SYBR® Green PCR and RT-PCR Reagents

Protocol

PE Applied Biosystems
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The SYBR® Green dye is sold under license from Molecular Probes, Inc.

Contents

Introduction

Purpose

The SYBR® Green PCR Core Reagents (P/N 4304886) are designed to detect genomic, plasmid, and cDNA with SYBR Green dye. Direct detection of polymerase chain reaction (PCR) product is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double-stranded (ds) DNA.

In RNA quantitation assays the SYBR Green PCR Core Reagents are used in the second step of a two-step reverse transcription-polymerase chain reaction (RT-PCR) protocol. The template in this case is the cDNA generated from a reverse transcription reaction.

The SYBR Green PCR Core Reagents are designed for use with the ABI PRISM® 7700 Sequence Detector. The excitation-emission profile for the SYBR Green dye is similar to that of the FAM dye. This allows the use of the ROX Passive Reference molecule.

For the best quantitation results, use the following:

- Primer Express™ software (P/N 402089) for primer design
- PE Applied Biosystems reagents
- PE Applied Biosystems universal thermal cycling conditions

Follow the instructions in “Amplifying Custom Target Sequences for Quantitation” on page 10.

Interpreting Results

Normalization

The Passive Reference is a dye included in the 10X SYBR Green PCR Buffer that does not participate in the PCR amplification. The Passive Reference provides an internal reference to which the SYBR Green-dsDNA complex signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations caused by changes in concentration or volume.

Multicomponenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescence spectra. The overlapping spectra from the Pure Dye components generate the composite spectrum. This spectrum represents one fluorescence intensity reading from one well. The ABI PRISM 7700 Sequence Detector should include in its Pure Dye spectra the SYBR Green and ROX spectra for multicomponenting data generated with the SYBR Green PCR Core Reagents.

To add SYBR Green to the Pure Dye spectra, use the Sequence Detection Systems Spectral Calibration Kit (P/N 4305822). Follow the detailed instructions in *ABI PRISM 7700 User Bulletin #4: Generating New Spectra Components* (P/N 4306234).

R_n and ΔR_n Values

Normalization is accomplished by dividing the emission intensity of SYBR Green by the emission intensity of the Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction:

- $R_n^+ = R_n$ value of a reaction containing all components including the template
- $R_n^- = R_n$ value of an unreacted sample

  This value can be obtained from the early cycles of a Real Time run, those cycles prior to a detectable increase in fluorescence. R_n^- can also be obtained from a reaction not containing template.
- $\Delta R_n = (R_n^+) - (R_n^-)$

  where:

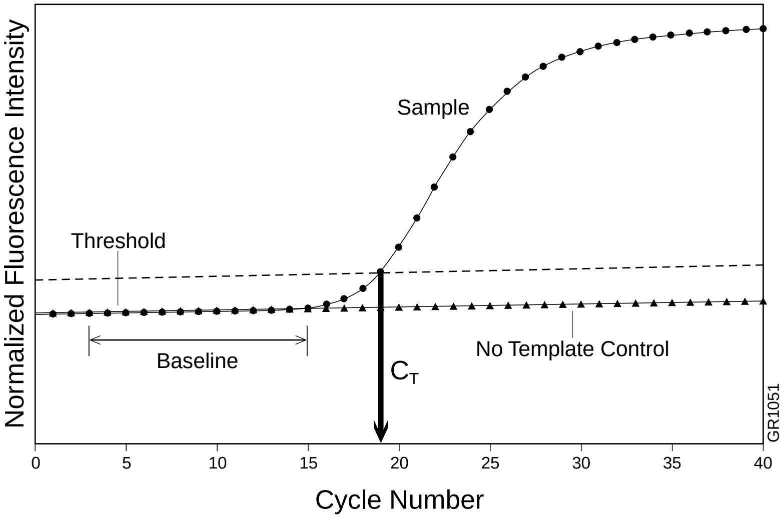
$$R_n^+ = \frac{\text{Emission Intensity of SYBR Green}}{\text{Emission Intensity of Passive Reference}} \qquad \text{(PCR with template)}$$

$$R_n- = \frac{\text{Emission Intensity of SYBR Green}}{\text{Emission Intensity of Passive Reference}} \qquad \text{(PCR without template or early cycles of a Real Time reaction)}$$

ΔR_n indicates the magnitude of the signal generated by the given set of PCR conditions.

Real Time Detection

The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor. On the graph shown below, the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.



Materials and Equipment

Kit Contents

The SYBR Green PCR Core Reagents (P/N 4304886) contain enough PCR reagents for up to 200 50-µL reactions.

Table 1. SYBR Green PCR Core Reagents

Reagent	Volume	Description
10X SYBR Green PCR Buffer	1.5 mL	One tube containing optimized 10X SYBR Green PCR Buffer, which includes Passive Reference 1
AmpErase® UNG	100 µL	One vial containing 1 U/µL uracil-N-glycosylase
AmpliTaq Gold® DNA Polymerase	50 µL	One vial containing 5 U/µL AmpliTaq Gold DNA Polymerase
dNTP Mix	1.0 mL	One tube containing 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 5.0 mM dUTP
25 mM $MgCl_2$	3.0 mL	Two tubes containing 25 mM $MgCl_2$, 1.5 mL each

Storage and Stability

Store the SYBR Green PCR Core Reagents at –15 to –25 °C. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

MSDS

For extra copies of Material Safety Data Sheets (MSDS), contact Customer Service at 1-800-345-5224.

Materials Required But Not Supplied

The following items are required in addition to the reagents supplied in the SYBR Green PCR Core Reagents.

Table 2. User-supplied Materials

Item	Source
ABI PRISM 7700 Sequence Detection System	See your local PE Applied Biosystems representative for the instrument or software best suited to meet your needs.
Primer Express™ software (single-use license)	PE Applied Biosystems (P/N 402089)
MicroAmp® Optical 96-Well Reaction Plate and Optical Caps	PE Applied Biosystems (P/N 403012)
MicroAmp Optical 96-Well Reaction Plate	PE Applied Biosystems (P/N N801-0560)

Table 2. User-supplied Materials *(continued)*

Item	Source
MicroAmp Optical Tubes	PE Applied Biosystems (P/N N801-0933)
MicroAmp Optical Caps	PE Applied Biosystems (P/N N801-0935)
Sequence Detection Systems Spectral Calibration Kit	PE Applied Biosystems (P/N 4305822)
TaqMan® Reverse Transcription Reagents	PE Applied Biosystems (P/N N808-0234)
Unlabeled primers: ♦ 40 nm ♦ 0.2 µM ♦ 1.0 µM	PE Applied Biosystems: ♦ P/N 450005 ♦ P/N 450004 ♦ P/N 450021
Centrifuge with adapter for 96-well plate	Major laboratory suppliers (MLS)
Microcentrifuge	MLS
NuSieve 4% (3:1) agarose gels, for DNA <1 kb	FMC BioProducts (P/N 54928)
Polypropylene tubes	MLS
Pipettors, positive-displacement or air-displacement	MLS
Pipette tips, with filter plugs	MLS
Vortexer	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Disposable gloves	MLS

Technical Support

To Reach Us On the World Wide Web

- Our Web site address is http://www.perkin-elmer.com/ab

  We strongly encourage you to visit our Web site for answers to frequently asked questions, and to learn more about our products. You can also order technical documents and/or an index of available documents and have them faxed to you through our site (see the “Fax-on-Demand” section below).
- Answers to Frequently Asked Questions: http://www2.perkin-elmer.com/ab/FAQ/frontend.html

To Reach Us by Telephone, Fax, or E-mail

- Phone: 1-800-762-4001. Choose PCR or Sequence Detection.
- Fax: 1-203-761-2542
- E-mail: pcrlab@perkin-elmer.com

Hours for Telephone Technical Support

In the United States and Canada, technical support is available between 5:30 a.m. and 5:00 p.m. Pacific Time.

Outside of the United States and Canada, call your local sales office (see the back cover of this protocol).

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- Order through our Web site on the Internet at http://www.perkin-elmer.com/fod

  Search for documents to order using keywords. Up to five documents can be faxed to you, if you already know the titles.
- Order by phone
  - In the United States and Canada, call 1-800-487-6809 from a touch-tone phone.
  - Outside the United States and Canada, call (650) 596-4419 from a touch-tone phone.

  Have your fax number ready. Press 1 to order an index of available documents and have it faxed to you. Press 2 to have up to five documents faxed to you.

Preventing Non-Specific Amplification

Overview

The DNA amplification capability of the PCR process and the non-specific detection format of SYBR Green chemistry make special laboratory practices necessary. Small levels of DNA carryover from samples with high DNA concentrations, from the DNA Template Controls, or from previous PCR amplifications can result in product even in the absence of added template DNA. Because the indicator dye will bind to any double-stranded DNA, unintended products will result in a positive signal.

See the references in Appendix B on page 20 for more information on the polymerase chain reaction and the prevention of unintended products.

Hot Start PCR

To improve PCR specificity and sensitivity by controlling mispriming events, the Hot Start technique was introduced (Faloona *et al.*, 1990). Hot Start PCR is a simple modification of the original PCR process where the amplification reaction is started at an elevated temperature. This was initially performed manually, by adding an essential component of the reaction to the reaction mixture only after that mixture had been heated to an elevated temperature. However, this approach was often cumbersome and time consuming, especially when using large numbers of samples.

AmpliTaq Gold DNA Polymerase

Recently, PE Applied Biosystems introduced a new PCR enzyme, AmpliTaq Gold® DNA Polymerase, to perform an automated, convenient, and efficient Hot Start. AmpliTaq Gold DNA Polymerase is a chemically modified form of AmpliTaq® DNA Polymerase. The modification renders the enzyme inactive.

Upon thermal activation, the modifier is released, resulting in active enzyme. The high-temperature incubation step required for activation ensures that active enzyme is generated only at temperatures where the DNA is fully denatured.

When AmpliTaq Gold DNA Polymerase is added to the reaction mixture at room temperature, the inactive enzyme is not capable of primer extension. Any low-stringency mispriming events that may have occurred will not be enzymatically extended and subsequently amplified.

AmpliTaq Gold DNA Polymerase can be introduced into existing amplification systems with only minimal alterations to the reaction protocol, resulting in higher specificity, sensitivity, and product yield.

AmpErase UNG

AmpErase uracil-N-glycosylase (UNG) is a pure nuclease-free, 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. This gene has been inserted into an *E. coli* host to direct expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UNG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo *et. al*., 1990).

AmpErase UNG treatment can prevent the reamplification of carryover PCR products. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50-µL reaction.

General PCR Practices

Please follow these recommended procedures:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipet or aerosol-resistant pipet tips
- Clean lab benches and equipment periodically with 10% bleach solution.

Fluorescent Contaminants

Since fluorescent contaminants can interfere with this assay and give false-positive results, it may be necessary to include a No Amplification Control tube that contains sample, but no enzyme. If the absolute fluorescence of the No Amplification Control is greater than that of the No Template Control after PCR, fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler.

Agarose Gels to Check PCR Product Purity

The absence of non-specific amplification can be confirmed by analyzing the PCR amplification products by agarose gel electrophoresis.

Step	Action
1	Load 12–15 µL of sample per well on an ethidium bromide-stained 4% NuSieve 3:1 agarose gel (FMC, P/N 54928).
2	♦ For PCR fragments <100 bp, run the gel at 80–100 V for 45–60 minutes. ♦ For PCR fragments 100–250 bp, run the gel at 100–115 V for 1–1.5 hours.
3	Run samples 1/3–1/2 the length of the gel, without letting the dye run off the bottom of the gel. Use a UV lamp to check the migration of the samples.

Amplifying Custom Target Sequences for Quantitation

Overview

We recommend the following steps for the development of quantitative PCR assays:

Step	Action	See page
1	Install Primer Express Software	–
2	Identify Target Sequence and Amplicon Size	10
3	Design Primers	10
4	Order Reagents	11
5	Quantitate Primers	11
6	Perform RT Reactions for Two-Step RT-PCR	12
7	Optimize Primer Concentrations for Two-Step RT-PCR	13

For best results, use Primer Express primer design software, PE Applied Biosystems reagents, and the universal thermal cycling parameters given on page 14.

Identify Target Sequence and Amplicon Size

A target template is a DNA, cDNA, or plasmid containing the nucleotide sequence of interest.

Design primers to amplify short segments of DNA within the target sequence. These short segments are called amplicons. Shorter amplicons work the most efficiently: consistent results are obtained for amplicon size ranges from 50–150 bp.

Design Primers

Design primers using Primer Express software as described in the "DNA PCR" section of the *Primer Express Applications-Based Primer Design Software User's Manual* (P/N 4303014).

Follow these guidelines:

- Primers can be designed as close as possible to each other provided that they do not overlap.
- Keep the GC content in the 20–80% range.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- When using Primer Express software, the T_m should be 58–60 °C.

- The five nucleotides at the 3´ end should have no more than two G and/or C bases.

Order Reagents

See “Materials Required But Not Supplied” on page 4 for a list of required reagents and equipment.

Quantitate Primers

Use a spectrophotometric method to determine the concentrations of the primers received:

- Measure the absorbance at 260 nm of a 1:100 dilution of each oligonucleotide in TE buffer.
- Calculate the oligonucleotide concentration (C) in µM using the method shown in Table 3.

Table 3. Extinction Coefficient Calculation

Chromophore	**Extinction Coefficient**	**Number**	**Extinction Coefficient Contribution**
A	15,200	1	15,200
C	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
Total	–	–	167,950

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette pathlength × oligonucleotide concentration/100

0.13 = 167,950 $M^{-1}cm^{-1} \times 0.3\ cm \times C/100$

C = 258 µM

Perform RT Reactions for Two-Step RT-PCR

If you are working with RNA, you will need to generate a cDNA by reverse transcription-PCR (RT-PCR). The procedure for generating cDNA using the TaqMan® Reverse Transcription Reagents (P/N N808-0234) is described below. Refer to the *TaqMan Gold RT-PCR Kit Protocol* (P/N 402876) for more information on RT-PCR.

RT Reaction Mix

Component	**Volume/Tube (µL)**	**Final Concentration**
10X TaqMan RT Buffer	10	1X
25 mM $MgCl_2$	22	5.5 mM
deoxyNTPs mixture	20	500 µM of each dNTP
Random Hexamer[a]	5	2.5 µM
RNase Inhibitor	2	0.4 U/µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	2.5	1.25 U/µL
RNA Sample + RNase-free H_2O	38.5	10 pg–2 µg
Total	100	–

[a] Random hexamers, oligo $d(T)_{16}$, or sequence-specific reverse primers can be used for primers of cDNA synthesis.

Thermal Cycling Parameters for RT Reactions

Step	Incubation[a]	Reverse Transcription	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Time	10 min	30 min	5 min
Temperature	25 °C	48 °C	95 °C

[a] If using random hexamers or oligo $d(T)_{16}$ primers for first-strand cDNA synthesis, a primer incubation step is necessary at 25 °C for 10 minutes.

Perform RT Reactions

Step	**Action**
1	Prepare the RT Reaction Mix by combining all the nonenzymatic components listed in “RT Reaction Mix” above.
2	Vortex briefly.

Perform RT Reactions *(continued)*

Step	Action
3	Add the enzymatic components (*e.g.*, MultiScribe® Reverse Transcriptase, RNase Inhibitor) and the RNA.
4	Mix the components by inverting the microcentrifuge tube.
5	Transfer the contents to a MicroAmp Optical tube or multiple wells of a MicroAmp Optical 96-Well Reaction Plate.
6	Using MicroAmp Optical Caps, cap the tubes and briefly centrifuge to remove air bubbles and collect the liquid at the bottom of the tube. Transfer the plates to the thermal cycler block.
7	Perform RT as described in “Thermal Cycling Parameters for RT Reactions” on page 12.
8	Remove the 96-Well Reaction Plate after thermal cycling is complete.

Optimize Primer Concentrations for Two-Step RT-PCR

The purpose of this procedure is to determine the minimum primer concentrations giving the maximum ΔR_n and minimum non-specific amplification. The reaction volumes are 50 µL. Use 10–100 ng of genomic DNA or 1–10 ng of cDNA template.

The ABI PRISM 7700 Sequence Detector can provide additional data for optimization using the minimum threshold cycle (C_T). See “Real Time Detection” on page 3 for more information regarding C_T.

PCR Master Mix for Primer Optimization

Component	Volume per 50-µL Reaction (µL)	Volume per 100 Reactions (µL)
10X SYBR PCR Buffer	5.0	500
25 mM $MgCl_2$	6.0	600
dNTP Blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5.0 mM dUTP)	4.0	400
AmpliTaq Gold (5 U/µL)	0.25	25
AmpErase UNG (1 U/µL)	0.50	50
Total Volume	15.75	1575

Plate Configuration for Primer Optimization

Wells	PCR Master Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template	Deionized Water (µL)
A1–A4	15.75	0.5	0.5	5.0	28.25
A5–A8	15.75	0.5	3.0	5.0	25.75
A9–A12	15.75	0.5	9.0	5.0	19.75
B1–B4	15.75	3.0	0.5	5.0	25.75
B5–B8	15.75	3.0	3.0	5.0	23.25
B9–B12	15.75	3.0	9.0	5.0	17.25
C1–C4	15.75	9.0	0.5	5.0	19.75
C5–C8	15.75	9.0	3.0	5.0	17.25
C9–C12	15.75	9.0	9.0	5.0	11.25
D1–D4	15.75	0.5	0.5	0	33.25
D5–D8	15.75	0.5	3.0	0	30.75
D9–D12	15.75	0.5	9.0	0	24.75
E1–E4	15.75	3.0	0.5	0	30.75
E5–E8	15.75	3.0	3.0	0	28.25
E9–E12	15.75	3.0	9.0	0	22.25
F1–F4	15.75	9.0	0.5	0	24.75
F5–F8	15.75	9.0	3.0	0	22.25
F9–F12	15.75	9.0	9.0	0	16.25

Thermal Cycling Parameters for Primer Optimization

Times and Temperatures			
AmpErase UNG Incubation	AmpliTaq Gold Activation	Each of 40 Cycles	
		Melt	Anneal/Extend
HOLD	HOLD	CYCLE	
2 min. 50 °C	10 min. 95 °C	15 sec. 95 °C	1 min. 60 °C

Determine the Optimal Primer Concentrations

<table>
<tr><th>Step</th><th colspan="4">Action</th></tr>
<tr><td rowspan="6">1</td><td colspan="4">Prepare a PCR master mix to run four replicates of each of the nine conditions as shown. The master mix is described in “PCR Master Mix for Primer Optimization” on page 13.</td></tr>
<tr><th rowspan="2">Reverse Primer (nM)</th><th colspan="3">Forward Primer (nM)</th></tr>
<tr><th>50</th><th>300</th><th>900</th></tr>
<tr><th>50</th><td>50/50</td><td>300/50</td><td>900/50</td></tr>
<tr><th>300</th><td>50/300</td><td>300/300</td><td>900/300</td></tr>
<tr><th>900</th><td>50/900</td><td>300/900</td><td>900/900</td></tr>
<tr><td>2</td><td colspan="4">Load the plate for both a template and a No Template Control (NTC) matrix as shown in “Plate Configuration for Primer Optimization” on page 14.</td></tr>
<tr><td>3</td><td colspan="4">Place the plate in the ABI PRISM 7700 Sequence Detector. Use the thermal cycling conditions in “Thermal Cycling Parameters for Primer Optimization” on page 14. Confirm that a SYBR Green Pure Dye spectrum is accessed by the SDS software before starting the run.</td></tr>
<tr><td>4</td><td colspan="4">At the end of the run:
♦ Tabulate the results for the yield. This analysis will identify the optimum concentrations of primers for PCR yield.
♦ Tabulate the results for the C_T value. This analysis will identify the optimum primer concentrations for C_T and for the absence of non-specific amplification.</td></tr>
</table>

Note The absence of non-specific amplification should be confirmed by analyzing the PCR products by agarose gel electrophoresis (see page 9).

Optimize Primer Concentrations for One-Step RT-PCR

One-step RT-PCR reactions with MultiScribe Reverse Transcriptase are optimized following a similar protocol. In this case, the “PCR Master Mix for Primer Optimization” on page 13 is replaced with the corresponding One-Step RT-PCR Master Mix. See the table below.

Note Procedures for one-step RT-PCR are the same as for two-step RT-PCR. See “Optimize Primer Concentrations for Two-Step RT-PCR” on page 13.

One-Step RT-PCR Master Mix for Primer Optimization

Component	Volume per 50-µL Reaction (µL)	Volume per 100 wells (µL)
10X SYBR PCR Buffer	5.0	500
25 mM $MgCl_2$	6.0	600
dNTP Blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5.0 mM dUTP)	6.0	600
AmpliTaq Gold (5 U/µL)	0.25	25
MultiScribe Reverse Transcriptase (50 U/µL)	0.25	25
RNase Inhibitor	1.0	100
Total Volume	18.50	1850

Appendix A. Absolute and Relative Quantitation

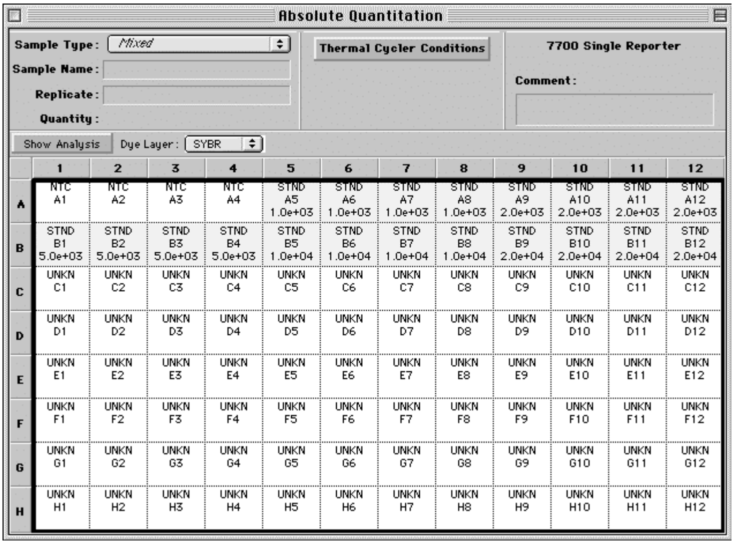
Overview

Two types of quantitation are possible with the SYBR Green reagents:

- Relative quantitation of a target against an internal standard is particularly useful for gene expression measurements.
- Absolute quantitation is possible if the isolation procedure and sample contents do not impact the PCR results. The quantitation of genomic DNA may lend itself for absolute quantitation against a standard curve.

Absolute Quantitation of Plasmids or Genomic DNA

Absolute quantitation compares the C_T of an unknown sample against a standard curve with known copy numbers. Absolute quantitation on the ABI PRISM 7700 Sequence Detector can be carried out with the plate configuration shown below:



Sample Type: Mixed
Sample Name:
Replicate:
Quantity:
Comment:
Dye Layer: SYBR

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC A1	NTC A2	NTC A3	NTC A4	STND A5 1.0e+03	STND A6 1.0e+03	STND A7 1.0e+03	STND A8 1.0e+03	STND A9 2.0e+03	STND A10 2.0e+03	STND A11 2.0e+03	STND A12 2.0e+03
B	STND B1 5.0e+03	STND B2 5.0e+03	STND B3 5.0e+03	STND B4 5.0e+03	STND B5 1.0e+04	STND B6 1.0e+04	STND B7 1.0e+04	STND B8 1.0e+04	STND B9 2.0e+04	STND B10 2.0e+04	STND B11 2.0e+04	STND B12 2.0e+04
C	UNKN C1	UNKN C2	UNKN C3	UNKN C4	UNKN C5	UNKN C6	UNKN C7	UNKN C8	UNKN C9	UNKN C10	UNKN C11	UNKN C12
D	UNKN D1	UNKN D2	UNKN D3	UNKN D4	UNKN D5	UNKN D6	UNKN D7	UNKN D8	UNKN D9	UNKN D10	UNKN D11	UNKN D12
E	UNKN E1	UNKN E2	UNKN E3	UNKN E4	UNKN E5	UNKN E6	UNKN E7	UNKN E8	UNKN E9	UNKN E10	UNKN E11	UNKN E12
F	UNKN F1	UNKN F2	UNKN F3	UNKN F4	UNKN F5	UNKN F6	UNKN F7	UNKN F8	UNKN F9	UNKN F10	UNKN F11	UNKN F12
G	UNKN G1	UNKN G2	UNKN G3	UNKN G4	UNKN G5	UNKN G6	UNKN G7	UNKN G8	UNKN G9	UNKN G10	UNKN G11	UNKN G12
H	UNKN H1	UNKN H2	UNKN H3	UNKN H4	UNKN H5	UNKN H6	UNKN H7	UNKN H8	UNKN H9	UNKN H10	UNKN H11	UNKN H12

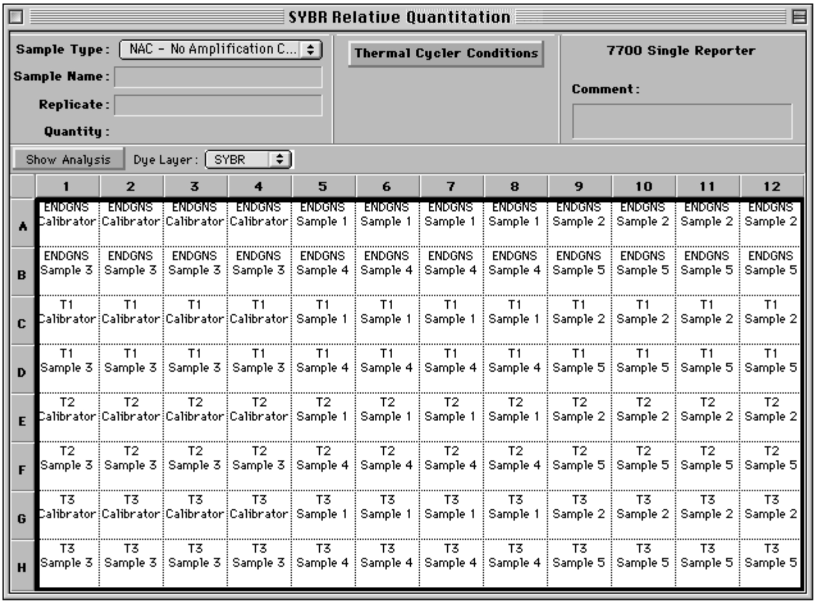
Each well in this plate receives 45 µL of a master mix with 3 mM Mg^{2+}, forward primer, and reverse primer concentrations and 5 µL of RNAse-free water (NTC wells), template standard (STND wells) or unknown template (UNKN wells).

Quantitation of cDNA Relative to a Calibrator Sample

Gene expression can be measured by the quantitation of cDNA converted from a messenger RNA corresponding to this gene relative to a calibrator sample serving as a physiological reference. In a typical experiment, gene expression levels are studied as a function of either a treatment of cells in culture, of patients, or of tissue type. The calibrator sample in each case is the cDNA from either the untreated cells or patients, or a specific tissue type.

All quantitations are also normalized to an endogenous control such as 18S rRNA to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. All amplicons in these determinations should follow the amplicon design criteria defined previously around the Primer Express software. Refer to *User Bulletin #2* (P/N 4303859) for additional information about relative quantitation.

The plate below shows a typical configuration for relative quantitation with SYBR Green reagents:



Sample Type: NAC – No Amplification C...
Sample Name:
Replicate:
Quantity:
Comment:
Dye Layer: SYBR

	1	2	3	4	5	6	7	8	9	10	11	12
A	ENDGNS Calibrator	ENDGNS Calibrator	ENDGNS Calibrator	ENDGNS Calibrator	ENDGNS Sample 1	ENDGNS Sample 1	ENDGNS Sample 1	ENDGNS Sample 1	ENDGNS Sample 2	ENDGNS Sample 2	ENDGNS Sample 2	ENDGNS Sample 2
B	ENDGNS Sample 3	ENDGNS Sample 3	ENDGNS Sample 3	ENDGNS Sample 3	ENDGNS Sample 4	ENDGNS Sample 4	ENDGNS Sample 4	ENDGNS Sample 4	ENDGNS Sample 5	ENDGNS Sample 5	ENDGNS Sample 5	ENDGNS Sample 5
C	T1 Calibrator	T1 Calibrator	T1 Calibrator	T1 Calibrator	T1 Sample 1	T1 Sample 1	T1 Sample 1	T1 Sample 1	T1 Sample 2	T1 Sample 2	T1 Sample 2	T1 Sample 2
D	T1 Sample 3	T1 Sample 3	T1 Sample 3	T1 Sample 3	T1 Sample 4	T1 Sample 4	T1 Sample 4	T1 Sample 4	T1 Sample 5	T1 Sample 5	T1 Sample 5	T1 Sample 5
E	T2 Calibrator	T2 Calibrator	T2 Calibrator	T2 Calibrator	T2 Sample 1	T2 Sample 1	T2 Sample 1	T2 Sample 1	T2 Sample 2	T2 Sample 2	T2 Sample 2	T2 Sample 2
F	T2 Sample 3	T2 Sample 3	T2 Sample 3	T2 Sample 3	T2 Sample 4	T2 Sample 4	T2 Sample 4	T2 Sample 4	T2 Sample 5	T2 Sample 5	T2 Sample 5	T2 Sample 5
G	T3 Calibrator	T3 Calibrator	T3 Calibrator	T3 Calibrator	T3 Sample 1	T3 Sample 1	T3 Sample 1	T3 Sample 1	T3 Sample 2	T3 Sample 2	T3 Sample 2	T3 Sample 2
H	T3 Sample 3	T3 Sample 3	T3 Sample 3	T3 Sample 3	T3 Sample 4	T3 Sample 4	T3 Sample 4	T3 Sample 4	T3 Sample 5	T3 Sample 5	T3 Sample 5	T3 Sample 5

In this configuration, endogenous controls are run for each sample in rows A and B and targets T1–T3 are run on rows C–H. Each row shows wells corresponding to a calibrator sample and up to five experimental samples. All wells are run in quadruplicate. The analysis of relative quantitation for a target template in samples 1–5 requires the following:

- The mean C_T value of the replicate wells run for each sample
- The difference (ΔC_T) between the mean C_T values of the samples in the target wells (T1–T3) and those of the endogenous controls in the ENDGNS wells
- The difference ($\Delta\Delta C_T$) between the ΔC_T values of the samples for each target and the mean C_T value of the Calibrator for that target

The relative quantitation value is expressed as $2^{-\Delta\Delta C_T}$.

A semilog plot of these values provides a graphical representation of the relative quantitation values obtained in this plate.

Appendix B. References

Ausubel, F.M., Brent, R., Kingstin, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds. 1987. *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York.

Delort, A.-M., Duplaa, A.-M., Molko, D., and Teoule, R. 1985. Excision of uracil residues in DNA: mechanism of action of *Escherichia coli* and *Micrococcus luteus* uracil-DNA glycosylases. *Nucleic Acids Res.* 13: 319–335.

Faloona, F., Weiss, S., Ferre, S., and Mullis, K. 1990. Abstract 1019, Sixth International Conference on AIDS, June 20–24, 1990, San Francisco, CA.

Gelfand, D.H., and White, T.J. 1990. Thermostable DNA Polymerases. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds. *PCR Protocols. A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, pp. 129–141.

Higuchi, R., Dollinger, G., Walsh, P.S., and Griffith, R. 1992. Simultaneous amplification and detection of specific DNA sequences. *Bio/Technology* 10: 413–417.

Kwok, S. 1990. Procedures to minimize PCR-product carry-over. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds. *PCR Protocols. A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, pp. 142–145.

Kwok, S., and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339: 237–238.

Longo, N., Berninger, N.S., and Hartley, J.L. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93: 125–128.

Mullis, K.B., and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. In: Wu, R., ed. *Methods in Enzymology*, Vol. 155, Academic Press, Inc., San Diego, CA, pp. 335–350.

Orrego, C. 1990. Organizing a laboratory for PCR work. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds. *PCR Protocols. A*

Guide to Methods and Applications, Academic Press, Inc., San Diego, CA, pp. 447–454.

PCR Technical Information. 1996. In: *Perkin-Elmer Systems, Reagents & Consumables. 1996-1997*, Foster City, CA.

Saiki, R.K., Scharf, S.J., Faloona, F.A., Mullis, K.B., Horn, G.T., Erlich, H.A., and Amheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350–1354.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487–491.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press.

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