assays.

- **TaqMan Gene Signature Array** – Preconfigured card. The TaqMan Gene Signature Arrays contain preselected TaqMan Gene Expression Assays that target specific gene classes (for example, human stem cell pluripotency markers). The TaqMan Gene Signature Arrays are manufactured and placed in inventory.
- **TaqMan PreAmp Pools** – Prepooled sets of TaqMan Gene Expression Assays ready for use with four specific TaqMan Gene Signature Arrays. TaqMan PreAmp Pools are designed for challenging RNA samples, such as very small quantities, or degraded material and genes that have low expression levels. Use PreAmp Pools to increase the quantity of your sample targets without introducing bias. Four pools are available corresponding to fixed TaqMan Human and Rat GPCR, and Human and Mouse Stem Cell Pluripotency Gene Signature Arrays.

A complete list of the TaqMan Gene Expression Assays that are available for each type of TaqMan Array card can be found on the Applied Biosystems web site. For ordering information, see "Assay and array ordering information" on page 23.

TaqMan Array Plates

TaqMan Array Plates are MicroAmp® Optical 96-Well Reaction Plates, Standard or Fast, that contain dried-down TaqMan Gene Expression Assays. The gene expression assays are a collection of predesigned, gene-specific primer and probe sets for performing quantitative gene expression studies on cDNA. The assays are available for multiple species.

Custom TaqMan Array Plates

TaqMan 96-Well Fast Plates and TaqMan Array 96-Well Plates complement individual TaqMan Gene Expression Assays and prespotted 384-Well TaqMan Arrays. The plates:

- Contain up to 95 gene expression assays.
- Are available in five different plate formats.
- Are available with or without candidate endogenous control genes (human, mouse, or rat).
- Contain one manufacturing control.

TaqMan Array Gene Signature Sets

TaqMan Array Gene Signature Sets:

- Contain preplated gene expression assays that detect genes to specific biological pathways, disease states, or have common biological function. The assays are available in predefined formats as described at **www.appliedbiosystems.com**.
- Contain at least three candidate control genes and one manufacturing control.
- Are available in Standard or Fast plate formats.

Assay and array ordering information

Gene expression assays and arrays	Part number
Individual Assays	
• TaqMan® Gene Expression Assays	
– Made to order (Extra Small)	4448892
– Inventoried (Small)	4331182
– Made to order (Small)	4351372
– Made to order (Medium)	4351370
– Made to order (Large)	4351368
• Custom TaqMan® Gene Expression Assays	
– Large-scale, 60X concentration (2900 x 20 µL) reactions	4332079
– Medium-scale, 20X concentration (750 x 20 µL) reactions	4332078
– Small-scale, 20X concentration (360 x 20 µL) reactions	4331348
• Custom Plus TaqMan® RNA Assays	
– Large-scale, 60X concentration (2900 x 20 µL) reactions	4441118
– Medium-scale, 20X concentration (750 x 20 µL) reactions	4441117
– Small-scale, 20X concentration (360 x 20 µL) reactions	4441114
• TaqMan® Non-coding RNA Assays	
– Made to order (Small)	4426961
– Made to order (Medium)	4426962
– Made to order (Large)	4426963
• TaqMan® Endogenous Controls	For a complete list of TaqMan® Endogenous Controls, go to **www.appliedbiosystems.com**. Click **Products** ▸ **Real-Time PCR** ▸ **Gene Expression Assays & Arrays** ▸ **Individual Assays** ▸ **TaqMan® Endogenous Controls**.
96-Well Arrays (Fast and Standard Plates)	
• TaqMan® Array Gene Signature Plates	For a complete list of TaqMan® Array Gene Signature Plates, go to **www.appliedbiosystems.com**. Click **Products** ▸ **Real-Time PCR** ▸ **Gene Expression Assays & Arrays** ▸ **96-Well Arrays (Fast & Standard Plates)** ▸ **TaqMan® Array Gene Signature Plates**.

Gene expression assays and arrays *(continued)*	**Part number** *(continued)*
• Custom TaqMan® Array Plates	
– TaqMan® Array Standard 96-Well Plates	Std_96
– TaqMan® Array Micro Fluidic Cards	MFC_384
– TaqMan® Arrays Fast 96-Well Plates	Fast_96
• TaqMan® Gene Sets	
– TaqMan® Array Micro Fluidic Cards	MFC_384
• TaqMan® PreAmp Pools	
– TaqMan® PreAmp Pool for Human Stem Cell Pluripotency Array	4405625
– TaqMan® PreAmp Pool for Mouse Stem Cell Pluripotency Array	4405626
– TaqMan® PreAmp Pool for Human GPCR Array	4405623
– TaqMan® PreAmp Pool for Rat GPCR Array	4405624
384-Well Arrays (Micro Fluidic Card)	For a complete list of 384-Well arrays, go to www.appliedbiosystems.com. Click **Products** ▸ **Real-Time PCR** ▸ **Gene Expression Assays & Arrays** ▸ **384-Well Arrays (Micro Fluidic Card)**.

Instruments and accessories

Instruments:

- Applied Biosystems 7300 Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7500 Fast Real-Time PCR System
- Applied Biosystems 7900 HT Fast Real-Time PCR System
- Applied Biosystems StepOne™ Real-Time PCR System
- Applied Biosystems StepOnePlus™ Real-Time PCR System

Reaction plates instruments:

Instrument	**Reaction plate**	**Part number**
Applied Biosystems StepOne™ System	MicroAmp® Fast Optical 48-Well Reaction Plate	4375816
Applied Biosystems StepOne Plus™ , 7500 Fast, and 7900HT Fast Systems	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 20 plates 0.1 ml	4346906
	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 200 plates 0.1 ml	4366932

Instrument	Reaction plate	Part number
Applied Biosystems 7000, 7300, 7500, and (96-Well) 7900HT Systems	• MicroAmp® Fast Optical 48-Well Reaction Plate	4375816
	• MicroAmp® Optical 96-Well Reaction Plate, 10 plates	N8010560
	• MicroAmp® Optical 96-Well Reaction Plate, 500 plates	4316813
	• MicroAmp® Optical 96-Well Reaction Plate with Barcode, 20 plates	4306737
	• MicroAmp® Optical 96-Well Reaction Plate with Barcode, 500 plates	4326659
	• MicroAmp® Optical 96-Well Reaction Plate with Barcode and Optical Caps, 20 plates	403012
	• MicroAmp® Optical 96-Well Reaction Plate with Barcode and Optical Adhesive Films, 100 plates	4314320
Applied Biosystems (384-Well) 7900HT System	MicroAmp® Optical 384-Well Reaction Plate with Barcode, 50 plates	4309849

Adhesive films for instruments:

Instrument	Adhesive film	Part number
Applied Biosystems StepOne™ System	• MicroAmp® 48-Well Optical Adhesive Film, 100 films	4375323
	• MicroAmp® 48-Well Optical Adhesive Film, 25 films	4375928
Applied Biosystems StepOnePlus™ 7000, 7300, 7500, 7500 Fast, and 7900HT Systems	• MicroAmp® Optical Adhesive Film - 25 films	4360954
	• MicroAmp® Optical Adhesive Film - 100 films	4311971
	• MicroAmp® Optical 384-Well Reaction Plate with Barcode, 500 plates	4326270
	• MicroAmp® Optical 384-Well Reaction Plate with Barcode, 1,000 plates	4326270
	• MicroAmp® Optical 384-Well Reaction Plate, 1,000 plates	4343370

Designing TaqMan® Assays, Primers, and Probes

Design Custom TaqMan primers

Primers can be designed using Primer Express software as described in the *Primer Express Software Version 3.0 Getting Started Guide* (PN 4362460). Follow these guidelines when designing primers:

- Choose the primers after selecting the probe.
- Design the primers as close as possible to the probe without overlapping the probe.
- Keep the G-C content in the 20% to 80% range.
- Avoid runs of an identical nucleotide, especially for guanine, where runs of four or more Gs should be avoided.
- The T_m of each primer should be 58 to 60 °C.
- The five nucleotides at the 3′ end of each primer should have no more than two G and/or C bases.
- Amplicon Length should be 50 to 150 bases for optimum PCR efficiency.

Design TaqMan probes

Applied Biosystems fluorogenic probes enable the detection of a specific PCR product as it accumulates during PCR cycles. The Applied Biosystems patented fluorogenic probe design, which incorporates the reporter dye on the 5′ end and the quencher dye on the 3′ end, has greatly simplified the design and synthesis of effective 5′ fluorogenic nuclease assay probes (Livak, Flood, et al., 1995).

TaqMan MGB probes

TaqMan® minor groove binder (MGB)probes contain:

- A reporter dye (for example, FAM™ dye) linked to the 5′ end of the probe.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe.

  Because the quencher does not fluoresce, Applied Biosystems real-time PCR systems can measure reporter dye contributions more accurately.
- MGB moiety attached to the NFQ.

  MGBs increase the melting temperature (T_m) without increasing probe length (Afonina et al., 1997; Kutyavin et al., 1997); they also allow for the design of shorter probes.

Guidelines for designing Custom TaqMan probes

Probes can be designed using Primer Express software, as described in the *Primer Express Software Version 3.0 Getting Started Guide* (PN 4362460). Follow these guidelines when designing probes:

- Probe length should be 13 to 25 bases (13 to 30 bases if using conventional TaqMan probes).
- The melting temperature (T_m) should be – 68 °C to 70 °C.
- For a primer, the guanine-cytosine (GC) content should be in the range of 30% to 80%.
- The 5′ end cannot be a guanosine residue. A guanosine residue adjacent to the reporter dye somewhat quenches the reporter fluorescence, even after cleavage.
- Avoid including long sequences of identical nucleotides.

Bibliography

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Documentation

Related documentation

Document	Part number	Description
Application Note: *TaqMan® PreAmp Master Mix Kit*	127AP09-01	Describes the TaqMan PreAmp Master Mix Kit, a tool for gene expression studies on biological samples of limited quantity.
TaqMan® Gene Expression Assays Protocol	4333458	Provides instructions for real-time reverse transcription-PCR (real-time RT-PCR) using TaqMan® Gene Expression Assays and TaqMan Non-coding RNA Assays.
TaqMan Gene Expression Assays Quick Reference Card	4401212	Provides instructions for real-time reverse transcription-PCR (real-time RT-PCR) using TaqMan Gene Expression Assays.
Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies	127AP08-01	Explains the use of TaqMan endogenous control assays to identify multiple candidate endogenous control genes that can be used to normalize gene expression data within a defined experimental study.
Online Ordering Guide for TaqMan Gene Expression Assays	127M107-05	Provides instructions for finding and selecting TaqMan Gene Expression Assays for your application or experiment.
Online Selection Guide for TaqMan Gene Expression Assays	127GU08-01	
Product Guide: *TaqMan Gene Expression Assays*	127GU01-04	Details all available TaqMan Gene Expression Assays products.
Application Note: *Amplification Efficiency of TaqMan Gene Expression Assays*	127AP05-03	Details the steps taken by Applied Biosystems to provide assays products with 100% amplification efficiency of the PCR reaction.
White Paper: *Product Stability Study, TaqMan Gene Expression Assays*	127WP03-01	Demonstrates the maximum storage time for TaqMan Gene Expression Assays.
White Paper: *TaqMan Gene Expression Assays for Validating Hits From Fluorescent Microarrays*	127WP01- 02	Illustrates how TaqMan Gene Expression Assays are the most accurate, precise, and cost-effective method for validating microarray results.

Related documentation *(continued)*

Document	Part number	Description
White Paper: *The Design Process for a New Generation of Quantitative Gene Expression Analysis Tools: TaqMan Probe-Based Assays for Human, Mouse, and Rat Genes*	127WP02- 02	Describes the Applied Biosystems bioinformatics pipeline, which integrates public and proprietary gene sequence information, and uses it to create the most specific and robust quantitative assays to mRNA transcripts.
Application Note: *Factors Influencing Multiplex Real-Time PCR*	136AP04-01	Outlines the optimization and validation of duplex PCR and provides recommendations for multiplex reactions with a greater number of targets.
User Bulletin: *ABI PRISM 7700 Sequence Detection System – Relative Quantitation Of Gene Expression*	4303859	Describes how relative quantitation with data from the ABI PRISM® 7700 Sequence Detection System can be performed using the standard curve method or the comparative method.

For additional documentation and for the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

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- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
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- Download software updates and patches.

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