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Product Information

Quantitative RT-PCR ReadyMix™

Catalog Number **QR0200**
Storage Temperature -20 °C

Product Description

Quantitative RT-PCR ReadyMix™ combines the necessary reagents for probe-based one step quantitative RT-PCR with high specificity and sensitivity, reduced risk of contamination, and reliable reproducibility. Users need to only provide template along with specific primers and appropriate probe toward their gene of interest.

This kit comes with M-MLV Reverse Transcriptase for efficient RNA to cDNA transcription, which is spiked into a 2x qRT-PCR ready mix containing buffer, JumpStart *Taq* DNA polymerase, 99% pure deoxynucleotides, $MgCl_2$, and RNase inhibitor. JumpStart *Taq* DNA polymerase allows for hot-start PCR through an antibody which binds *Taq* polymerase at low temperatures, thus minimizing primer-dimer and nonspecific product formation. The kit also contains additional $MgCl_2$, 10x PCR Buffer, and Reference Dye that users can augment with to meet their specific RT-qPCR needs.

Reagents provided

Kit contains sufficient reagent for 5 mL total reactions (for example, 100 reactions of 50 µL each, or 250 reactions of 20 µL each).

- **P5871** 2x qRT-PCR ReadyMix, 2 x 1.25 mL
  0.1 U/µL Taq DNA Polymerase, 20 mM Tris-HCl pH 8.3, 100 mM KCl, 6 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), stabilizers and glass passivator, 1 U/µL RNase Inhibitor, JumpStart Taq mAb
- **M1427** M-MLV Reverse Transcriptase
  5000 units, 200 units/µL
- **M8787** $MgCl_2$ solution, 25 mM, 1.5 mL
- **P2192** 10x PCR Buffer, 1.5 mL
  100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin
- **R4526** Reference Dye for Quantitative PCR
  100x dye, 0.3 ml

Storage/Stability

The M-MLV RT should be stored at -20 °C. All other components can be stored at -20 °C, or may be stored at 2-8 °C for up to 6 months. Protect reference dye from light.

Reagents and equipment required but not provided

- Sample containing RNA template
- Specific Primers
- Specific qPCR-appropriate fluorescent probe
- Plastic PCR tubes, plates, or glass capillary tubes recommended by the PCR instrument manufacturer
- Real-time qPCR instrument

Quick Start Protocol

Assemble reaction mixtures with the following contents:

Reagent	Final Concentration	20 µL Reaction	50 µL Reaction
2x qRT-PCR ReadyMix	0.05 U/µL Taq 10 mM Tris-HCl 50 mM KCl 3 mM $MgCl_2$ 0.2 mM each dNTP 0.5 U/µL RNase inhibitor	10 µL	25 µL
M-MLV Reverse Transcriptase	1 U/µL (dilute in 10x PCR buffer if necessary)	0.1 µL	0.25 µL
RNA template	10-100 ng/µL total RNA or 0.5-10 ng mRNA	variable	variable
Forward Primer	500 nM (optimizable)	variable	variable
Reverse Primer	500 nM (optimizable)	variable	variable
Probe	250 nM (optimizable)	variable	variable
25 mM $MgCl_2$ (optional)	Supplement to increase beyond 3.0 mM	variable	variable
Reference Dye (optional)	1x	0.2 µL	0.5 µL
Nuclease-Free Water		Fill to 20 µL	Fill to 50 µL

Typical cycling parameters for 100-600 bp fragments:

Reverse Transcription		42–44 °C	30 min
Initial denaturation		94 °C	2 min
40	Denaturation	94 °C	15 sec
40	Annealing, extension, and detection	60 °C or 5 °C below lowest primer T_m	1 min
(Optional) Hold		4 °C (for further analysis)	

Preliminary Considerations

RNA Preparation

The single most important step in assuring success with RT-PCR is high quality RNA preparation. Integrity and purity of RNA template is essential. RNA must be entirely free of RNase contamination. Probe Based RT-PCR involves multiple cycles of enzymatic reactions and is therefore more sensitive to impurities such as proteins, phenol/chloroform, salts, EDTA, and other chemical solvents. Contaminants can also interfere with fluorescence detection. The ratio between absorbance values at 260 nm and 280 nm gives an estimate of RNA purity. Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1. Lower ratios indicate the presence of contaminants such as proteins. Either total or poly(A) RNA can be used as template for the reverse transcription reaction. All RNA preparations should be DNA-free to assure that quantification is based on RNA. If using primers/probe that are additionally complementary to DNA potentially found in samples, DNase I (cat no. AMPD1) is strongly recommended for the digestion of contaminating DNA before quantitative RT-PCR. Purified RNA should be stored at -20 °C or -70 °C.

Primer and Probe Design

Well-designed specific primers and probes are recommended for this system to ensure the highest possible specificity. Specific primers for RT and PCR should be designed with the aid of primer design software, or have been previously validated, to eliminate the complications introduced with primer-dimers and secondary structures. Lower primer concentrations decrease the accumulation of primer- dimer formation and nonspecific product formation, which is critical to maintain high efficiency in quantitative RT-PCR. If genomic DNA contamination is possible, primers spanning an intron will reduce the possibility of amplifying DNA and overestimating RNA message. Scientific literature and online databases can be useful source of previously validated primers and probes.

Magnesium Chloride Concentration

Supplemented Magnesium Chloride can sometime boost PCR sensitivity, however lower $MgCl_2$ concentrations usually result in the formation of fewer nonspecific products. The Ready-Mix solution is provided at a 2x concentration of 6 mM $MgCl_2$ (final concentration 3 mM). A vial of 25 mM $MgCl_2$ is provided for further optimization of the final magnesium chloride concentration if necessary.

Internal Reference Dye

A ROX-like proprietary internal reference dye is included in a separate vial for reaction normalization when using real-time thermal cyclers that recommend addition of a reference dye. Dye is provided as 100x stock, or 1000x stock for Low-ROX instruments. Maximum excitation of this dye is 586 nm and maximum emission is 605 nm. Standard instrument settings for ROX reference dye are satisfactory for the measurement of the internal reference dye. Check the fluorescent properties of your probe to ensure it uses a distinct emission wavelength and is therefore compatible with the reference dye. See Reference Dye Requirements on page 4 for thermocycler dye compatibility.

Controls

A positive control is always helpful to make sure all of the kit components are working properly. Two negative controls, no template and no reverse transcriptase, are necessary to determine if contamination is present. A signal in the no template control suggests the presence of DNA contamination or primer dimer formation. A signal in the no reverse transcriptase reaction indicates the presence of significant DNA that can be amplified. See Lovatt *et al.* for a thorough discussion of qRT-PCR controls.[1]

Data Analysis

Follow the recommendations of the real time instrument used to perform quantitative RT-PCR. Generally, fluorescence is plotted on a logarithmic scale against the cycle number. Instrument software will often determine a fluorescence threshold that indicates a positive signal; this value can also be set manually by users. The cycle number in which fluorescence for a given sample crosses the threshold, referred to as the Threshold cycle (C_T), is used to determine the template amount in each sample. For reliable interpretation of data, the fluorescence threshold should fall on the log-linear range of all positive sample and not be near the baseline or plateau; users are encouraged to visually confirm and adjust the threshold if necessary to meet this requirement.

Methods of Quantification

Standard Curves

Standard curves are necessary for both absolute and relative quantification. When generating standard curves, different concentrations of RNA (typically at least four) should be used to generate a standard curve that will bracket the concentration of the unknown. Each concentration should be run in duplicate.

Absolute and Relative Quantification

This quantitative RT-PCR kit may be used to quantify target RNA using either absolute or relative quantification. Absolute quantification techniques are used to determine the amount of target RNA in the initial sample, while relative quantification determines the ratio between the amount of target RNA and a reference amplicon. The ideal reference amplicon should have invariant, constitutive expression. Often a housekeeping gene is chosen for this function,

although other reference choices may better adhere to the above requirements.[2]

Absolute quantification uses external standards to determine the absolute amount of target nucleic acid of interest. To remove the differences in quantification due to annealing, the primer binding sites of the external standards must be the same as those in the target sequence. The ideal external standard contains sequences that are the same as the target sequence or which vary only slightly from the target sequence. Equivalent amplification efficiencies between the target and external standard are necessary for absolute quantification. Once a suitable construct or amplicon is identified, a standard curve of external standard dilutions is generated and used to determine the concentrations of unknown target samples.[3]

Relative quantification allows calculation of the ratio between the amount of target template and a reference template in a sample. Since this method measures the amount of target relative to a presumably invariant control, relative quantification is useful for measuring changes in gene expression, for example between different tissues or in healthy versus diseased samples. Use of an internal standard is critical towards normalizing for variations in sample preparation and handling.

Determination of RT-PCR Reaction Efficiencies
The RT-PCR efficiency between a reference sample and a target sample is determined by preparing a dilution series for each target. The C_T values of the reference are subtracted from the target and this difference in C_T values is plotted against the logarithm of the template amount. If the resulting slope of the straight line is less than ±0.1 the amplification efficiencies are judged to be similar.

Procedures
For best results, optimal concentrations of primers, probes, $MgCl_2$, KCL and PCR enhancers need to be determined.

Testing various combinations of primer concentrations (50-1000 nM) while keeping the probe concentration constant (250 nM) is most efficient for primer optimization. The same method could be used to optimization probe concentrations by varying probe concentrations (50-250 nM) and keeping optimal primer concentrations constant. Primer/probe combinations with the lowest C_t and the highest fluorescence will give the most sensitive and reproducible assays. If maximum sensitivity is not required and your PCR target is abundant, satisfactory results for probe-based qPCR are often obtained with final concentrations of both primers at 500 nM and probe at 250 nM.

Other additives intended to enhance RT-PCR can similarly be tested at varying amounts to find the optimal concentration of each. The use of up to 5% (v/v) dimethyl sulfoxide (DMSO) will not disturb the enzyme-antibody complex. Other co-solvents, solutes (salts) and extremes in pH or other reaction conditions may reduce the affinity of the JumpStart *Taq* antibody for the *Taq* polymerase and thereby compromise its effectiveness.

Procedure for Routine Analysis

1. Preparation of a reaction master mix is highly recommended to give best reproducibility. Mix all reagents but template in a common mix, using ~10% more than needed. Distribute appropriate amount of master mix amongst RNase-free PCR tubes or plate wells recommended by the qPCR instrument manufacturer.

2. Add template to corresponding tubes or plate well containing master mix.

3. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.

4. Perform thermal cycling.

See Page 1, Quick Start Protocol, for typical master mix and thermal cycling parameters

Note:
Initial reverse transcription at 42-44 °C is recommended, but poorly transcribing templates may benefit from a 55 °C reaction temperature. Such cases will need to be empirically determined. For primers with a T_m below 55 °C, a lower ramp time is recommended.

Optimal cycling parameters vary with primer design and thermal cycler. Consult your thermal cycler manual. It may be necessary to optimize the cycling parameters to achieve maximum product yield and/or quality.

References
1. Lovatt, A., et al. Validation of Quantitative PCR Assays, *BioPharm*, March 2002, p. 22-32.
2. Bustin, S. A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, *J. Molecular Endocrinology*, **29**, 23-29 (2002).
3. Morrison, T. B., et al., Quantification of Low-Copy Transcripts by Continuous SYBR® Green I Monitoring during Amplification. *BioTechniques,* **24,** 954-962 (1998).

Related products

- DNase I, Amplification Grade, for removing DNA from RNA preps, Catalog Number AMPDI
- GenElute Direct mRNA Miniprep Kit, for isolating mRNA from cells or tissue, Catalog Numbers DMN10 and DMN70
- GenElute Mammalian Total RNA Miniprep Kit, for isolating total RNA from tissue or cells, Catalog Numbers RTN10, RTN70, and RTN350
- GenElute mRNA Miniprep Kit, for isolating mRNA from total RNA, Catalog Numbers MRN10 and MRN70
- TRI Reagent®, for isolating total RNA from tissue Catalog Number T9424
- TRI Reagent BD, for isolating total RNA from whole blood, Catalog Number T3809
- TRI Reagent LS, for isolating total RNA from fluid samples, Catalog Number T3934

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Reference Dye Requirements

ROX compatible instruments

- Applied Biosystems® 5700
- Applied Biosystems 7000
- Applied Biosystems 7300
- Applied Biosystems 7700
- Applied Biosystems 7900
- Applied Biosystems 7900 HT Fast
- Applied Biosystems 7900 HT
- Applied Biosystems StepOnePlus™
- Applied Biosystems StepOne™

Low-ROX compatible instruments

- Applied Biosystems® 7500
- Applied Biosystems 7500 Fast
- Applied Biosystems ViiA 7
- Applied Biosystems QuantStudio
- Agilent® AriaMx
- Douglas Scientific IntelliQube
- QIAGEN® Rotor-Gene® Q
- Stratagene Mx3000P®
- Stratagene Mx3005P®
- Stratagene Mx4000™

No ROX required instruments

- Bio-Rad® CFX384™
- Bio-Rad CFX96™
- Bio-Rad MiniOpticon™
- Bio-Rad/MJ Chromo4™
- Bio-Rad/MJ Opticon 2
- Bio-Rad/MJ Opticon™
- Cepheid SmartCycler®
- Eppendorf Mastercycler® ep realplex
- Eppendorf Mastercycler ep realplex2 s
- Illumina Eco qPCR
- Qiagen/Corbett Rotor-Gene® 3000
- Qiagen/Corbett Rotor-Gene 6000
- Qiagen/Corbett Rotor-Gene Q
- Roche LightCycler® 480

Precautions and Disclaimer

This product is for R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

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Troubleshooting Guide

Problem	Possible Cause	Solution
No RT-PCR product (signal) is observed, RT-PCR product is detected late in PCR, or only primer-dimers are detected.	The RNA is degraded.	Check the RNA by denaturing agarose gel electrophoresis. Poly(A)$^+$ RNA should appear as a smear between 0.5 kb and 2 kb. The total RNA should have two sharp ribosomal RNA bands without notable degradation. For purifying RNA, use TRI Reagent$^{\vee}$ or GenElute RNA isolation kits (See Related Products section).
	There is not enough RNA template.	After increasing the number of cycles has shown no success, repeat the reaction with a higher concentration of template.
	There is incomplete removal of guanidinium during RNA isolation.	For any procedure using guanidinium-based lysis solution, remove as much of the residual liquid as possible after the first precipitation and then wash once with 70% alcohol.
	There is incomplete removal of the protease (such as Proteinase K) during RNA isolation.	Proteases used during RNA isolation may be removed by phenol/chloroform extraction and alcohol precipitation.
	Reverse transcription reaction temperature is incorrect.	The reverse transcription reaction temperature is usually best between 42-44 °C, variation from this range is not usually helpful.
	The design or concentration of the probe is sub-optimal	Check to ensure the probe does not hybridize significantly with either primer. Test a range of probe concentrations.
	RT-PCR product is too long.	The best results are obtained when RT-PCR products are between 100-150 bp and do not exceed 500 bp.
	Primer concentration is not optimal.	Lower concentrations of primers give less non-specific products and primer-dimers.
	Primers are degraded.	Check for possible degradation of primers on a denaturing polyacrylamide gel.
	A PCR component is missing or degraded.	A positive control should always be run to ensure components are functioning. Check concentrations and storage conditions of reagents, including primers and template RNA. A checklist is also recommended when assembling reactions.
	JumpStart *Taq* activated too early.	Complete the RT reaction before the denaturation step, which inactivates the JumpStart antibody.
	There are too few cycles performed.	Increase the number of cycles (3-5 additional cycles at a time). Some spectrofluorometric thermal cyclers including the Roche LightCycler allow extra cycles to be added during the run.
	The annealing temperature is too high.	Decrease the annealing temperature in 1 °C increments.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of the primer sequence to non-target sequences.
	The denaturation temperature is too high or too low.	Optimize the denaturation temperature by increasing or decreasing the temperature in 1 °C increments.
	The denaturation time is too long or too short.	Optimize the denaturation time by increasing or decreasing it in 10 second increments. The Roche LightCycler only recommends a 0 second denaturation time for normal templates and should only be increased in 5 second or less increments.
	Magnesium concentration is not optimal.	Start with the magnesium concentration provided in the quantitative RT-PCR master mix (3 mM final concentration). A 25 mM vial of magnesium chloride is provided if increased magnesium concentration is needed for optimal results.
	Detection was not activated.	Make sure the fluorescence detection is activated and correct for the probe being used (FAM, HEX, etc.)
	The correct detection parameters were not activated.	Perform fluorescence detection during the extension or extra detection step of the PCR cycling program.

Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
Multiple RT-PCR products	Reactions set up at room temperature.	Set up RT-PCR reactions on ice to avoid premature cDNA synthesis from nonspecific primer annealing.
	JumpStart *Taq* activated too early.	Complete the reverse transcription reaction before the denaturation step, which inactivates the JumpStart antibody.
	Magnesium concentration is not optimal.	Start with the magnesium concentration provided in quantitative RT-PCR master mix (3 mM final concentration). A 25 mM vial of magnesium chloride is provided if increased magnesium concentration is needed for optimal results.
	The annealing temperature is too low.	Increase the annealing temperature in increments of 1 °C.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information and the specificity of primer sequence to non-target sequences.
	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase I, Catalog Number AMPD1. Try and use primers that span an intron so amplification from genomic DNA is minimized.
	Primer-dimers are co-amplified	Include an additional detection step in the cycling program to avoid detection of primer-dimers.
	Primer concentration is too high.	Reduce the primer concentration in a series of two-fold dilutions (i.e. 0.1 µM, 0.05 µM, 0.025 µM) and test in a trial set of PCR reactions.
	Reverse transcription reaction temperature too low.	Start out at a reaction temperature between 42-50 °C. The reverse transcription reaction temperature may be increased if mispriming is detected.
	Primers are degraded.	Check for primer degradation on a polyacrylamide gel.
No linearity in ratio of C_T value to log of the template amount.	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase I, Catalog Number AMPD1.
	Template amount is too high.	Do not exceed the maximum recommended amounts of template RNA.
	Template amount is too low.	Increase amount of template RNA.
	Primer-dimers were co-amplified.	Include an additional detection step in the cycling program to avoid detection of primer-dimers, or redesign primers that do not produce primer-dimers.

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