May 2010

PyroMark OneStep RT-PCR Handbook

For highly sensitive and accurate one-step RT-PCR, optimized for Pyrosequencing® analysis

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Contents

Kit Contents

PyroMark OneStep RT-PCR Kit	(50)	(200)
Catalog no.	**978801**	**978803**
Reaction volume	**25 µl**	**25 µl**
QIAGEN OneStep RT-PCR Enzyme Mix (contains the QIAGEN products Omniscript® Reverse Transcriptase, Sensiscript® Reverse Transcriptase, and HotStarTaq® DNA Polymerase)	50 µl	200 µl
QIAGEN OneStep RT-PCR Buffer, 5x*	250 µl	1.0 ml
CoralLoad® Concentrate, 10x	550 µl	550 µl
Q-Solution®, 5x	400 µl	2.0 ml
dNTP Mix (10 mM of each dNTP)	50 µl	200 µl
RNase-Free Water	1.9 ml	2 x 1.9 ml
Handbook	1	1

* Contains 12.5 mM $MgCl_2$.

Shipping and Storage

The PyroMark OneStep RT-PCR Kit is shipped on dry ice and should be stored immediately upon receipt at –20°C in a constant temperature freezer. When stored under these conditions and handled correctly, this product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

Product Use Limitations

The PyroMark OneStep RT-PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the PyroMark OneStep RT-PCR Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Product Specifications

Enzymes

The QIAGEN OneStep RT-PCR Enzyme Mix contains a specially formulated enzyme blend for both reverse transcription and PCR. The unique combination of Omniscript and Sensiscript Reverse Transcriptases, with their high affinity for RNA templates, ensures highly efficient and sensitive transcription of RNA amounts from as little as 1 pg up to 2 µg.

Omniscript and Sensiscript Reverse Transcriptases are unique enzymes, and are different from the reverse transcriptases of Moloney murine leukemia virus (MMLV) or avian myeloblastosis virus (AMV). Omniscript and Sensiscript Reverse Transcriptases are recombinant heterodimeric enzymes expressed in *E. coli*.

HotStarTaq DNA Polymerase is a chemically modified form of a recombinant 94-kDa DNA polymerase (deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7), originally isolated from *Thermus aquaticus*, expressed in *E. coli*.

Buffers and reagents

Storage buffer containing QIAGEN OneStep RT-PCR Enzyme Mix:	20 mM Tris·Cl, 100 mM KCl,1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% (v/v) Nonidet® P-40, 0.5% (v/v) Tween® 20,50% glycerol (v/v), stabilizer; pH 9.0 (20°C)
QIAGEN OneStep RT-PCR Buffer:	5x concentrate. Contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 12.5 mM $MgCl_2$,DTT; pH 8.7 (20°C)
Q-Solution:	5x concentrate
CoralLoad Concentrate	10x concentrate. Contains gel loading reagent, orange dye, red dye
dNTP Mix:	10 mM each of dATP, dCTP, dGTP, and dTTP; PCR-grade
RNase-free water:	Ultrapure quality, PCR-grade

Quality Control

QIAGEN OneStep RT-PCR Enzyme Mix:	(See quality-control label inside kit lid for lot-specific values)
RT-PCR specificity and reproducibility assay:	RT-PCR specificity and reproducibility are tested in reproducibility assay: parallel 50 µl reactions containing 100 pg or 10 pg of human total RNA and 0.6 µM primers (specific for β-actin). After 35 cycles, a 0.3 kb product is detectable as a single, specific band.
Exonuclease activity assay:	Reactions are prepared using QIAGEN OneStep RT-PCR Enzyme Mix components and linear DNA. Exonuclease activity is indicated under "Exo.".
Endonuclease activity assay:	Reactions are prepared using QIAGEN OneStep RT-PCR Enzyme Mix components and DNA. Endonuclease activity is indicated under "Endo".
RNase activity assay:	Reactions are prepared using QIAGEN OneStep RT-PCR Enzyme Mix components and RNA. RNase activity is indicated under "RNase".
Protease activity assay:	QIAGEN OneStep Enzyme Mix components are incubated in storage buffer. Protease activity is indicated under "Protease".

Buffers and reagents

QIAGEN OneStep RT-PCR Buffer, 5x:	Conductivity, performance in RT-PCR, and pH are tested.
Q-Solution, 5x:	Performance in PCR and pH are tested.
CoralLoad Concentrate, 10x:	Conductivity and dye concentrations are tested.
dNTP Mix:	Concentration and purity are verified by UV spectroscopy. Performance in PCR is tested.
RNase-free water:	Conductivity, pH, and RNase activities are tested.

Introduction

The PyroMark OneStep RT-PCR Kit is specifically optimized for Pyrosequencing analysis enabling precise reverse transcription of the RNA template and subsequently sensitive and unbiased amplification of the cDNA. The entire process takes place in one tube, thus affording speed and simplicity of use. The RT-PCR product can be used for various Pyrosequencing applications such as virus detection and genotyping, allele-specific gene expression analysis, and detection of mRNA splicing isoforms.

QIAGEN OneStep RT-PCR Enzyme Mix

The QIAGEN OneStep RT-PCR Enzyme Mix contains a specially formulated enzyme blend specific for both reverse transcription and PCR amplification (see Table 1).

- **Omniscript and Sensiscript Reverse Transcriptases** are included in the QIAGEN OneStep RT-PCR Enzyme Mix and provide highly efficient and specific reverse transcription. Both reverse transcriptases exhibit a higher affinity for RNA, facilitating transcription through secondary structures that inhibit other reverse transcriptases. Omniscript Reverse Transcriptase is specially designed for reverse transcription of RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase is optimized for use with very small amounts of RNA (<50 ng). This special enzyme combination in the QIAGEN OneStep RT-PCR Enzyme Mix provides highly efficient and sensitive reverse transcription of any RNA quantity from 1 pg to 2 µg.
- **HotStarTaq DNA Polymerase** included in the QIAGEN OneStep RT-PCR Enzyme Mix provides hot-start PCR for highly specific amplification. During reverse transcription, chemically modified HotStarTaq DNA Polymerase is completely inactive and does not interfere with the reverse-transcriptase reaction. After reverse transcription by Omniscript and Sensiscript Reverse Transcriptases, reactions are heated to 95°C for 15 minutes to activate HotStarTaq DNA Polymerase and to simultaneously inactivate the reverse transcriptases. This hot-start procedure using HotStarTaq DNA Polymerase eliminates extension from nonspecifically annealed primers and primer-dimers in the first cycle ensuring highly specific and reproducible PCR.

Although all of the enzymes are present in the reaction mix, the use of HotStarTaq DNA Polymerase ensures the temporal separation of reverse transcription and PCR allowing both processes to be performed sequentially in a single tube. Only one reaction mix needs to be set up: no additional reagents are added after the reaction starts.

QIAGEN OneStep RT-PCR Buffer

QIAGEN OneStep RT-PCR Buffer is designed to enable both efficient reverse transcription and specific amplification.

The unique buffer composition allows reverse transcription to be performed at high temperatures (50°C). This high reaction temperature improves the efficiency of the reverse-transcriptase reaction by disrupting secondary structures and is particularly important for one-step RT-PCR performed with limiting template RNA amounts.

It has been reported that one-step RT-PCR may exhibit reduced PCR efficiency compared to two-step RT-PCR. The combination of QIAGEN enzymes and the unique formulation of the QIAGEN OneStep RT-PCR Buffer ensures high PCR efficiency in one-step RT-PCR.

The buffer contains the same balanced combination of KCl and $(NH_4)_2SO_4$ included in QIAGEN PCR Buffer. This formulation enables specific primer annealing over a wider range of annealing temperatures and Mg^{2+} concentrations than conventional PCR buffers.* The need for optimization of RT-PCR by varying the annealing temperature or the Mg^{2+} concentration is therefore minimized.

CoralLoad Concentrate

PyroMark OneStep RT-PCR Kit is supplied with CoralLoad Concentrate. We strongly recommend its use with the RT-PCR master mix for highly specific RT-PCR and high yields of amplified RNA. Furthermore, because CoralLoad Concentrate contains a gel loading reagent and two marker dyes (an orange and a red dye), RT-PCR products amplified in the presence of CoralLoad Concentrate can be loaded directly onto an agarose gel. These components enable estimation of DNA migration distance and optimization of agarose gel run time. CoralLoad Concentrate does not affect amplification sensitivity or specificity. RT-PCR fragments amplified in the presence of CoralLoad Concentrate have been successfully tested for Pyrosequencing.

Q-Solution

The PyroMark OneStep RT-PCR Kit is provided with Q-Solution, an innovative additive that enables amplification of difficult templates by modifying the melting behavior of nucleic acids. This unique reagent will often enable or improve a suboptimal RT-PCR caused by difficult templates that, for example, have a high degree of secondary structure or templates that are GC-rich. Unlike

* For further information see our comprehensive brochure "*Critical success factors and new technologies for PCR and RT-PCR*". To obtain a copy, visit the QIAGEN web site at www.qiagen.com or call one of the QIAGEN Technical Service Departments or local distributors listed on the last page (see back cover).

other commonly used additives such as DMSO, Q-Solution is used at just one working concentration, it is nontoxic, and RT-PCR purity is guaranteed. RT-PCR fragments amplified in the presence of Q-Solution have been successfully tested for Pyrosequencing.

Specificity and sensitivity

The QIAGEN OneStep RT-PCR Buffer, with its balanced potassium and sodium salts, promotes specific primer-template annealing and simultaneously reduces nonspecific annealing. Maximum yields of specific products are obtained even when using extremely low template amounts.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Reaction tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- Mineral oil (only if the thermal cycler does not have a heated lid)
- Primers: Primers should be purchased from an established oligonucleotide manufacturer. Lyophilized primers should be dissolved in TE to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry. Primer stock solutions should be stored in aliquots at –20°C (see Appendix B, page 31)

  IMPORTANT: The PyroMark OneStep RT-PCR Kit is designed for use with gene-specific primers. We do not recommend the use of random oligomers or oligo-dT primers.
- RNase inhibitor* (optional): RNase inhibitor is a 50 kDa protein that strongly inhibits RNases A, B, and C, as well as human placental RNases. It helps to minimize the risk of RNA degradation during experimental setup.

* The use of an RNase inhibitor is optional. The composition of QIAGEN OneStep RT-PCR Buffer has an inhibitory effect on RNases.

Protocol: One-Step RT-PCR Using PyroMark OneStep RT-PCR Master Mix

This protocol serves as a guideline for one-step RT-PCR to generate template DNA for subsequent Pyrosequencing analysis. Reverse transcription and PCR are carried out sequentially in the same tube. All components required for both reactions are added during setup, and there is no need to add additional components once the reaction has started. The protocol has been optimized for 1 pg – 2 µg of total RNA.

Important points before starting

- One PCR primer must be biotinylated at its 5′ end in order to prepare a single-stranded RT-PCR product for use in the subsequent Pyrosequencing procedure. We recommend HPLC or an equivalent procedure to purify the biotinylated primer.
- For primer design we recommend using PyroMark Assay Design Software 2.0.
- The optimal RT-PCR amplicon length for Pyrosequencing is between 80 and 200 bp, although products up to 500 bp may work well for Pyrosequencing assays.
- HotStarTaq DNA Polymerase, contained in the QIAGEN OneStep RT-PCR Enzyme Mix requires an activation step of 15 min at 95°C (see Table 2, page 15). This incubation also inactivates the reverse transcriptases. Do not heat activate the HotStarTaq DNA Polymerase until the reverse-transcriptase reaction is finished.
- Set up all reactions on ice.
- Ensure the thermal cycler is preheated to 50°C before placing samples in it.
- QIAGEN OneStep RT-PCR Buffer provides a final concentration of 2.5 mM $MgCl_2$ in the reaction mix, which typically produces satisfactory results.
- An RNase-free environment should be maintained during RNA isolation and reaction setup.
- Set up all reaction mixtures in an area separate from that used for RNA or DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

Procedure

1. **Thaw template RNA, primer solutions, dNTP Mix, QIAGEN OneStep RT-PCR Buffer, CoralLoad Concentrate, and RNase-free water, and place them on ice.**

It is important to mix the solutions completely before use to avoid localized differences in salt concentration.

2. **Prepare a master mix according to Table 1.**

   The master mix typically contains all the components required for RT-PCR except the template RNA. Prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

   Note: If another reaction volume is used, adjust the amount of each component accordingly.

Table 1. Components of the PyroMark OneStep RT-PCR Master Mix

Component	Volume/reaction	Final concentration
Reaction mix		
QIAGEN OneStep RT-PCR Buffer, 5x*	5 µl	1x
dNTP Mix	1.0 µl	400 µM each
CoralLoad Concentrate, 10x	2.5 µl	1x
QIAGEN OneStep RT-PCR Enzyme Mix	1.0 µl	–
Primer A	Variable	0.6 µM†
Primer B	Variable	0.6 µM†
RNase-free water	Variable	–
RNase Inhibitor‡	Variable	5–10 U/reaction
Template RNA		
Template RNA, added at step 4	Variable	1 pg – 2 µg/reaction
Total volume	25 µl	

* Contains 12.5 mM $MgCl_2$.

† A final primer concentration of 0.6 µM is optimal for most primer-template systems. However, in some cases using other primer concentrations (i.e., 0.5–1.0 µM) may improve amplification performance.

‡ The use of RNase inhibitor is optional because the buffer composition has an inhibitory effect on RNases.

3. **Gently pipet the master mix up and down to mix thoroughly, and dispense appropriate volumes into PCR tubes.**
4. **Add template RNA (≤2 µg/reaction) to the individual PCR tubes.**

   PyroMark OneStep RT-PCR Kit can be used with total RNA, messenger RNA, or viral RNA.
5. **If using a thermal cycler without a heated lid, overlay each reaction tube with approximately 50 µl mineral oil. Otherwise, proceed directly to step 6.**
6. **Program the thermal cycler according to the program outlined in Table 2, page 15.**
7. **Start the RT-PCR program while PCR tubes are still on ice. Wait until the thermal cycler has reached 50°C. Then place the PCR tubes in the thermal cycler.**

   Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

Table 2. Thermal Cycling Program

			Additional comments
Reverse transcription	30 min	50°C	A reverse-transcription reaction temperature of 50°C is recommended. However, if satisfactory results are not obtained using 50°C, the reaction temperature may be increased up to 60°C.
Initial PCR activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step. Omniscript and Sensiscript Reverse Transcriptases are inactivated and the cDNA template is denatured.
3-step cycling			
Denaturation	30 s	94°C	
Annealing	30 s	60°C	Recommended annealing temperature when using primers designed with PyroMark Assay Design Software 2.0. In all other cases, begin with 5°C below the calculated T_m of the primers. Use the annealing temperature with the highest specificity for the desired RT-PCR product.
Extension	30 s	72°C	
Number of cycles	45		
Final extension	10 min	72°C	

8. Use 5–20 µl of a 25 µl PCR for subsequent Pyrosequencing analysis.

In most cases, 5–10 µl of the RT-PCR product gives satisfactory Pyrosequencing results when using the PyroMark Q96 MD and PyroMark Q24, and 10–20 µl when using the PyroMark Q96 ID. Adjust the volume of the RT-PCR according to the instructions in the user manual of the specific instrument as required.

We recommend checking your RT-PCR product prior to Pyrosequencing analysis by fast analysis on the QIAxcel® or by agarose gel analysis. RT-PCR products can be directly loaded onto an agarose gel without prior addition of a loading buffer and gel tracking dyes when using CoralLoad Concentrate. The QIAxcel enables fully automated loading and analysis of up to 96 samples per run.

CoralLoad Concentrate contains a gel loading reagent and gel tracking dyes. Refer to Table 3 below to identify the dyes according to migration distance and agarose gel percentage and type. Distances given in parenthesis correspond to TBE agarose gels.

Note: Due to the viscosity of the solution, apply the solution slowly into the wells of the agarose gel.

Table 3. Migration Distances of Gel-Tracking Dyes in CoralLoad

%TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Protocol: One-Step RT-PCR Using PyroMark OneStep RT-PCR Master Mix and Q-Solution

This protocol is designed for one-step RT-PCR using Q-Solution. Q-Solution changes the melting behavior of nucleic acids and can be used for RT-PCR systems that do not work well under standard conditions. When using Q-Solution the first time with a particular primer-template system, always perform parallel reactions with and without Q-Solution. This recommendation should also be followed if another RT-PCR additive (such as DMSO) was previously used for a particular primer–template system.

When using Q-Solution, the following effects may be observed depending on the individual RT-PCR assay (Figure 1):

Case A: Q-Solution enables an amplification reaction that previously failed.

Case B: Q-Solution increases RT-PCR specificity in certain primer–template systems.

Case C: Q-Solution has no effect on RT-PCR performance.

Case D: Q-Solution causes reduced efficiency or failure of a previously successful amplification reaction. In this case, addition of Q-Solution disturbs the previously optimal primer–template annealing. Therefore, when using Q-Solution for the first time for a particular primer–template system, always perform reactions in parallel with and without Q-Solution.

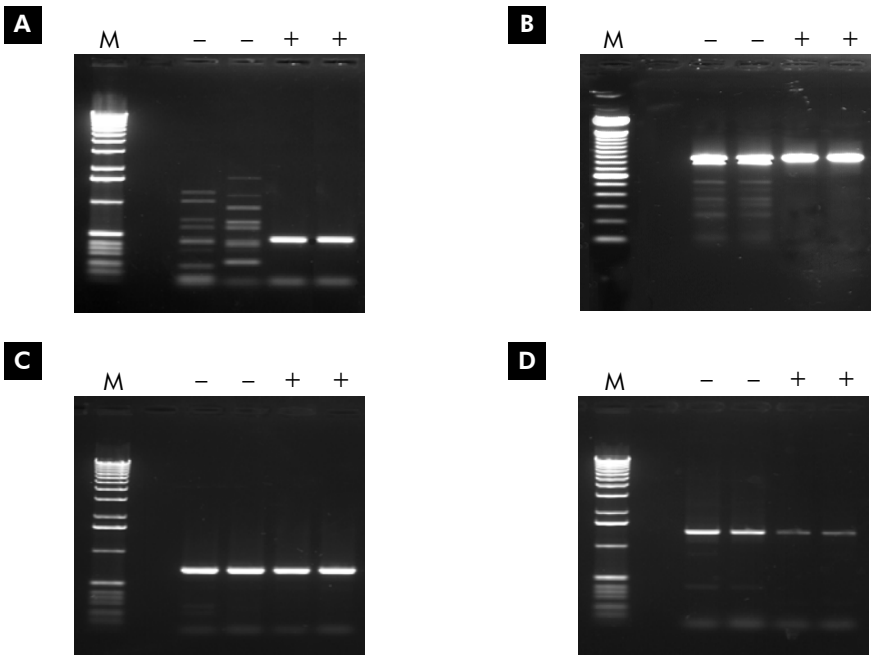

Figure 1. Effects of using Q-Solution. M: markers; –: without Q-Solution; +: with Q-Solution.

Important points before starting

- When using Q-Solution for the first time in a particular primer–template system, it is important to perform parallel amplification reactions with and without Q-Solution.
- One PCR primer must be biotinylated at its 5' end in order to prepare a single-stranded RT-PCR product for use in the subsequent Pyrosequencing procedure. We recommend HPLC or an equivalent procedure to purify the biotinylated primer.
- For primer design we recommend using PyroMark Assay Design Software 2.0.
- The optimal RT-PCR amplicon length for Pyrosequencing is between 80 and 200 bp, although products up to 500 bp may work well for Pyrosequencing assays.
- HotStarTaq DNA Polymerase, contained in the QIAGEN OneStep RT-PCR Enzyme Mix requires an activation step of 15 min at 95°C (see Table 4, page 19). This incubation also inactivates the reverse transcriptases. Do not heat activate the HotStarTaq DNA Polymerase until the reverse-transcriptase reaction is finished.
- Set up all reactions on ice.
- Ensure the thermal cycler is preheated to 50°C before placing samples in it.
- QIAGEN OneStep RT-PCR Buffer provides a final concentration of 2.5 mM $MgCl_2$ in the reaction mix, which typically produces satisfactory results.
- An RNase-free environment should be maintained during RNA isolation and reaction setup.
- Set up all reaction mixtures in an area separate from that used for RNA or DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

Procedure

1. **Thaw template RNA, primer solutions, dNTP Mix, QIAGEN OneStep RT-PCR Buffer, CoralLoad Concentrate, Q-Solution, and RNase-free water, and place them on ice**

   It is important to mix the solutions completely before use to avoid localized differences in salt concentration.

2. Prepare a master mix according to Table 4.

The master mix typically contains all the components required for RT-PCR except the template RNA. Prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

Note: If another reaction volume is used, adjust the amount of each component accordingly.

Table 4. Components of the PyroMark OneStep RT-PCR Master Mix with Q-Solution

Component	Volume/reaction	Final concentration
Master mix		
QIAGEN OneStep RT-PCR Buffer, 5x*	5 µl	1x
dNTP Mix	1.0 µl	400 µM each
Q-Solution, 5x	5.0 µl	1x
CoralLoad Concentrate, 10x	2.5 µl	1x
QIAGEN OneStep RT-PCR Enzyme Mix	1.0 µl	–
Primer A	Variable	0.6 µM†
Primer B	Variable	0.6 µM†
RNase-free water	Variable	–
RNase Inhibitor (optional)‡	Variable	5–10 U/reaction
Template RNA		
TemplateRNA, added at step 4	Variable	1 pg – 2 µg/reaction
Total volume	25 µl	

* Contains 12.5 mM $MgCl_2$

† A final primer concentration of 0.6 µM is optimal for most primer–template systems. However, in some cases using other primer concentrations (i.e., 0.5–1.0 µM) may improve amplification performance.

‡ The use of RNase inhibitor is optional because the buffer composition has an inhibitory effect on RNases.

3. **Gently pipet the master mix up and down to mix thoroughly, and dispense appropriate volumes into PCR tubes.**
4. **Add template RNA (≤2 µg/reaction) to the individual PCR tubes.**

   The PyroMark OneStep RT-PCR Kit can be used with total RNA, messenger RNA, or viral RNA.
5. **If using a thermal cycler without a heated lid, overlay each reaction tube with approximately 50 µl mineral oil. Otherwise, proceed directly to step 6.**
6. **Program the thermal cycler according to the program outlined in Table 5, page 21.**
7. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

Table 5. Thermal Cycling Program

			Additional comments
Reverse Transcription	30 min	50°C	A reverse-transcription reaction temperature of 50°C is recommended. However, if satisfactory results are not obtained using 50°C, the reaction temperature may be increased up to 60°C.
Initial PCR activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step. Omniscript and Sensiscript Reverse Transcriptases are inactivated and the cDNA template is denatured.
3-step cycling			
Denaturation	30 s	94°C	
Annealing	30 s	60°C	Recommended annealing temperature when using primers designed with PyroMark Assay Design Software 2.0. In all other cases, begin with 5°C below the calculated T_m of the primers. Use the annealing temperature with the highest specificity for the desired RT-PCR product.
Extension	30 s	72°C	
Number of cycles	45		
Final extension	10 min	72°C	

8. **Use 5–20 µl of a 25 µl PCR for subsequent Pyrosequencing analysis.**

   In most cases, 5–10 µl of the RT-PCR product gives satisfactory Pyrosequencing results when using the PyroMark Q96 MD and PyroMark Q24, and 10–20 µl when using the PyroMark Q96 ID. Adjust the volume of the RT-PCR according to the instructions in the user manual of the specific instrument as required.

   We recommend checking your RT-PCR product prior to Pyrosequencing analysis, by fast analysis on the QIAxcel or by agarose gel analysis. RT-PCR products can be directly loaded onto an agarose gel without prior addition of a loading buffer and gel tracking dyes when using CoralLoad Concentrate. The QIAxcel enables fully automated loading and analysis of up to 96 samples per run.

   CoralLoad Concentrate contains a gel loading reagent and gel tracking dyes. Refer to Table 6 below to identify the dyes according to migration distance and agarose gel percentage and type. Distances given in parenthesis correspond to TBE agarose gels.

   Note: Due to the viscosity of the solution, apply the solution slowly into the wells of the agarose gel.

Table 6. Migration Distances of Gel-Tracking Dyes

%TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Little or no RT-PCR product	
a) Pipetting error or missing reagent	Check the concentrations and storage conditions of reagents, including primers and dNTP mix. Repeat the RT-PCR.
b) HotStarTaq DNA Polymerase not activated	Ensure that the cycling program included the HotStarTaq DNA Polymerase activation step (15 min at 95°C) as described in Tables 2 and 5 of the protocols (pages 15 and 21).
c) HotStarTaq DNA Polymerase activated too early	Check the cycling program. Ensure that the reverse-transcription reaction is complete (30 min at 50°C) before activating HotStarTaq DNA Polymerase (15 min at 95°C).
d) Reverse-transcription reaction temperature incorrect	We recommend a temperature of 50°C for reverse transcription. However, if desired results are not obtained using 50°C, reaction temperatures of 45–60°C may be used.
e) Primer concentration not optimal or primers degraded	We strongly recommend a primer concentration of 0.6 µM. However, if desired results are not obtained using this concentration, repeat the RT-PCR with different primer concentrations from 0.5–1.0 µM in 0.1 µM increments. In particular, when performing highly sensitive RT-PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.*

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

	Comments and suggestions
f) RT-PCR conditions not optimal	Using the same cycling conditions, repeat the RT-PCR using Q-Solution. Follow the protocol on page 17.
g) Incorrect nucleotide concentration	Use 0.4 mM of each dNTP. Different nucleotide concentrations can reduce the amount of RT-PCR product.
h) Problems with starting template	Check the concentration, integrity, purity, and storage conditions of the starting RNA template (see Appendix A, page 29). If necessary, make new serial dilutions of template RNA from stock solutions. Repeat the RT-PCR using the new dilutions.
i) Enzyme concentration too low	Ensure that 1 µl of PyroMark OneStep RT-PCR Enzyme Mix was used per 25 µl reaction.
j) Incorrect PCR annealing temperature or time	Decrease annealing temperature in 2°C steps. Annealing time should be 30 s. Extending the annealing time to 60 s can be beneficial with certain complex RT-PCR systems. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing a temperature-gradient of the annealing temperature.
k) Incorrect denaturation temperature or time	Denaturation should be at 94°C for 30 s. Extending the denaturation time to 60 s can be beneficial with certain complex RT-PCR systems. Ensure that the cycling program included the HotStarTaq DNA Polymerase activation step (15 min at 95°C) as described in Tables 2 and 5 of the protocols (pages 15 and 21).
l) Insufficient starting template	Increase the template amount. If this is not possible, perform a second round of PCR using a nested-PCR approach.
m) Primer design not optimal	Review primer design (Appendix B, page 31). Only use gene-specific primers. Do not use random oligomers or oligo-dT primers. We strongly recommend use of the PyroMark Assay Design Software 2.0 for primer design.

	Comments and suggestions
n) Reactions overlaid with mineral oil when using a thermal cycler with a heated lid	When using a thermal cycler with a heated lid that is switched on, do not overlay the reactions with mineral oil as this may decrease the yield of RT-PCR product.
o) Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.
Product is multibanded	
a) Reactions were set up at room temperature	Be sure to set up the RT-PCR on ice to avoid premature cDNA synthesis.
b) Starting conditions for reverse-transcriptase reaction incorrect	Make sure that the thermal cycler is preheated to 50°C before placing samples in it.
c) Reverse-transcription reaction temperature too low	We recommend a temperature of 50°C for reverse transcription. However, if the desired results are not obtained using 50°C, the reaction temperature may be increased in increments of 2°C up to 60°C.
d) RT-PCR cycling conditions not optimal	Using the same cycling conditions, repeat the RT-PCR using Q-Solution. Follow the protocol on page 17.
e) PCR annealing temperature too low	Increase annealing temperature in 2°C steps. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing a temperature-gradient of the annealing temperature.
f) Primer concentration not optimal or primers degraded	We strongly recommend a primer concentration of 0.6 µM. However, if desired results are not obtained using this concentration, repeat the RT-PCR with different primer concentrations from 0.5–1.0 µM in 0.1 µM increments. In particular, when performing highly sensitive RT-PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.*

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

	Comments and suggestions
g) Primer design not optimal	Review primer design (Appendix B, page 31). Only use gene-specific primers. Do not use random oligomers or oligo-dT primers. We strongly recommend use of the PyroMark Assay Design Software 2.0 for primer design.
h) Contamination with genomic DNA	Pretreat starting RNA template with DNase I. Alternatively, use primers located at splice junctions of the target mRNA to avoid amplification from genomic DNA.
Product is smeared	
a) Too much starting template	Check the concentration of the starting RNA template (see Appendix A, page 29). If necessary, make new serial dilutions of template RNA from stock solutions. Repeat the RT-PCR using the new dilutions.
b) Carryover contamination	If the negative control (without template RNA) shows a RT-PCR product or a smear, exchange all reagents. Use disposable pipet tips containing hydrophobic filters to minimize cross-contamination. Set up all reaction mixtures in an area separate from that used for RNA preparation or PCR product analysis.
c) Reactions set up at room temperature	Be sure to set up the RT-PCR on ice to avoid premature cDNA synthesis.
d) Starting conditions for reverse-transcription reaction incorrect	Make sure that the thermal cycler is preheated to 50°C before placing samples in it.
e) RT-PCR cycling conditions not optimal	Using the same cycling conditions, repeat the RT-PCR using Q-Solution. Follow the protocol on page 17.
f) Enzyme concentration too high	Ensure that 1 µl of PyroMark OneStep RT-PCR Enzyme Mix was used per 25 µl reaction.
g) Too many cycles	Reduce the number of cycles in steps of 3 cycles.

	Comments and suggestions
h) Primer design not optimal	Review primer design (Appendix B, page 31). Only use gene-specific primers. Do not use random oligomers or oligo-dT primers. We strongly recommend use of the PyroMark Assay Design Software 2.0 for primer design.
i) Primer concentration not optimal or primers degraded	We strongly recommend a primer concentration of 0.6 µM. However, if desired results are not obtained using this concentration, repeat the RT-PCR with different primer concentrations from 0.5–1.0 µM in 0.1 µM increments. In particular, when performing highly sensitive RT-PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.*

Low or missing peaks in the Pyrosequencing run

a) Primer is insufficiently or not biotinylated	Ensure that RT-PCR primers are of high quality (use HPLC-purified biotinylated primers). One of the primers must be biotin-labeled to enable immobilization to streptavidin-coated beads during preparation of a single-stranded Pyrosequencing template.
b) Sequencing primer does not match to the biotinylated strand	Check that the sequencing primer binds to the biotinylated strand.
c) Low-quality Pyrosequencing template	Recommended RT-PCR conditions will result in high-quality Pyrosequencing templates in most cases. Optimize RT-PCR if required. However, we recommend inspection of your RT-PCR product on agarose gels or by fast analysis on the QIAxcel. Be sure to only use single-banded templates with a sufficient yield for Pyrosequencing.
d) Assay design not optimal	We strongly recommend use of PyroMark Assay Design Software 2.0 for designing RT-PCR and sequencing primers to ensure optimal primer design and orientation.

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	Comments and suggestions
e) Sample preparation and Pyrosequencing workflow-related errors	Please refer to the user manual for the PyroMark instrument being used.

Poor or faulty Pyrosequencing run

a) Background of contaminating sequence from RT-PCR	Check your RT-PCR product by fast analysis on the QIAxcel or by agarose gel analysis to confirm that you only have one specific product. Additional sequence signals can be caused by miscellaneous events such as: ■ Duplex-formation of sequencing primer and/or biotinylated primer ■ Hairpin formation of biotinylated primer and/or template loop on Pyrosequencing template ■ Annealing between the sequencing primer and the biotinylated PCR primer ■ Nonspecific annealing of the sequencing primer to Pyrosequencing template Be sure to include following controls when you run an assays the first time: ■ RT-PCR without RNA ■ Sequencing primer on its own ■ Template without sequencing primer For more information, see the user manual for the specific Pyrosequencing instrument being used. We strongly recommend using PyroMark Assay Design Software 2.0 for designing RT-PCR and sequencing primers suitable for Pyrosequencing.

Appendix A: Starting Template

The efficiencies of reverse transcription and PCR are highly dependent on the quality and quantity of the starting RNA template.

It is important to have intact RNA as starting template. Even trace amounts of contaminating RNases in the RNA sample can cause RNA cleavage, resulting in shortened cDNA products. Chemical impurities, such as protein, poly-anions (e.g., heparin), salts, EDTA, ethanol, phenol, and other solvents, can affect the activity and processivity of the reverse transcriptases and the *Taq* DNA polymerase. To ensure reproducible and efficient RT-PCR, it is important to determine the quality and quantity of the starting RNA.

For best results, we recommend starting with RNA purified using silica-membrane technology. For example, RNeasy® Kits, QIAamp® Viral RNA Kits, and the QIAamp RNA Blood Mini Kit can be used to isolate RNA from a variety of starting materials and provide high-quality RNA ideal for use in reverse-transcription and RT-PCR applications. Alternatively, RNA can be isolated from whole blood collected in PAXgene® Blood RNA Tubes using the PAXgene Blood RNA Kit.

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable for at least 1 year.

Determining concentration and purity of RNA

- The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. Note that absorbance measurements cannot discriminate between DNA and RNA.
- To determine RNA concentration, we recommend dilution of the sample in a pH-neutral buffer, since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA with neutral pH. To ensure significance, readings should be between 0.1 and 1.0.
- The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. To determine RNA purity, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5.* Pure RNA has an

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A_{260}/A_{280} ratio of 1.9–2.1[†] in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer using the same solution.

Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose-gel electrophoresis and ethidium bromide[‡] staining. The respective ribosomal bands (Table 7) should appear as sharp bands on the stained gel. The intensity of the 28S ribosomal RNA band should be approximately twice that of the 18S rRNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear of lower molecular weight species, it is likely that the RNA sample suffered major degradation during preparation.

Table 7. Size of Ribosomal RNAs From Various Sources

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

[†] Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

[‡] Ethidium bromide is toxic and/or mutagenic. Take appropriate safety measures.

Appendix B: Assay and Primer Design, Handling, and Storage of Primers

Assay and primer design

Primers should be designed using PyroMark Assay Design Software 2.0. The program automatically generates primer sets that include both gene-specific RT-PCR and sequencing primers. Each primer set is given a quality score based on several performance parameters that are specific for Pyrosequencing analysis.

Gene-specific RT-PCR primer

RT-PCR primers should be 18 to 24 bases in length, with annealing temperatures that are similar and typically in the range of 50°C to 68°C (nearest neighbor method). When using PyroMark Assay Design Software 2.0 for primer design, an annealing temperature of 60°C gives good results in most cases. If you are not using PyroMark Assay Design Software 2.0, 5°C below the calculated T_m of the primers is a suitable temperature to start with. However, the annealing temperature of highest specificity for the desired PCR product should be used.

One of the PCR primers must be biotin-labeled to enable immobilization of the RT-PCR product to streptavidin-coated beads during the preparation of single-stranded Pyrosequencing template. The orientation of the assay can either be forward or reverse. The primer that needs to be biotinylated is indicated by PyroMark Assay Design Software 2.0.

The primers should not form strong hairpin loops or dimers with themselves or other primers. The biotinylated primer should be carefully checked for hairpin loops and duplexes with the sequencing primer, as excess biotinylated primer might cause background in Pyrosequencing assays. If possible, avoid placing primers over polymorphic positions.

The biotinylated primer should be purified by HPLC or an equivalent procedure since free biotin will compete with the biotinylated RT-PCR product for binding on streptavidin-coated beads.

Location of RT-PCR

For RT-PCR using mRNA as template, design primers so that one half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon (see Figure 2A, page 32). Primers will anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA. Thus, amplification of contaminating DNA is eliminated.

Alternatively, RT-PCR primers should be designed to flank a region that contains at least one intron (see Figure 2B). Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). Size difference in products is used to detect the presence of contaminating DNA.

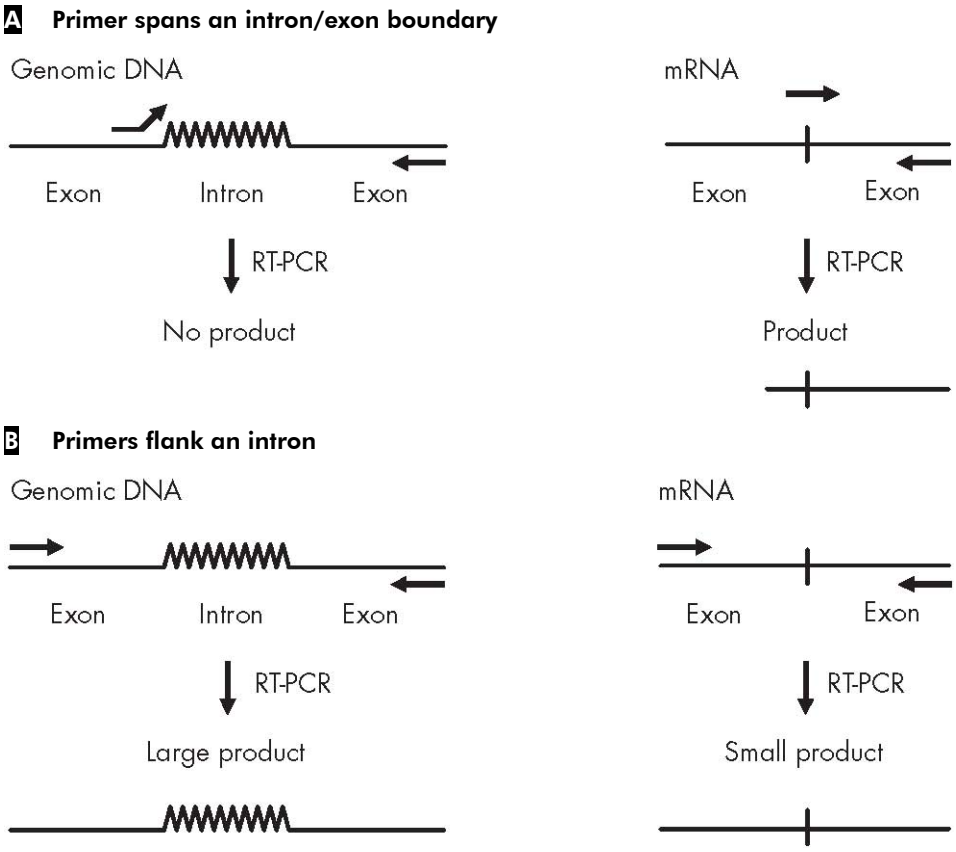

Figure 2. Primer design for RT-PCR. Primer design to **A** eliminate or **B** detect amplification from contaminating genomic DNA.

PCR amplicon length

The optimal amplicon length is between 80 and 200 bp, although products up to 500 bp may work well.

Sequencing primer

The sequencing primer should be 15 to 20 bases long and have an annealing temperature in the range of 45°C to 55°C. Ideally, the sequencing primer should differ from the RT-PCR primer by at least one additional and specific base at the 3' end.

Handling and storage of primers

Determining primer concentration and quality

Primer quality is crucial for successful RT-PCR. Problems encountered with RT-PCR are frequently due to the use of incorrect primer concentrations. If you observe problems in yield of amplification products, check that primers were used at the correct concentration.

Storage buffer

Lyophilized primers should be dissolved in a small volume of low-salt buffer* to give a concentrated stock solution (e.g., 100 µM). We recommend using TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0).*

Dissolving primers

Before opening a tube containing lyophilized primer, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer, add the required volume of sterile, nuclease-free TE buffer, mix, and leave for 20 minutes to allow the primer to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.

We do not recommend dissolving primers in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.

Concentration

Spectrophotometric conversion for primers:

$1\ A_{260}$ unit = 20–30 µg/ml

To check primer concentration, the molar extinction coefficient (ε_{260}) can be used:

$A_{260} = \varepsilon_{260}$ x molar concentration of primer or probe

If the ε_{260} value is not given on the data sheet supplied with the primers, it can be calculated from the primer sequence using the following formula:

$$\varepsilon_{260} = 0.89 \times [(A \times 15{,}480) + (C \times 7340) + (G \times 11{,}760) + (T \times 8850)]$$

Example

Concentration of diluted primer: 1 µM = 1×10^{-6} M

Primer length: 24 nucleotides with 6 each of A, C, G, and T bases

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Calculation of expected A_{260}: 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] x (1 x 10^{-6}) = 0.232

The measured A_{260} should be within ±30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers, or having the primers resynthesized.

Storage of primers

Primers should be stored in TE *in small aliquots at –20°C. Primers are stable under these conditions for at least one year. Prepare small aliquots of working solutions containing 10 pmol/µl to avoid repeated thawing and freezing. Primer quality can be checked on a denaturing polyacrylamide gel;* a single band should be seen.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Appendix C: Control of Contamination

It is extremely important to include at least one negative control that lacks the template nucleic acid in every RT-PCR setup to detect possible contamination.

General physical precautions

- Separate the working areas for setting up the RT-PCR master mix and RNA handling, including the addition of starting template, RT-PCR product analysis, or plasmid preparation. Ideally, use separate rooms.
- Use a separate set of pipets for the RT-PCR master mix. We strongly recommend uisng pipet tips with hydrophobic filters.
- Prepare and freeze small aliquots of primer solutions. We strongly recommend use of TE buffer.
- In case of contamination, laboratory benches, apparatus, and pipets can be decontaminated by cleaning them with a 1/10 dilution of a commercial bleach solution.*† Afterwards, the benches and pipets should be rinsed with distilled water.

General chemical precautions

- RT-PCR stock solutions can also be decontaminated using UV light. This method is laborious, however, and its efficiency is difficult to control and cannot be guaranteed. We recommend storing solutions in small aliquots and using fresh aliquots for each RT-PCR.
- Another approach to preventing amplification of contaminating DNA is to treat individual reaction mixtures with DNase I or restriction enzymes that cut between the binding sites of the amplification primers used, before adding the template DNA sample.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

† Most commercial bleach solutions are approximately 5.25% sodium hypochlorite.

Ordering Information

Product	Contents	Cat. no.
PyroMark OneStep RT-PCR Kit (50)	For 50 reactions: PyroMark OneStep RT-PCR Master Mix (includes HotStarTaq DNA Polymerase and optimized Reaction Buffer containing $MgCl_2$), 10x CoralLoad Concentrate, 5x Q-Solution, dNTP Mix, and RNase-Free Water	978801
PyroMark OneStep RT-PCR Kit (200)	For 200 reactions: PyroMark OneStep RT-PCR Master Mix (includes HotStarTaq DNA Polymerase and optimized Reaction Buffer containing $MgCl_2$), 10x CoralLoad Concentrate, 5x Q-Solution, dNTP Mix, and RNase-Free Water	978803
Related Products		
PyroMark systems, software, and accessories — for Pyrosequencing analysis		
PyroMark Q96 ID	Instrument, installation, and software for genetic analysis using Pyrosequencing technology	9001525
PyroMark Q96 MD	Instrument, installation, and software for genetic analysis using Pyrosequencing technology	9001526
PyroMark Q24	Instrument, installation and software for genetic analysis using Pyrosequencing technology	9001514
PyroMark Assay Design Software 2.0	Software for convenient design of RT-PCR and sequencing primers, optimized for Pyrosequencing analysis	9019077
PyroMark Q96 Cartridge (3)	For delivery of nucleotides and reagents; for use with PyroMark Q96 ID	979004
PyroMark Q24 Cartridge (3)	For delivery of nucleotides and reagents; for use with PyroMark Q24	979202

Product	Contents	Cat. no.
PyroMark Q96 Plate Low (100)	For sample analysis of DNA template on PyroMark Q96 ID	979002
PyroMark Q96 HS Plate (100)	For analysis of DNA template on PyroMark Q96 MD	979101
PyroMark Q96 HS Dispensing Tip Holder	Reusable holder for tips, nucleotide dispensing tips, and reagent dispensing tips; for use with PyroMark Q96 MD	9019075
PyroMark Q96 HS Capillary Tip Holder	Reusable holder for tips, capillary dispensing tips, and reagent dispensing tips; for use with PyroMark Q96 MD	9019076
PyroMark Q96 HS Reagent Tip (4)	Reusable tips (4 in each package); for dispensing reagents (RDT); for use with PyroMark Q96 MD	979102
PyroMark Q96 HS Nucleotide Tip (8)	Reusable tips (8 in each package); for dispensing nucleotides (NDT); for use with PyroMark Q96 MD	979103
PyroMark Q96 HS Capillary Tip (8)	Reusable capillary tips (8 in each package); for dispensing nucleotides (CDT); for use with PyroMark Q96 MD	979104
PyroMark Q96 HS Tip Storage Box	For dust-free and safe storage of tips in between runs; for use with PyroMark Q96 MD	9019074
PyroMark Q96 Vacuum Workstation	Vacuum workstation for preparing 96 samples in parallel from PCR product to single-stranded template	varies*
PyroMark Q24 Vacuum Workstation	Vacuum workstation for preparing 24 samples in parallel from PCR product to single-stranded template	varies†
PyroMark Gold Reagents — for performing Pyrosequencing reactions		
PyroMark Gold Q24 Reagents (5 x 24)	Nucleotides, enzyme, and substrate solutions, intended for use with PyroMark Q24	978002

* 9001529 (220 V); 9001528 (110 V); 9001740 (100 V).

† 9001518 (220 V); 9001516 (110 V); 9001519 (100 V).

Product	Contents	Cat. no.
PyroMark Gold Q96 Reagents (5 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 ID (5 x 96) and PyroMark Q96 MD (15 x 96)	972804
PyroMark Gold Q96 Reagents (50 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 MD and the PyroMark Q96 ID	972807
PyroMark Gold Q96 CDT Reagents (6 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 MD in combination with the capillary dispensing tips (CDT)	972824
PyroMark Gold Q96 SQA Reagents (1 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 ID	972812
QIAGEN sample preparation solutions – For efficient isolation of RNA		
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
QIAamp Viral RNA Mini Kit (50)	For 50 RNA preps: 50 QIAamp Mini Spin Columns, Carrier RNA, Collection Tubes (2 ml), RNase-free Buffers	52904
QIAamp RNA Blood Mini Kit (50)	For 50 RNA preps: 50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	52304
PAXgene Blood RNA Kit (50)	For 50 RNA preps: 50 PAXgene Spin Columns, 50 PAXgene Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-Free Reagents and Buffers. To be used in conjunction with PAXgene Blood RNA Tubes	762174

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