USER GUIDE

SYBR® Select Master Mix for CFX

Catalog Numbers 4472937, 4472942, 4472952, 4472953, 4472954, and 4472947

Revision Date 23 April 2012 (Rev A)

Publication Part Number 4474514

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Contents

Product Information

About the Reagent

The SYBR® Select Master Mix for CFX, is formulated to provide superior specificity and sensitivity. It is supplied in a convenient 2X concentration premix to perform real-time PCR using SYBR® GreenER™ dye. The master mix contains:

- SYBR® GreenER™ Dye
- AmpliTaq® DNA Polymerase, UP (Ultra Pure) with a proprietary hot start mechanism
- Heat-labile Uracil-DNA Glycosylase (UDG)
- dNTP blend containing dUTP/dTTP
- Optimized buffer components

The user only needs to provide primers, template, and water.

Hot Start

The AmpliTaq® DNA Polymerase, UP is provided in an inactive state to automate the hot start PCR technique and allow flexibility in the reaction setup, including pre-mixing of PCR reagents at room temperature.

The polymerase is equipped with a proprietary hot start mechanism that provides improved specificity. The polymerase is re-activated after a 2 minute incubation at 95°C.

UDG

SYBR® Select Master Mix for CFX contains heat-labile uracil-DNA glycosylase (UDG). UDG is also known as uracil-N-glycosylase (UNG).

Treatment with heat-labile UDG can prevent the reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded amplicons (Longo et al., 1990). Heat-labile UDG prevents reamplification of carryover PCR products in an assay if all previous PCR for that assay was performed using a dUTP-containing master mix. See “Prevent Contamination and Nonspecific Amplification” on page 10 for more information about UDG.

PCR products are stable for up to 72 hours post-amplification using master mixes containing heat-labile UDG. Unlike standard UDG, heat-labile UDG is completely inactivated prior to amplification.

dUTP/dTTP

A blend of dUTP/dTTP is included to enable UDG activity and maintain optimal PCR results.

SYBR® GreenER™

The SYBR® GreenER™ dye detects PCR products by binding to double stranded DNA formed during PCR (see Chemistry Overview section). The SYBR® GreenER™ dye provides both higher sensitivity and lower PCR inhibition than SYBR® Green I dye. It can be used on real-time PCR instruments calibrated for SYBR® Green I dye without any change of filters or settings.

Real-Time Instruments

SYBR® Select Master Mix for CFX can be used to run experiments with the CFX Real-Time PCR Detection Systems:

- CFX96 Touch™ Real-Time PCR Detection System
- CFX384 Touch™ Real-Time PCR Detection System

About This Protocol

This protocol provides:

- Background information about gene quantification assays
- A list of equipment and materials for using the SYBR® Select Master Mix for CFX
- Procedures for using the SYBR® Select Master Mix for CFX

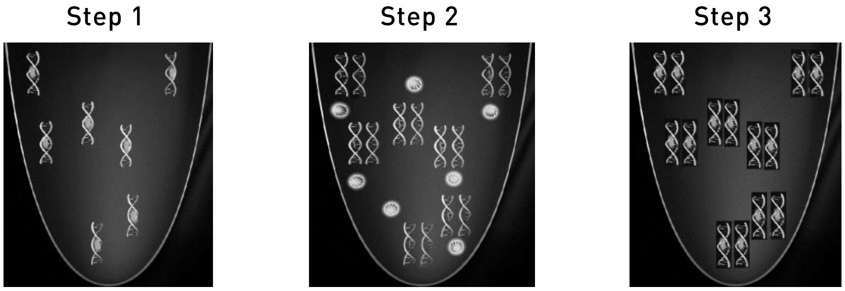
Chemistry Overview

How the SYBR® GreenER™ Dye Chemistry Works

The SYBR® GreenER™ dye is used to detect PCR products by binding to double-stranded DNA formed during PCR. The process works as follows:

1. When SYBR® Select Master Mix for CFX is added to a sample, SYBR® GreenER™ dye immediately binds to all double-stranded DNA.
2. During the PCR, AmpliTaq® DNA Polymerase, UP amplifies the target sequence, which creates the PCR product, or "amplicon."
3. The SYBR® GreenER™ dye then binds to each new copy of double-stranded DNA.
4. As the PCR progresses, more amplicon is created. Because the SYBR® GreenER™ dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportional to the amount of double-stranded PCR product produced.

The following figure illustrates this process.



The SYBR® GreenER™ dye within the SYBR® Select Master Mix for CFX immediately binds with all double-stranded DNA

During PCR, AmpliTaq® DNA Polymerase, UP amplifies each target.

The SYBR® GreenER™ dye then binds to each new copy of double-stranded DNA.

Figure 1 Representation of how the SYBR® GreenER™ dye acts on double-stranded DNA during one extension phase of PCR

Using the Master Mix in Two-Step RT-PCR

When performing a two-step RT-PCR reaction, total or mRNA must first be transcribed into cDNA:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the High-Capacity cDNA Reverse Transcription Kit or SuperScript® VILO™ cDNA Synthesis Kit (see page 9).
2. In the PCR step, PCR products are synthesized from cDNA samples using the SYBR® Select Master Mix for CFX.

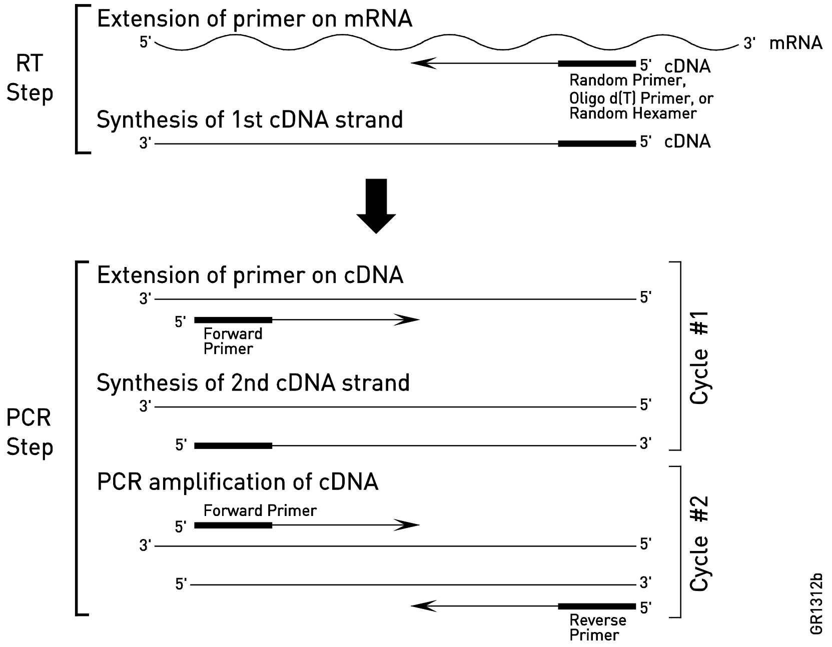


Figure 2 Two-step RT-PCR

Contents and Storage

Contents

The SYBR® Select Master Mix for CFX is supplied in a 2X concentration.

Item	Part Number	Contents
Mini-Pack	4472937	One 1-mL tube (100 × 20-µL reactions)
1-Pack	4472942	One 5-mL tube (500 × 20-µL reactions)
2-Pack	4472952	2 × 5-mL tubes (1000 × 20-µL reactions)
5-Pack	4472953	5 × 5-mL tubes (1500 × 20-µL reactions)
10-Pack	4472954	10 × 5-mL tubes (5000 × 20-µL reactions)
Bulk Pack	4472947	One 50-mL tube (5000 × 20-µL reactions)

Storage

Store the SYBR® Select Master Mix for CFX at 2°C to 8°C.

Required Materials

Plates and Optical Seals

Refer to the manual supplied by the instrument manufacturer for details on selecting the plate appropriate for your real-time instrument. Seal plates with the appropriate optical adhesive film.

Other Kits

Item	Catalog number
High Capacity cDNA Reverse Transcription Kit:	
• 200 reactions • 200 reactions with RNase Inhibitor • 1000 reactions • 1000 reactions with RNase Inhibitor	4368814 4374966 4368813 4374967
SuperScript® VILO™ cDNA Synthesis Kit:	
• 50 reactions • 250 reactions	4453650 4453651

Other Consumables

Item	Source
Centrifuge with adapter for 96-well plates *or* Centrifuge with adapter for 384-well plates	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement or air-displacement	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Prevent Contamination and Nonspecific Amplification

Overview

PCR assays require special laboratory practices to avoid false positive amplifications. The high throughput and repetition of these assays can lead to amplification of a single DNA molecule.

Using UDG to Minimize Reamplification Carryover Products

SYBR® Select Master Mix for CFX contains heat-labile uracil-DNA glycosylase (UDG). UDG is also known as uracil-N-glycosylase (UNG). Treatment with heat-labile UDG is useful in preventing the reamplification of carryover PCR products.

The heat-labile UDG used in the SYBR® Select Master Mix for CFX is a 26-kDa recombinant enzyme derived from the thermolabile UDG gene isolated from marine bacteria, and expressed in *E. coli*.

UDG 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).

Using NTC Controls

No Template Control (NTC) reactions can be used to identify PCR contamination. NTC reactions contain all reaction components (SYBR® Select Master Mix for CFX, primers, water) except sample, and therefore should not return a C_T value.

Design Primers to Avoid Primer-Dimers

Use primers that contain dA nucleotides near the 3´ ends so that any primer-dimer generated is efficiently degraded by UDG at least as well as any dU-containing PCR products. The farther a dA nucleotide is from the 3´ end, the more likely partially degraded primer-dimer molecules can serve as templates for a subsequent PCR amplification.

Production of primer-dimers could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, consider using primers with 3´ terminal dU-nucleotides. Single-stranded DNA with terminal dU nucleotides are not substrates for UDG (Delort et al., 1985) and, therefore, the primers are not degraded. Biotin-dUMP derivatives are not substrates for UDG.

For more information about designing primers, see "Guidelines for Designing Primers" on page 26.

Do not use UDG in subsequent amplifications of dU-containing PCR template, such as in nested PCR protocols. The UNG degrades the dU-containing PCR product, preventing further amplification.

PCR Good Laboratory Practices

When preparing samples for PCR amplification:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves.
- 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

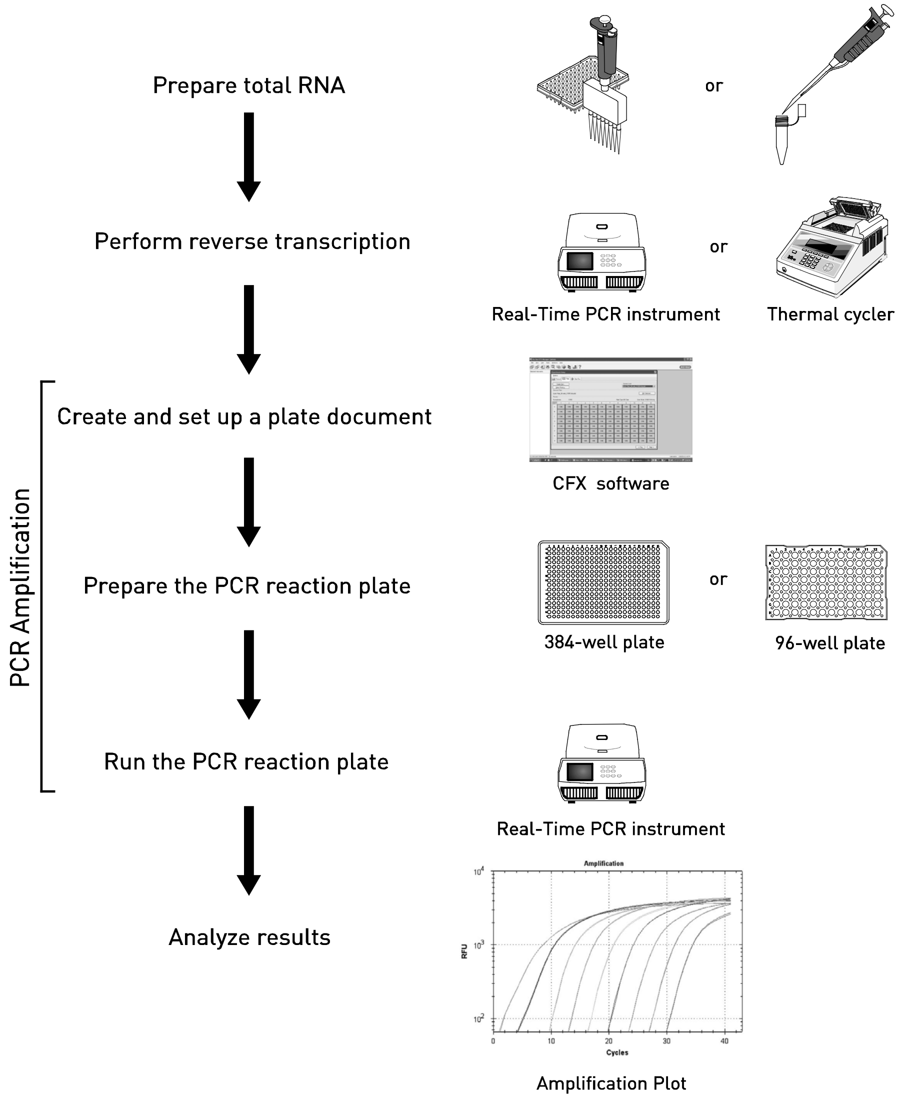
PCR Good Laboratory Practices, Continued

- 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 pipette or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with a 10% bleach solution.

Methods

Procedural Overview

This diagram is an overview of the procedures for performing gene expression experiments.



Prepare the Template

Examine RNA Template Quality

After isolating the template, examine its quality and quantity and store it properly.

Before using the SYBR® Select Master Mix for CFX, you need to synthesize single-stranded cDNA from total RNA or mRNA samples. For optimal performance, the RNA should be:

- Between 0.002 and 0.2 µg/µL
- Less than 0.005% of genomic DNA by weight
- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer
- Free of RNase activity

  IMPORTANT! If you suspect that the RNA contains RNase activity, add RNase inhibitor to the reverse transcription reaction at a final concentration of 1.0 U/µL. Adding RNase inhibitor to the reverse transcription reaction is not necessary if the RNA is purified using the 6100 Nucleic Acid PrepStation and nucleic acid purification reagents.
- Nondenatured
- **IMPORTANT!** It is not necessary to denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some gene targets.

Examine DNA Template Quality

Use both of the following methods to examine DNA quality:

- **Agarose gel electrophoresis –** Purified DNA should run as a single band on an agarose gel. Agarose gels reveal contaminating DNAs and RNAs, but not proteins.
- **Spectrophotometry –** The A_{260}/A_{280} ratio should be 1.8 to 2.0. Smaller ratios usually indicate contamination by protein or organic chemicals. Spectrophotometry can reveal protein contamination, but not DNA or RNA contamination.

Quantitate the Template

Template quantitation is critical for successful PCR reactions. The most common way to determine DNA quantity is to measure the absorbance (optical density or O.D.) of a sample at 260 nm in a spectrophotometer.

One O.D. unit is the amount of a substance dissolved in 1.0 mL that gives an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. The wavelength is assumed to be 260 nm unless stated otherwise. A260 values can be converted into µg/µL using Beer´s Law:

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

The following formulas are derived from Beer's Law (Ausubel et al., 1998):

- Concentration of single-stranded DNA = $A_{260} \times 33$ µg/µL
- Concentration of double-stranded DNA = $A_{260} \times 50$ µg/µL
- Concentration of single-stranded RNA = $A_{260} \times 40$ µg/µL

Note: Absorbance measurements of highly concentrated (O.D. > 1.0) or very dilute (O.D. < 0.05) DNA or RNA samples can be inaccurate. Dilute or concentrate the DNA/RNA to obtain a reading within the acceptable range.

Store the Template

Store the templates as follows:

- Store purified RNA templates at −20°C or −70°C in RNase-free water.
- Store purified DNA templates at −20°C or −70°C in TE, pH 8.0.

Set Up the Plate Document

Select a Plate for PCR

Refer to the manual supplied by the instrument manufacturer for details on selecting the plate appropriate for your real-time instrument.

Configure the Plate Document

For information about configuring plate documents when performing real-time quantification, refer to the appropriate user guide supplied by the manufacturer.

Prepare the PCR Reaction Plate

General Guidelines

- For best results, it is recommended to perform four replicates of each reaction.
- Reaction mixes can be prepared depending upon your experimental requirements. Scale the components to be included in the reaction mix according to the number of reactions to be performed. Include an additional 10% of the reaction mix volume to account for variations in pipetting.
- If using smaller reaction volumes, scale all components of the reaction mix proportionally. Reaction volumes <10 µL are not recommended.

Reminder About Your Primers

Refer to page 26 for information about identifying target sequences and designing primers.

Note: Separate PCR thermal-cycling conditions are required for primers with a T_m <60°C

Reagent Handling and Preparation

Follow these guidelines to ensure optimal PCR performance. Prior to use:

- Mix the SYBR® Select Master Mix for CFX thoroughly by swirling the bottle.
- Place frozen cDNA samples and primers on ice to thaw. After the samples are thawed, vortex them, then centrifuge the tubes briefly.

CAUTION **CHEMICAL HAZARD**. SYBR® Select Master Mix for CFX (2X) may cause eye, skin and respiratory tract irritation. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Prepare the PCR Reactions

1. Prepare the appropriate number of reactions according to the volumes in the following table:

Component	384-Well Plate (10 µL/well)	96-Well Plates (20 µL/well)
SYBR® Select Master Mix for CFX (2X)	5 µL	10 µL
Forward and Reverse Primers[‡]	Variable	Variable
cDNA template + RNase-free water[§]	Variable	Variable
Total Volume	10 µL	20 µL

[‡] For optimal performance, use a range from 150–400 nM of each primer.

[§] For optimal performance, use up to 100 ng of cDNA for each reaction.

2. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
3. Transfer the appropriate volume of each reaction to each well of an optical plate.
4. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.

   Note: PCR can be performed on the reaction plate at any time up to 72 hours after completing the reaction setup when kept at room temperature.

Run the PCR Reaction Plate

Run the plate with the CFX real-time PCR system. See the appropriate instrument user guide for help with programming the thermal-cycling conditions or with running the plate.

To run the plate:

1. Place the reaction plate in the instrument.
2. Set the thermal cycling conditions using the default PCR thermal-cycling conditions specified in the following tables according to the melting temperature of your primers:

Standard Cycling Mode (Primer T_m ≥60°C)

Step	Temperature	Duration	Cycles
UDG Activation	50°C	2 min	Hold
AmpliTaq® DNA Polymerase, UP Activation	95°C	2 min	Hold
Denature	95°C	15 sec	40
Anneal/Extend	60°C	1 min	

Standard Cycling Mode Primer T_m <60°C

Step	Temperature	Duration	Cycles
UDG Activation	50°C	2 min	Hold
AmpliTaq® DNA Polymerase, UP Activation	95°C	2 min	Hold
Denature	95°C	15 sec	40
Anneal	55–60°C*	15 sec	
Extend	72°C	1 min	

*Anneal temperature should be set to the melting point for your primers.

3. Set the instrument to perform a default dissociation step.

   Note: A melt curve can be performed up to 72 hours after the real-time PCR run if the plate is stored in the dark, or up to 24 hours later if the plate is stored exposed to light.
4. Set the reaction volume appropriate for the type of plate being used for your PCR reaction.
5. Start the run.

Analyze Your Results

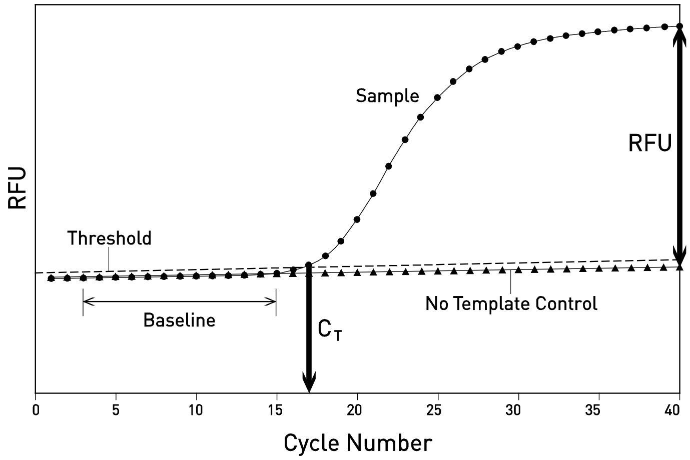
The general process for analyzing the data from gene expression assays requires that you:

- View the amplification plots.
- Adjust the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
- Use the standard curve method or the relative quantification ($\Delta\Delta C_T$) method to analyze the results.

Baseline and Threshold Values

Use the software provided with your instrument to automatically calculate or manually set the baseline and threshold for the amplification curves.

- Baseline refers to the initial cycles of PCR in which there is little change in fluorescence signal.
- The intersection of the threshold with the amplification plot defines the C_T in real-time PCR assays. The threshold is set above the background and within the exponential growth phase of the amplification curve.

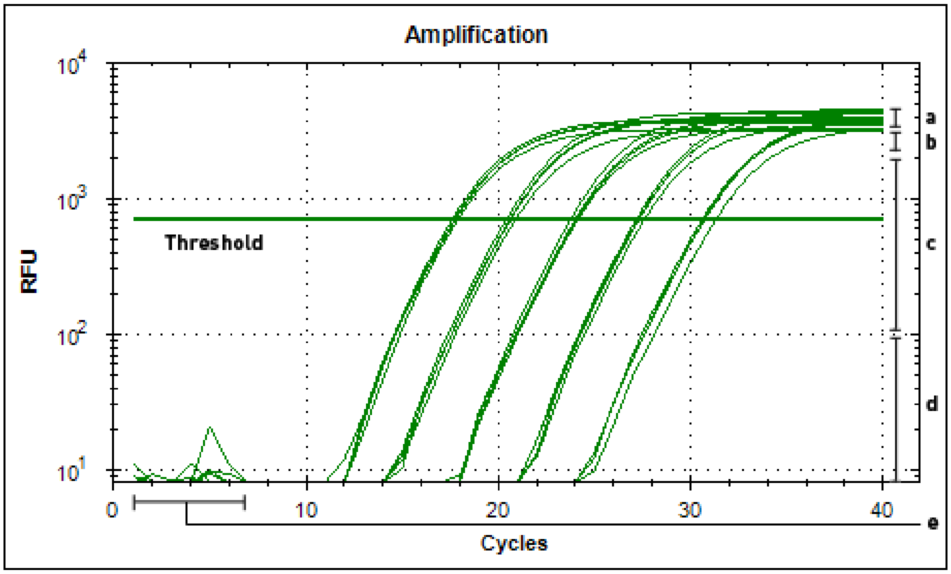


View the Amplification Plots

The instrument software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the “typical” amplification curve.

A typical amplification curve, as shown below, has a:

- Plateau phase (a)
- Linear phase (b)
- Exponential (geometric) phase (c)
- Background (d)
- Baseline (e)



Manually Adjust the Baseline and Threshold

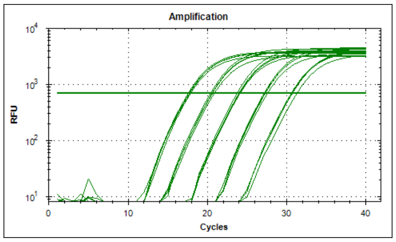
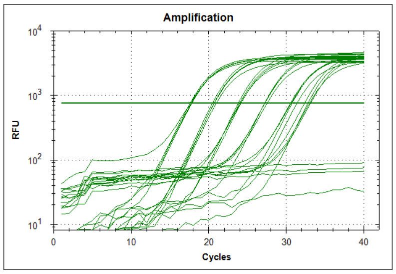
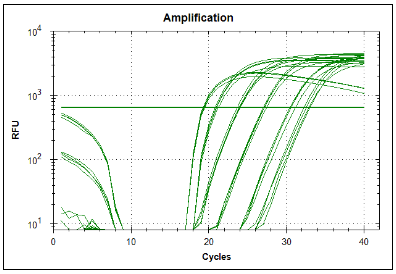
Experimental error (such as contamination or inaccurate pipetting) can produce data that deviate significantly from data for typical amplification curves. Such atypical data cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

Reviewing all baseline and threshold values after analysis of the study data is recommended. If necessary, adjust the values manually as described in the appropriate instrument user manual.

IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots.

Baseline Settings

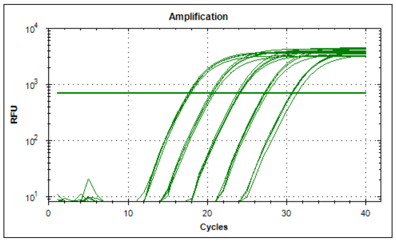
See the example amplification plots below to determine whether or not the baseline and threshold settings were correctly set.

Baseline Set Correctly The amplification curve begins after the maximum baseline. No adjustment necessary.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

Threshold Settings

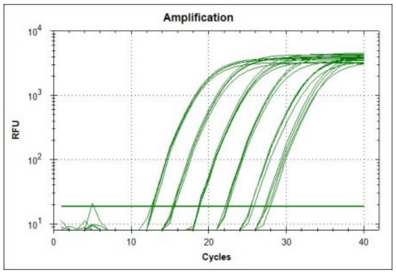
Threshold Set Correctly

The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.



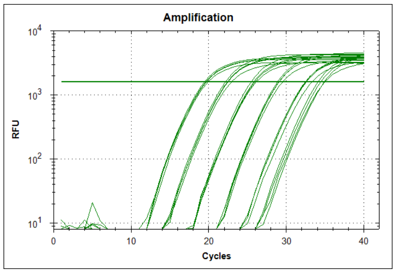
Threshold Set Too Low

The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Set the threshold up into the exponential phase of the curve.



Threshold Set Too High

The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Set the threshold down into the exponential phase of the curve.



Analyzing the Results

Using the SYBR® Select Master Mix for CFX, you can perform two types of quantitation: relative and absolute.

- Relative quantitation compares a target against an internal standard. You can perform relative quantitation using either the standard curve method or the comparative C_T method.
- Absolute quantitation compares the C_T of an unknown sample against a standard curve with known copy numbers.

Relative Quantitation Method

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

All quantitations are also normalized to an endogenous control (such as GAPDH) to account for variability in the initial concentration and quality of the total RNA, and in the conversion efficiency of the reverse transcription reaction.

Resources for Data Analysis

For more information about analyzing your data, refer to the appropriate instrument manual available from the instrument manufacturer.

Detect Nonspecific Amplification

Because SYBR® GreenER™ dye detects any double-stranded DNA, check for nonspecific product formation by using melt-curve or gel analysis.

Melt Curves

A melt curve is a graph that displays melt data from the amplicons of quantitative PCR runs. Change in fluorescence, due to a dye or probe interacting with double-stranded DNA, is plotted against temperature.

When to Generate Melt Curves

Note: Because of the presence of heat-labile UDG, you can generate a melt curve up to 72 hours after the real-time PCR run.

An Example

The melt curves below show typical primer-dimer formation. The specific product is shown with a melting temperature (T_m) of 80.5°C, but the primer-dimer has a characteristically lower T_m of 75°C.

Primer-dimers are most prevalent in NTC wells and sample wells containing a low concentration of template.

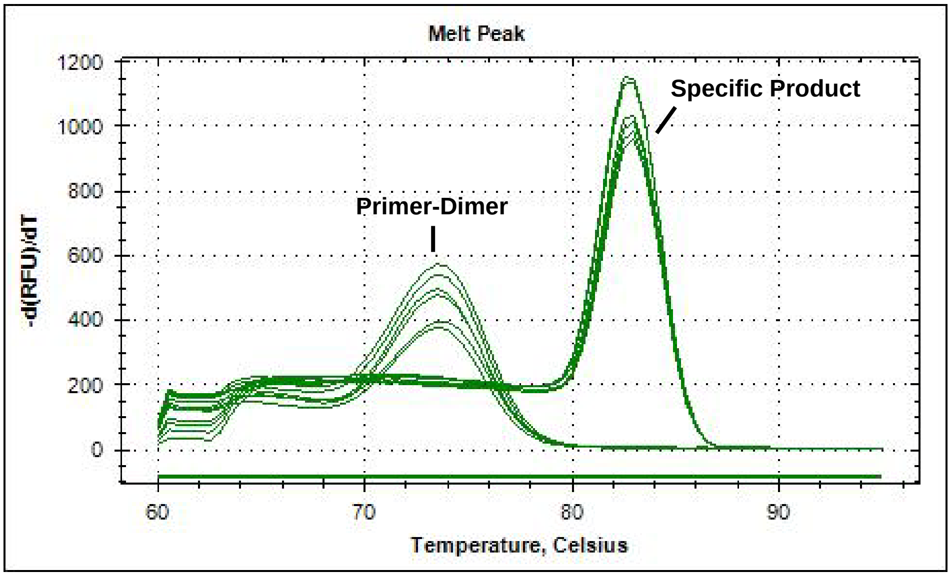


Figure 3 Example of two melt curves

(*Optional*) Check PCR Product Purity by Agarose Gel Electophoresis

Note: Because of the presence of heat-labile UDG, you can verify the absence of nonspecific amplification using agarose gel electrophoresis up to 72 hours after amplification.

1. Load 12 to 15 µL of sample per well on an ethidium bromide-stained agarose gel made with UltraPure™ Agarose 1000 (Cat. no. 16550-100):

PCR Fragment Size	% Agarose in TBE Buffer	% Agarose in TAE Buffer
<100 bp	5%	6%
100–250 bp	3%	4%

CHEMICAL HAZARD. Ethidium bromide causes eye, skin, and respiratory tract irritation and is a known mutagen (that is, it can change genetic material in a living cell and has the potential to cause cancer). Always use adequate ventilation such as that provided by a fume hood. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

2. Run the gel:

   For PCR fragments <100 bp, use 80 to 100 V for 45 to 60 min.

   For PCR fragments 100 to 250 bp, use 100 to 115 V for 1 to 1.5 h.

3. Run samples 1/3 to 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.

Troubleshoot

Observation	Possible Cause	Action
High C_T values/poor precision or failed PCR reactions	Insufficient cDNA template is present	Use up to 100 ng of cDNA template per 20-µL reaction.
	Quality of cDNA template is poor	• Quantify the amount of cDNA template. • Test the cDNA template for the presence of PCR inhibitors.
	Sample degradation	Prepare fresh cDNA, then repeat the experiment.
	Incorrect pipetting.	Prepare the reactions as described on page 16.
	Reduced number of PCR cycles in the thermal cycler protocol	Increase the number of PCR cycles to the default setting of 40 (see page 17).
	Primer-dimer formation and residual polymerase activity	• Prepare the reaction mixes and the reaction plate on ice. • To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, store the reaction plate at 4°C.
Low RFU values	Extension time is too short	Use the default thermal profile settings (see page 17).
	Primer-dimer formation and residual polymerase activity	• Prepare the reaction mixes and the reaction plate on ice. • To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, store the reaction plate at 4°C.

Observation	Possible Cause	Action
Extremely high RFU values	Evaporation	Make sure that the reaction plate is sealed completely, especially around the edges.
Lower RFU values obtained in early cycles	C_T value is less than 15	Adjust the upper baseline range to a value less than 15.
High variability across the reaction plate	Evaporation	Make sure that the reaction plate is sealed completely, especially around the edges.
High variability across replicates	Reaction mix was not mixed well	Mix the reaction mix gently by inversion, then centrifuge briefly before aliquoting to the reaction plate.

Appendix A

Identify Target Sequences and Design Primers

Identify Target Sequence and Amplicon Size

A target template is a DNA sequence, including cDNA, genomic DNA, or plasmid nucleotide sequence that you want to amplify.

Using Primer Express Software, you design primers to amplify amplicons (segments of DNA) within the target sequence. Shorter amplicons work best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Guidelines for Designing Primers

Using Primer Express® Software

Design primers using Primer Express Software as described in the P*rimer Express® Version 3.0 Getting Started Guide* (PN 4362460) and *Online Help*.

General Guidelines

- Do not overlap primer and probe sequences. The optimal primer length is 20 bases.
- Keep the GC content in the 30–70% range.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- Make sure the last five nucleotides at the 3´ end contain no more than two G and/or C bases.

If the template is...	Then...
DNA	Design the primers as described above.
plasmid DNA	
genomic DNA	
cDNA	Design the primers as described above. Also see "Select an Amplicon Site for cDNA" on page 27.
RNA	Design the primers as described above.

Select an Amplicon Site for cDNA

Selecting a good amplicon site ensures amplification of the target cDNA without co-amplifying the genomic sequence, pseudogenes, and related genes.

Guidelines

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair must be specific to the target gene; the primer pair does not amplify pseudogenes or other related genes.
- Design primers according to Primer Express Software guidelines.
- Test the amplicons, then select those that have the highest signal-to-noise ratio (that is, low C_T with cDNA and no amplification with no template control or genomic DNA).
- If no good sequence is found, you may need to examine the sequence and redesign the amplicon or to screen for more sites.

If the gene you are studying does not have introns, then you cannot design an amplicon that amplifies the mRNA sequence without amplifying the genomic sequence. In this case, you may need to run RT minus controls.

Optimize Primer Concentrations for PCR

Overview

By independently varying the forward and reverse primer concentrations, you can identify the primer concentrations that provide optimal assay performance. The primer concentrations you select should provide a low C_T and a high RFU when run against the target template, but should not produce nonspecific product formation with NTCs.

Quantitate the Primers

1. Measure the absorbance (at 260 nm of a 1:100 dilution) of each primer oligonucleotide in TE buffer.
2. Calculate the sum of extinction coefficient contributions for each primer:

   extinction coefficient contribution = Σ(extinction coefficient × number of bases in oligonucleotide sequence)

   See "An Example Calculation of Primer Concentration" on page 28 for an example calculation.
3. Calculate the oligonucleotide concentration in µM for each primer:

   absorbance at 260 nm = sum of extinction coefficient contribution × cuvette pathlength × concentration/100

   Rearrange to solve for concentration:

   concentration = 100[absorbance at 260 nm / (sum of extinction coefficient contribution × cuvette pathlength)]

An Example Calculation of Primer Concentration

In this example, the concentration of a primer (in TE buffer, diluted 1:100), with the sequence CGTACTCGTTCGTGCTGC is calculated using the following values:

Chromophore	Extinction Coefficient	Number of Specific Chromophores in Example Sequence	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
Total	—	—	167,950

measured absorbance at 260 nm = 0.13

sum of extinction coefficient = 167,950 $M^{-1}cm^{-1}$ contributions for probe

cuvette pathlength = 0.3 cm

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

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

$C = 258\ \mu M$

Determine the Optimal Primer Concentration

⚠ **WARNING** **CHEMICAL HAZARD**. SYBR® Select Master Mix for CFX is a combustible liquid and vapor (keep away from heat and flame). It may cause eye, skin, and respiratory tract irritation. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing and gloves.

To optimize primer concentrations for PCR:

1. Prepare a 96-well reaction plate as described below.

   Use 10 to 100 ng of genomic DNA or 1 to 10 ng of cDNA template.

   The final concentration of SYBR® Select Master Mix for CFX is 1X.

   Note: The plate configuration accounts for four replicates of each of the following nine variations of primer concentration applied to both template and NTC wells:

Reverse Primer (nM)	Forward Primer (nM)		
	150	200	400
150	150/150	200/150	400/150
200	150/200	200/200	400/200
400	150/400	200/400	400/400

2. Calibrate your instrument for SYBR Green Dye, if necessary. Refer to the instrument user manual for calibration instructions.

   Note: It is recommended to calibrate your instrument every 6 months.
3. Load the plate into the CFX real-time PCR detection system.
4. Program the thermal-cycling conditions according to the information in step 2 on page 17.
5. Run the plate.
6. Compile the results for RFU and C_T, then select the minimum forward and reverse primer concentrations that yield the maximum RFU values and low C_T values.

Confirm the Absence of Nonspecific Amplification

Melt curves help you select the optimal primer concentrations for your SYBR quantification assays.

1. Review the linear view of the amplification plot in your NTC wells.

   Note: In Figure A-1 on page 30, part a, the strong amplification of the NTC wells indicates that significant nonspecific amplification is occurring.
2. Generate a melt curve with your Real-Time PCR System.

   Note: In the example melt curve data shown in Figure A-1 on page 30, part b, the melting temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template. This variation is typical of primer-dimer formation, and it indicates that lower primer concentration may provide optimal results.

Example of Nonspecific Amplification

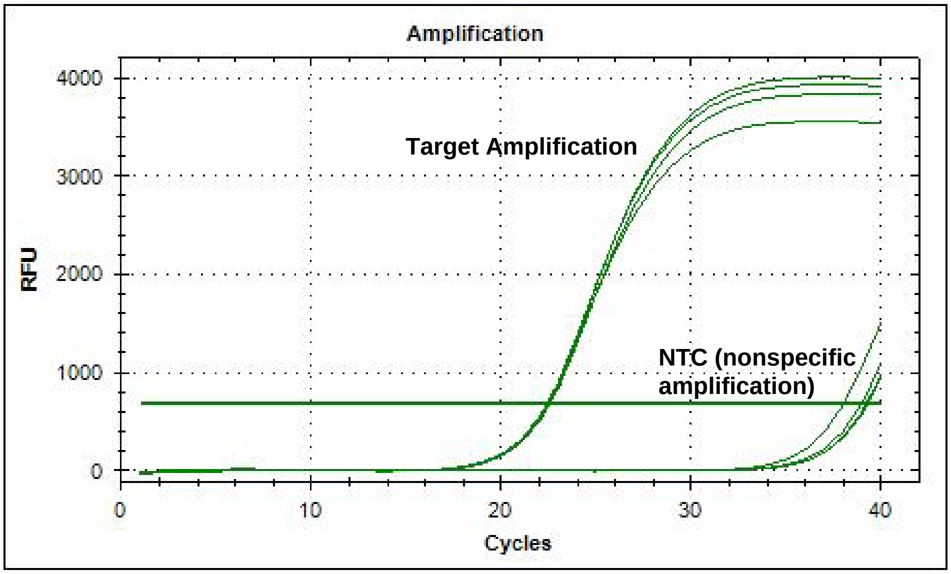


Figure A-1 Amplification data using SYBR® Green dye chemistry

(a) Amplification plot (linear view) demonstrating suspected nonspecific amplification in NTC wells.

(b) Melt curve analysis confirming that product in NTC wells has a melting temperature different from the specific product.

Appendix B

Safety

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page **Error! Bookmark not defined.**.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

⚠ **WARNING** **BIOHAZARD**. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories

  http://bmbl.od.nih.gov
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

Documentation and Support

Support Documents

You can download the following documents from the Life Technologies website at: **www.lifetechnologies.com**

Document	Part number
High-Capacity cDNA Reverse Transcription Kit Protocol	4375575
Primer Express® Software Version 3.0 Getting Started Guide	4362460

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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