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TaqMan® Environmental Master Mix 2.0

Real-time PCR Master Mix for inhibited samples

Protocol

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About This Guide

Purpose

The *TaqMan® Environmental Master Mix 2.0 Protocol* describes the primary application of the TaqMan Environmental Master Mix 2.0 (EMM 2.0): quantitative real-time PCR. Although the EMM 2.0 can be used in a broad variety of PCR applications, this document describes the use of the master mix with pre-optimized TaqMan® assays.

Because analysis methods vary greatly between applications, this protocol provides general guidelines for the analysis of data generated from experiments that use EMM 2.0 and TaqMan® assays. For detailed information about data analysis or the procedures outlined in this protocol, consult the appropriate documentation for your instrument (see "Related documentation" on page 39).

Safety information

Note: For general safety information, see this section and Appendix D, "Chemical Safety" on page 35. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 36.

IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

TaqMan® Environmental Master Mix 2.0

Product information

Purpose of the product

TaqMan® Environmental Master Mix 2.0 (EMM 2.0) is a convenient mix of components (not including primers, probes, template, and water) necessary to perform a real-time polymerase chain reaction (PCR). EMM 2.0 offers accurate, real-time PCR-based pathogen detection in the presence of high levels of inhibitors. Use this Master Mix to amplify and analyze complementary DNA (cDNA) and DNA targets in a variety of environmental, food, and other challenging samples.

About the kit

TaqMan® Environmental Master Mix 2.0 has been optimized for use with primers and TaqMan® probes that have been designed according to Applied Biosystems guidelines. The master mix can be used with custom TaqMan® assays available from the Applied Biosystems custom assay service, or with pre-optimized TaqMan® assays.

See "Reagents not supplied" on page 9 for a list of recommended assays.

Kit contents

The TaqMan® Environmental Master Mix 2.0 is supplied in a 2X concentration and contains:

- AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)
- dNTPs with dUTP
- ROX™ Passive Reference
- Optimized buffer components

EMM 2.0 is available in the following volumes:

Master mix	Item	Volume	50-µL reactions	Part number
TaqMan® Environmental Master Mix 2.0, no UNG	1-Pack	1 × 5-mL bottle	200	4396838
	2-Pack	1 × 10-mL bottle	400	4398021
	4-Pack	1 × 20-mL bottles	800	4398044

Compatible real-time instruments

The TaqMan® Environmental Master Mix 2.0 may be used for real-time or plate read (endpoint) detection of DNA or cDNA. Analysis is performed using any of the following real-time PCR systems:

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

Storage

Upon receipt, store the TaqMan® Environmental Master Mix 2.0 at –15 to –25 °C before first use, protected from light. Store at 2 to 8 °C after first use.

Applied Biosystems does not recommend storing EMM 2.0 at temperatures other than 2 to 8 °C after first use or using EMM 2.0 after the date printed on the package and bottle label.

Before use, thoroughly mix the EMM 2.0.

Materials and equipment required

Reagents not supplied

The reagents below are not supplied with TaqMan® Environmental Master Mix 2.0.

Item	Source
TaqMan® Gene Expression Assays	Applied Biosystems‡ • Inventoried assays: 4331182 • Made to Order assays: 4351372
Custom TaqMan® Gene Expression Assays	Applied Biosystems‡
Custom TaqMan® MGB probes	Applied Biosystems§
Custom TaqMan® TAMRA™ probes	
Sequence Detection Primers	
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free sterile-filtered water)	Ambion PN AM9849
DNAZap™ Solution, 2 × 250-mL bottles	Ambion PN AM9890
DNase-free water	Ambion PN AM9914G

‡ For information on new assays and updated product information, go to **www.appliedbiosystems.com**
§ For information on the latest available products and specific product uses, go to **www.appliedbiosystems.com**

Plastics not supplied

See "Real-time PCR systems, PCR systems, and consumables" on page 26 for a list of compatible real-time PCR system consumables.

Consumables and equipment not supplied

See "Reagents, consumables, and equipment" on page 27 for a list of required laboratory consumables and equipment.

Workflow

Prepare the premix solution (page 12)

Determine the number of controls and test samples to assay

↓

Prepare the premix solution

↓

Prepare the PCR reaction plate (page 12)

Add the premix solution to the plate

↓

Add the controls and test samples to the plate

↓

Set up the experiment or plate document (page 13)

Create the experiment or plate document

↓

Set the thermal cycling parameters

↓

Run the plate (page 13)

Load the reaction plate into the instrument

↓

Start the run

↓

Analyze the results (page 14)

Set the baseline and threshold values

↓

Perform analysis

↓

View the amplification plots

↓

View the results (page 16)

Check sample signal and interpret the results

↓

(Optional) Reanalyze the results

Before you begin

Prevent contamination

Review Appendix B, "PCR Good Laboratory Practices" on page 29.

Select an instrument and reaction plate

IMPORTANT! Use TaqMan® Environmental Master Mix 2.0 with *Standard* mode thermal cycling conditions only.

You can perform PCR amplification with any of the instruments and compatible plates listed in Appendix A, "Ordering Information" on page 25.

Fast reagents and thermal cycling conditions

IMPORTANT! TaqMan® Environmental Master Mix 2.0 is not supported for use with Fast Mode thermal cycling conditions. When using EMM 2.0 on the StepOne™, StepOnePlus™, 7500 Fast, or 7900HT Fast instruments, use Standard mode thermal cycling conditions. If you use assays other than TaqMan® assays, or use thermal cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal cycling conditions as needed. Refer to *Real-Time PCR Systems Reagent Guide* (PN 4387787) for more information on selecting thermal cycling conditions.

Reagent preparation guidelines

- Keep all TaqMan® assays in the freezer, protected from light, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Mix the TaqMan® Environmental Master Mix 2.0 thoroughly by swirling the bottle.
  - Thaw frozen TaqMan® assays by placing them on ice. When thawed, resuspend the assays by vortexing, then centrifuge the tubes briefly.
  - Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by briefly vortexing and then centrifuge the tubes.
  - Prepare the PCR reaction mix before transferring to the reaction plate for thermal cycling and fluorescence analysis.

Procedure

Prepare the premix solution

1. Determine the number of controls and test samples whose DNA content you will assay.

2. In an appropriate tube, prepare a premix solution for an assay mix using the components listed below.

Component	Volume (µL) per 30-µL reaction	Final conc.
TaqMan® Environmental Master Mix 2.0 (2X)	15.0	1X
TaqMan® assay mix‡	3.0	1X
Total Volume	18.0	—

‡ If using custom assays, formulate to 10X initial concentration.

   - Multiply the volume of each component by the number of reactions for the assay mix.
   - Use 10% excess volume to compensate for pipetting losses.

   IMPORTANT! EMM 2.0 must be from the same lot for all reactions containing the same assay mix.

3. Repeat step 2 for each assay mix.

4. Cap the tube(s) and vortex briefly to mix the solution.

5. Centrifuge the tube(s) briefly to spin down the contents and eliminate any air bubbles from the solution.

Prepare the PCR reaction plate

1. Transfer 18 µL of premix solution to the appropriate well(s) of an optical plate.

2. Transfer the appropriate volume of control and test sample components into each well to be used, gently pipetting up and down to mix the solution.

Component	Volume (µL) per 30-µL reaction	
	Test sample or Positive control	Negative control
DNA template	2.0 - 12.0	—
Nuclease-free water	As required	12.0
Total Volume	12.0	12.0

3. Cover the plate with a MicroAmp® Optical Adhesive Film. For standard 96-well plates, you may use MicroAmp® Optical Caps.

4. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

5. Apply a compression pad to the plate if required by your real-time PCR system.

Set up the experiment or plate document

Refer to your instrument documentation for instructions on how to configure the plate document/experiment. See "Related documentation" on page 39 for a list of documentation for Applied Biosystems real-time PCR systems.

When creating plate documents/experiments, use the following parameters:

- Thermal Cycling Parameters:

Step	AmpliTaq Gold®, UP enzyme activation‡	PCR	
	Hold	Cycle (40 cycles)	
		Denature	Anneal/extend
Temp. (°C)	95	95	60
Time (min:sec)	10:00	00:15	1:00

‡ The 10-minute, 95 °C step is required to activate the AmpliTaq Gold®, UP enzyme. See "About AmpliTaq Gold® DNA Polymerase, (UP) Ultra Pure" on page 31 for more information.

- Run Mode: **Standard** (Default)
- Sample Volume: **30** µL
- Auto Increment Settings: Accept default values (default is 0)
- Ramp Rate Settings: Accept default values (default is Standard)
- Data Collection: Accept default values (default is 60 °C)

Run the plate

Refer to the appropriate instrument user guide for detailed instructions on loading and running the PCR plates (see "Related documentation" on page 39).

To run the plate:

1. In the real-time PCR system software, open the experiment or plate document that corresponds to the reaction plate.

2. Load the reaction plate into the instrument.

3. Start the run.

Analyze the results

Overview

Data analysis varies depending on the real-time PCR system that you use. Refer to the appropriate instrument documentation for instructions on how to analyze your data. See "Related documentation" on page 39 for a list of applicable documents.

General process

The general process for analyzing the real-time amplification results involves:

- Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
- Performing analysis using the system software (refer to your real-time PCR system documentation for instructions).
- Viewing the amplification plots for the entire plate.

Baseline and threshold values

When using a real-time PCR system, you can use the software to set the baseline and threshold for the amplification curves either automatically or manually.

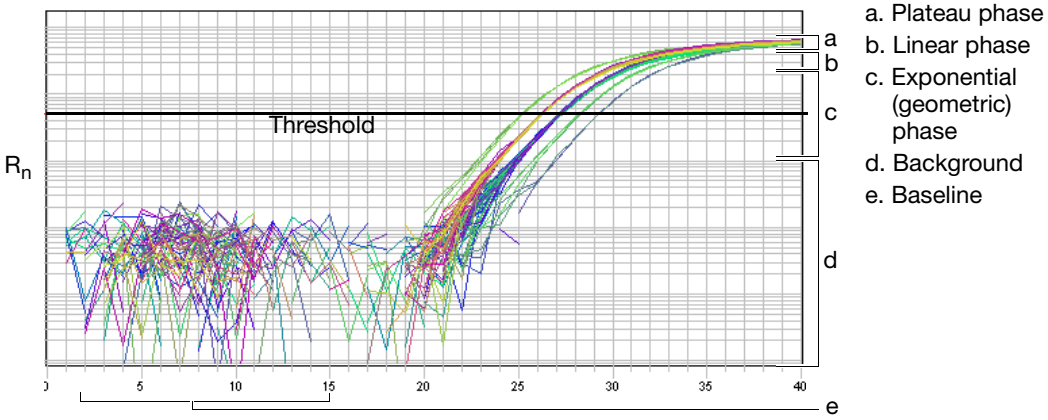
- The *baseline* refers to the initial cycles of PCR in which there is slight change in fluorescence signal.
- The intersection of the *threshold* with the amplification plot defines the C_T in real-time PCR assays. The threshold is set above the background and within the exponential growth phase of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

Automatic calculation of the baseline and threshold

The system software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the "typical" amplification curve shown in Figure 1. Experimental error (such as contamination or pipetting errors) can produce atypical data that can cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

IMPORTANT! After an analysis, verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots, and adjust the values manually if necessary.

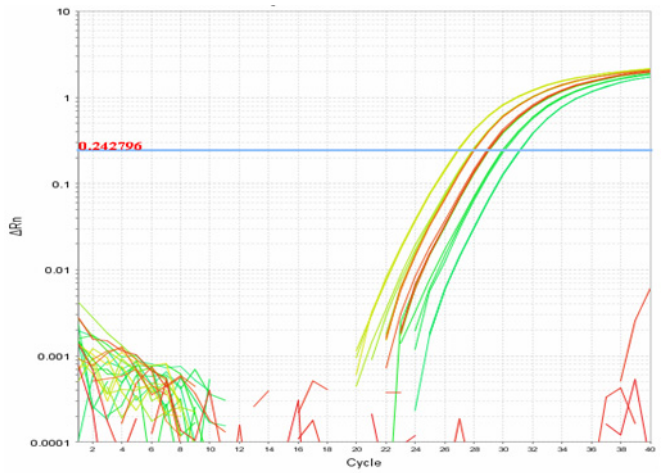
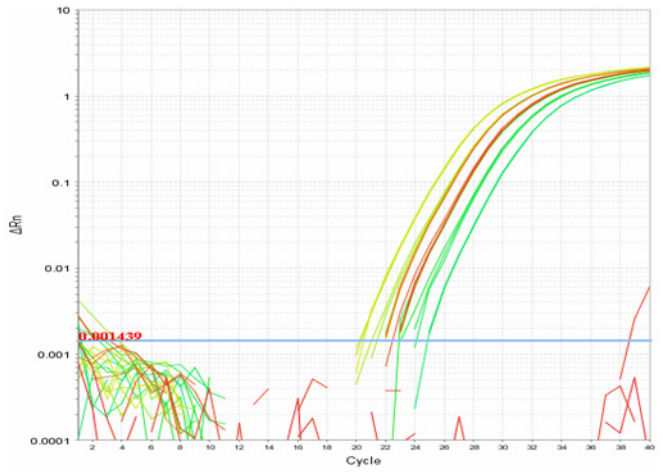
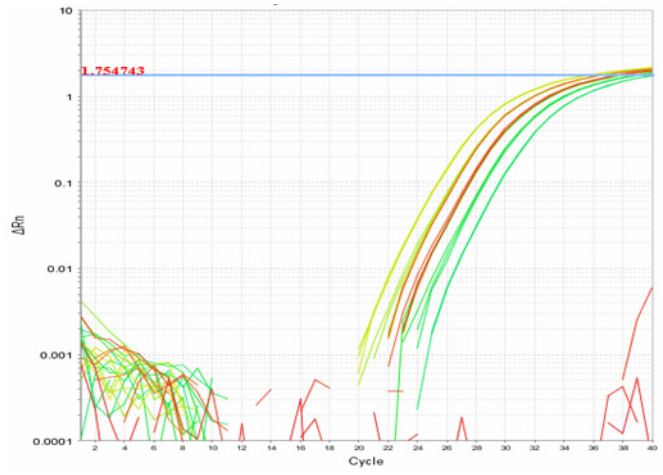
Figure 1 Typical amplification curve



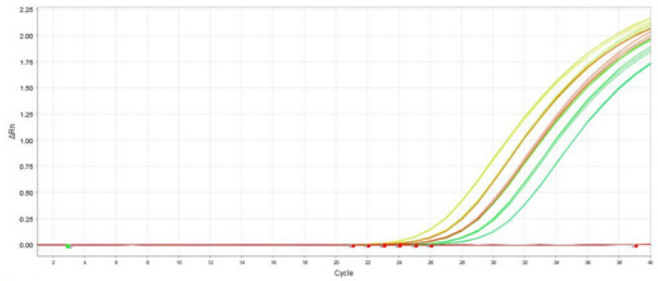
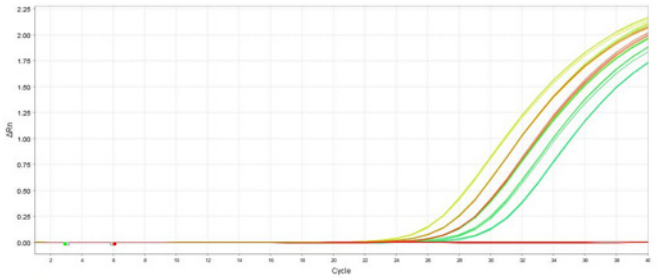
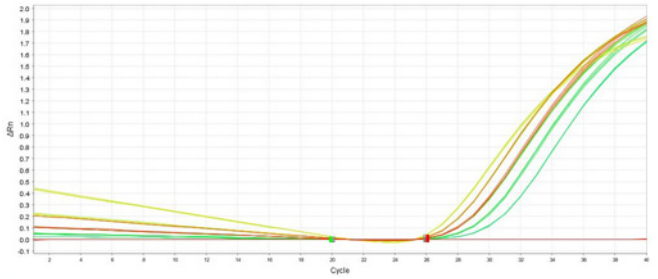
Manual setting of the baseline and threshold

If you use the system software to set the baseline and threshold values manually for any detector/assay in the plate, perform an adjustment procedure for each detector/assay. Refer to your real-time PCR system documentation for guidance on manually setting and adjusting your threshold and baseline.

Correct and incorrect 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.	

Correct and incorrect baseline settings

Baseline set correctly The amplification curve begins after the maximum baseline.	
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.	

View the results

Overview

Viewing results varies depending on the instrument you use. Refer to the appropriate instrument user guide for instructions on how to view your results. See "Related documentation" on page 39 for a list of applicable documents.

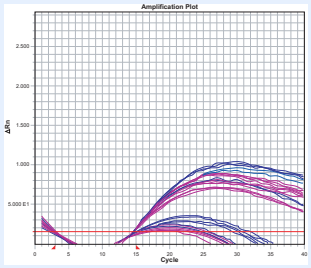
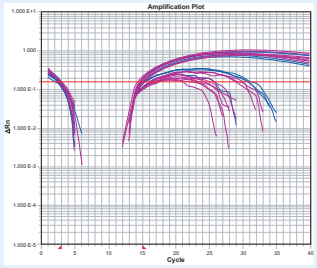
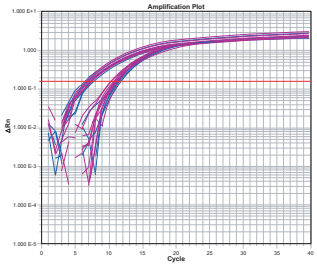
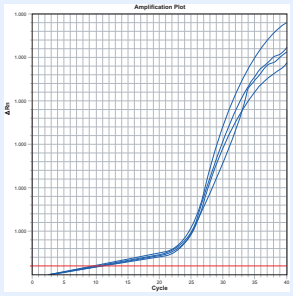
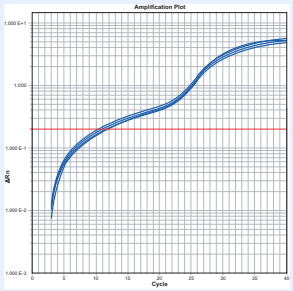
General process

The general process for viewing results from TaqMan® detection kits involves:

- Checking each sample for target specific signal, then interpreting the results.
- If required, reanalyzing the results.

Applied Biosystems does not recommend using the same method to both screen the samples and confirm the results. When you use TaqMan® detection kits to screen samples, biochemical methods are recommended to confirm the result.

Troubleshooting

Observation	Possible cause	Recommended action
Amplification curve shows abnormal plot and/or low ΔR_n values. Linear view:  Log view: 	The baseline was set improperly (some samples have C_T values lower than the baseline stop value)	Refer to your real-time PCR system user guide for procedures on setting the baseline. Switch from manual to automatic baselining, or move the baseline stop value to a lower C_T (2 cycles before the amplification curve for the sample crosses the threshold). Log view corrected: 
	An amplification signal is detected in the early cycles (no baseline can be set because the signal is detected too early)	Dilute the sample to increase the C_T value.
Amplification curve shows a rising baseline. Linear view:  Log view: 	Primer and probe interaction	• Adjust the threshold manually. • Select another assay from the same gene.

Observation	Possible cause	Recommended action
Multicomponent signal for ROX™ dye is not linear.	Pure dye components spectra are incorrect	Rerun the pure dye spectra.
	Incorrect dye components were selected	Select the correct dyes for the data analysis.
Amplification curve shows weak amplification.	Sequence mismatches between target and assay sequences	Perform bioinformatics. For more information, refer to the: • *Custom TaqMan® Genomics Assays Protocol: Submission Guidelines* (PN 4367671) • *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial* (from **www.appliedbiosystems.com**)
	Degraded reagents and/or probe	• Check the expiration date of the reagents. • Verify that you follow the correct handling and storage conditions. • Avoid excessive freeze-thaw cycles.
	Degraded or contaminated template	• Improve the sample integrity (extraction methods). • Check each template preparation by agarose gel electrophoresis or bioanalyzer to determine the: – Purity (only one product should be formed) – Level of degradation
	Inhibitors present in the reaction	• Verify the presence of an inhibitor: Create a serial dilution of your sample. If an inhibitor is present, low concentrations yield higher-than-expected C_T values. (High concentration means more inhibition because the sample is not diluted.) Rerun the assay with purified template. • Improve sample integrity (extraction methods).
Amplification curve shows low ROX™ dye (passive reference dye).	Inaccurate pipetting: Little or no TaqMan® Environmental Master Mix 2.0	Follow accurate pipetting practices.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_T = 40) across all assays or in an unusually large number of assays.	One or more of the reaction components was not added	Verify that the sample, assay mix, and TaqMan® Environmental Master Mix 2.0 were added to the reaction plate. (If the master mix is missing, the passive reference fails.)
	Incorrect dye components were selected	Check the dye components settings and reanalyze the data.
	The annealing temperature on the thermal cycler was too high for the primers and/or probe	Verify that the thermal cycler is set to the correct annealing and extension temperatures. Ensure that the thermal cycler is calibrated and maintained regularly.
	Inappropriate reaction conditions were used	Troubleshoot the PCR optimization.
	Degraded template	• Determine the quality of the template. • Rerun the assay with fresh template.
	Inhibitors present in the reaction	Verify the presence of an inhibitor: 1. Create a serial dilution of your sample. 2. If an inhibitor is present, low concentrations yield higher-than-expected C_T values. (High concentration means more inhibition because the sample is not diluted.) 3. Rerun the assay with purified template.
	The baseline and/or threshold was improperly set	Refer to your real-time PCR system user guide for procedures on setting the baseline and threshold: • Switch from automatic to manual baselining, or from manual to automatic. • Lower the threshold value to within the appropriate range.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_T = 40) across all assays or in an unusually large number of assays. *(continued)*	Assay design or synthesis failure: The wrong sequence was submitted to Applied Biosystems	• Verify that the sequence that you submitted is correct. • Check for an alternative transcript or a splice variant.
	Assay is designed in a variable region of the gene transcript	Verify that the location targeted by the assay is not within the 5′ untranslated region (UTR), which can be highly variable between transcripts. If the assay is designed within the 5′ UTR, select a different assay that is within the coding region of the transcript. Otherwise, select an assay for an alternative transcript or splice variant.
Amplification curve shows samples within the same assay that have differently shaped curves.	The baseline was set improperly	Refer to your real-time PCR system user guide for procedures on setting the baseline: • Switch from automatic to manual baselining, or from manual to automatic. • Increase the upper or lower value of the baseline range.
	Sample quality is poor	1. Perform a quality check on the sample. 2. If necessary, reextract the sample.
	Imprecise pipetting: different concentrations	Follow accurate pippetting practices.
	Contamination	Be sure your workspace and equipment are properly cleaned.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_T = 40) in the target assay.	One or more of the reaction components was not added	Check your pipetting equipment and/ or technique.
	Incorrect dye components were selected	Check the settings of the dye components before data analysis.
	The gene is not expressed in the tested sample	• Verify by: – Rerunning the sample using the same assay – Running the sample using an alternative assay for the same gene • Verify the known expression of the gene in the sample type. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	The reaction may not have enough copies of the target gene	Verify by: • Rerunning the sample using the same assay • Rerunning the assay using more sample • Running the sample using an alternative assay for the same gene **Note:** If the recommended actions do not resolve the problem, the result may be correct.
Decrease in ROX™ dye fluorescence (passive reference dye).	Precipitation in the TaqMan® buffers	• Be sure to mix the tubes well. • Use TaqMan® Environmental Master Mix 2.0. Be sure to mix thoroughly to produce a homogenous solution.
	Degraded TaqMan® buffers	Verify that the kits have been stored according to the instructions on the packaging and have not expired.
R_n on R_n-vs.-Cycle plot is very high.	ROX™ dye was not selected as the passive reference when the plate document/ experiment was set up	Select the ROX™ dye as the passive reference, then reanalyze the data.
Small ΔR_n.	PCR efficiency is poor	Recheck the concentration of the reagents.
	Quantity of starting target is low (low copy number of target)	Increase the quantity of the starting target.

Observation	Possible cause	Recommended action
No template control (NTC) shows amplification.	Contaminated reagents (contaminated with gDNA, amplicon, or plasmid clones)	• Rerun the assay using new reagents. • Be sure your workspace and equipment are cleaned properly. • Use UNG. • (*gDNA contamination only*) Design an assay that spans an exon-exon boundary.
Simultaneous increase in fluorescence from both the passive reference ROX™ dye and the reporter dye(s).	Evaporation	Check the seal of the optical adhesive cover for leaks.
High standard deviation of replicates (inconsistent data, C_T varies).	Inefficient mixing of reagents	• Increase the length of time that you mix the reagents. • Make a master mix for each dilution point on the curve, then transfer to the reaction plate. • Validate your mixing process by running a replicate plate.
	Inaccurate pipetting	• Check the calibration of the pipettes. • Pipette more than 5 µL of sample.
	Threshold was set improperly	Set the threshold above the noise and where the replicates are tightest. Refer to your real-time PCR system documentation for procedures on setting the threshold.
	Low concentration of target	Rerun the assay using more template.
	Template absorption (adhering to the tube)	Add a carrier (for example, yeast tRNA). Be sure the carrier does not interfere with assay performance.

Observation	Possible cause	Recommended action
C_T value is lower than expected.	gDNA contamination	• Perform bioinformatics: Design the assay to span an exon-exon junction. For more information, refer to the: – *Custom TaqMan® Genomics Assays Protocol: Submission Guidelines* (PN 4367671) – *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial* (from **www.appliedbiosystems.com**) • Treat the sample with DNase.
	More sample added than expected	• Reduce the amount of sample. • Quantitate and normalize the sample.
	Template or amplicon contamination	Follow established PCR good laboratory practices.
Shifting R_n value during the early cycles of the PCR (cycles 0 to 5).	Fluorescence did not stabilize to the buffer conditions of the reaction mix **Note:** This condition does not affect PCR or the final results.	• Reset the lower value of the baseline range. • Use automatic baselining.
Noisy signal above the threshold.	Evaporation	Check the seal of the optical adhesive cover for leaks.
	Empty well due to inaccurate pipetting	• Check the calibration of the pipettes. • Pipette more than 5 µL of sample.
	Well is labeled with a detector/assay in the plate document/experiment, but the well is empty	• Be sure your plate document/ experiment is set up correctly. • Exclude the well and reanalyze the data.

Ordering Information

How to order

The consumables, reagents and equipment in this appendix are for use with the TaqMan® Environmental Master Mix 2.0.

This appendix contains ordering information for the following:

Materials and equipment not included

Real-time PCR systems, PCR systems, and consumables

The following table lists real-time PCR systems, thermal cyclers and consumables that can be used with TaqMan® Environmental Master Mix 2.0 and TaqMan® assays. For a complete list of PCR systems and consumables, go to: **www.appliedbiosystems.com**

System	Reaction plates and accessories
7300 system	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical Film Compression Pad (PN 4312639) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (PN 4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (PN 4323032)
7500 system	
7500 Fast system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film (PN 4311971)
7900HT Fast system, standard 96-well block	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical Film Compression Pad (PN 4312639) for use with one plate • MicroAmp® Snap-On Optical Film Compression Pad (PN 4333292) for use with automation accessory
7900HT Fast system, Fast 96-well block	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical Film Compression Pad (PN 4312639) for use with one plate • MicroAmp® Snap-On Optical Film Compression Pad (PN 4333292) for use with automation accessory
9700 instrument	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Clear Adhesive Films, 100 films (PN 4306311) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (PN 4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (PN 4323032)
StepOne™ system	• MicroAmp® Fast Optical 48-Well Reaction Plate, 20 plates (PN 4375816) • MicroAmp® 48-Well Optical Adhesive Film (PN 4375323)

System	Reaction plates and accessories
StepOnePlus™ system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film (PN 4311971)

Reagents, consumables, and equipment

The following table includes required and optional laboratory reagents, consumables, and equipment. Unless otherwise noted, items listed are available from major laboratory suppliers.

Materials		Source
AmpErase® Uracil-N glycosylase (UNG), 100 µL		N8080096‡
Accessories for tubes of assay mixes • Decapper for single caps • Decapper for eight caps • TPE cap cluster for simultaneously capping 96 individual polypropylene tubes, 50 capmats/bag		Micronic BV§#
Centrifuge with plate adapter		MLS‡‡
Disposable gloves		MLS
Microcentrifuge		MLS
Heat block, water bath, or thermal cycler that heats to 95 °C		MLS
Microcentrifuge tubes, 1.5-mL		AM12400
Barrier (Filter) Tips	10 µL size - Pipetman™ (Ten 8 × 12 racks)	AM12640
	10 µL size - Eppendorf® (Ten 8 × 12 racks)	AM12635
	20 µL size (Ten 8 × 12 racks)	AM12645
	1000 µL size (Ten 100 ct racks)	AM12665
	200 µL size (Ten 8 x 12 racks)	AM12655
Pipettors	Positive-displacement	MLS
	Air-displacement	
	Multichannel	
Vortexer		MLS

‡ For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

§ Other vendors supply similar products.

Micronic BV, PO Box 604 8200, AP Lelystad, Netherlands: Telephone: 0031.320.277.090, Fax: 0031.320.277.088; United States: Telephone: 724.941.6411, Fax: 724.941.8662; Website: **www.micronic.com**

‡‡ Major laboratory supplier (MLS).

PCR Good Laboratory Practices

Sample preparation

When preparing samples for PCR amplification:

- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation).
- 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. Centrifuge tubes before opening. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution. Use DNA*Zap*™ Solution (PN AM9890).

Preventing contamination

PCR assays require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these assays can lead to amplification of a single DNA molecule (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

Since fluorescent contaminants may 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.

Chemistry Overview

About TaqMan® MGB Probes

The TaqMan® MGB probes contain:

- A reporter dye (for example, FAM™ dye) linked to the 5′ end of the probe.
- A minor groove binder (MGB) at the 3′ end of the probe.

  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.
- 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.

About AmpliTaq Gold® DNA Polymerase, (UP) Ultra Pure

The AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure) enzyme is identical to AmpliTaq Gold® DNA Polymerase, but the enzyme is further purified through a proprietary process to reduce bacterial DNA introduced from the host organism. The purification process ensures that non-specific, false-positive DNA products due to bacterial DNA contamination are minimized during PCR.

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. A thermal incubation step is required for activation to ensure that active enzyme is generated only at temperatures where the DNA is fully denatured.

About ROX™ passive reference

The ROX™ Passive Reference dye provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

About the 5′ nuclease assay

The 5′ nuclease assay process (Figure 3 through Figure 6) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

Figure 2 Legend for Figure 3 through Figure 6

NFQ = Nonfluorescent quencher
MGB = Minor groove binder
R = Reporter
P = Hot-start DNA polymerase

During PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 3).

When the probe is intact (Figure 3 and Figure 4), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983).

Figure 3 Polymerization

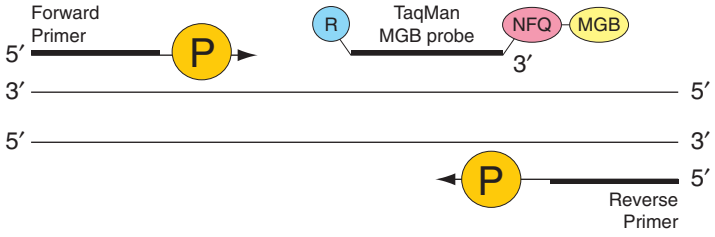
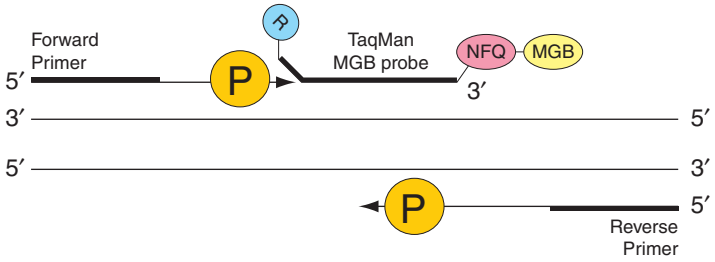
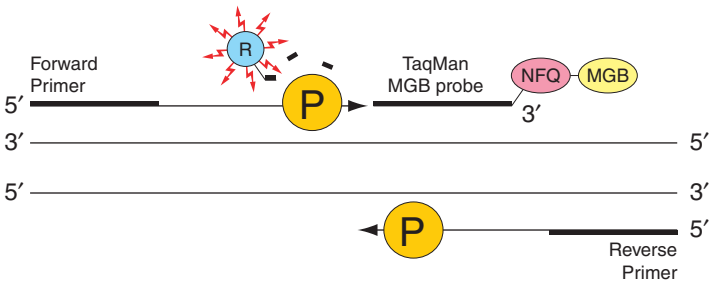


Figure 4 Strand displacement



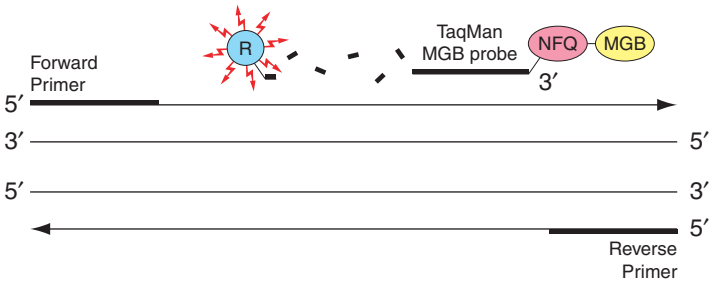
The DNA polymerase cleaves only probes that are hybridized to the target (Figure 5). Cleavage separates the reporter dye from the quencher dye; the separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter. The increase in fluorescence occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Figure 5 Cleavage



Polymerization of the strand continues, but because the 3′ end of the probe is blocked, no extension of the probe occurs during PCR (Figure 6).

Figure 6 Completion of polymerization



Chemical Safety

This appendix covers:

Chemical safety

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

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 36.)
- 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.

SDSs

About SDSs

Chemical manufacturers supply current Safety Data Sheets (SDSs) with shipments of hazardous chemicals to new customers. They also provide SDSs with the first shipment of a hazardous chemical to a customer after an SDS has been updated. SDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new SDS packaged with a hazardous chemical, be sure to replace the appropriate SDS in your files.

Obtaining SDSs

The SDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.

3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

Chemical waste safety

Chemical waste hazards

CAUTION! HAZARDOUS WASTE. Refer to Safety Data Sheets (SDSs) and local regulations for handling and disposal.

WARNING! CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

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 a 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

General biohazard

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* (**www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29CFR1910.1030; **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:

www.cdc.gov

Safety alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page 5.

Documentation and Support

Related documentation

The following related documents are available from Applied Biosystems:

Document	Part number	Description
Real-Time PCR Systems Reagent Guide	4387787	Provides an introduction to the reagents that you can use on the Applied Biosystems Real-Time PCR Systems, including descriptions and design guidelines for the following experiment types: quantitation, genotyping, and presence/absence.
TaqMan® Environmental Master Mix 2.0 Protocol	4448845	Describes the use of the TaqMan® Environmental Master Mix 2.0 (EMM 2.0) and provides general guidelines for the analysis and troubleshooting of data generated from experiments that use EMM 2.0.

Note: To open the portable document format (PDF) versions of this guide and other user documentation, use the Adobe® Reader® software available from **www.adobe.com**

Note: For additional documentation, see "Obtaining support" on page 39.

Obtaining support

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

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- 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.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

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Glossary

amplification

The process of making copies of, and thereby increasing the amount of, a specific DNA sequence.

assay mix

A reaction component that contains specific primers and probes for the target. The assay mix is used to prepare the premix solution.

ΔR_n value

The difference between the R_n+ value and the R_n– value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

$$\Delta R_n = (R_n+) - (R_n-)$$

where:

$$R_n+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$ PCR with template

$$R_n- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$ PCR without template or early cycles of a real-time reaction

Environmental Master Mix (EMM)

A common reagent for all pathogen detection assays. The EMM is used to prepare the premix solution. It contains the polymerase enzyme that initiates PCR in the presence of the necessary primers and DNA sample.

multicomponenting

The term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the dye components generate the composite spectrum, which represents one reading from one well.

negative control

A reaction mixture that lacks a target sequence. It indicates contamination if amplification occurs. At least one negative control is required for each target assay.

normalization

The emission intensity of the reporter dye divided by the emission intensity of the Passive Reference, used to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

normalized reporter (R_n)

Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference.

passive reference

A dye (ROX™) included in the TaqMan® Environmental Master Mix 2.0 that produces fluroescence signal but does not participate in the 5′ nuclease PCR. The passive reference provides an internal reference to which the reporter dye signal can be normalized during data analysis.

Polymerase Chain Reaction (PCR)	Technology used to amplify, or increase the amount of, a DNA sequence.
positive control	A control that establishes the expected amplification of a target. The lack of a target signal in a positive control well indicates a pipetting error or a problem with amplification. A positive control is provided by the investigator and is recommended but not required for each run.
premix solution	A solution you prepare that contains Environmental Master Mix (EMM) and TaqMan® assay mix.
primer	A segment of DNA that is complementary to the target DNA sequence. It is needed to start amplification.
probe	A segment of DNA that is complementary to the target DNA sequence. The probe is labeled with a reporter dye. When the probe binds to the target during the amplification step, fluorescence is emitted. The Sequence Detection System (SDS) or Real-Time PCR System detects the fluorescence, indicating the presence of the target DNA sequence.
replicate	Identical reactions that contain identical components and volumes and evaluate the same sample.
R_n–	The R_n value of an unreacted sample. This value may be obtained from the early cycles of a real-time run (those cycles prior to a detectable increase in fluorescence). This value may also be obtained from a reaction not containing template.
R_n+	The R_n value of a reaction containing all components including the template.
reporter	Fluorescent dye used to detect amplification.
target	The nucleic acid sequence that you want to amplify and detect.
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
test sample	A DNA sample that you test for the presence or absence of one or more targets.

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