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LiQuant™ Green qPCR Master Mix

Cat. #: M0026 Size: 5 ml/25 ml

Introduction

LiQuant™ Green qPCR master mix utilizes a special performance-enhanced Taq DNA polymerase protected via a hot-start activation technique, and optimized qPCR buffer system to perform SYBR Green I based quantitative PCR (qPCR). The mix, prepared at 2× reaction concentration, can be directly used for robust and low-template quantitative PCR with high sensitivity, specificity and reliability.

Package Information

Components	M0026-05 (500 rxn/ 20 µl/ rxn)	M0026-25 (2,500 rxn/ 20 µl/ rxn)
LiQuant™ Green qPCR Master Mix*	5 ml	25 ml
50× ROX Reference Dye 1**	200 µl	1000 µl
50× ROX Reference Dye 2**	200 µl	1000 µl

* Contain hot-start DNA Polymerase, dNTPs, Mg^{2+}, and SYBR Green I dye

** Used to rectify the error of fluorescence signals between different wells. Select appropriate ROX according to the Real-time PCR instrument used:

DO NOT USE ROX Reference Dye	Bio-Rad CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, MiniOpticon™; Opticon®, Opticon 2, Chromo4™; Cepheid SmartCycler®; Eppendorf Mastercycler® ep realplex, realplex 2 s; Illumina Eco qPCR; Qiagen/Corbett Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000; Roche Applied Science LightCyclerTM 480; Thermo Scientific PikoReal Cycler.
USE ROX Reference Dye 1 (final 1×)	Applied Biosystems 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOnePlus™
USE ROX Reference Dye 2 (final 1×)	Applied Biosystems 7500, 7500 Fast, ViiA™7; Stratagene MX4000™,

Storage

This product should be stored at -20°C protecting from light for 12 months. Repetitive freeze-thaw cycles while using. It is recommended to pre-aliquot the mix into small batches for frequent usage. The quality of this product is guaranteed for 12 months under proper storage conditions.

Attention Points in Operation (Please Read Carefully)

1. Avoid repetitive freeze-thaw cycles to prevent polymerase activity from decreasing. Aliquot the mix into small batches for frequent usage.

2. Gently invert the tube upside down several times before use. DO NOT vortex. Brief centrifugation prior to use is recommended.

3. Keep the mix from bright light during storage and usage due to the fact that the fluorescent SYBR Green I dye may fade under light over time, resulting in a decrease in performance sensitivity.

4. Due to the high sensitivity nature of the qPCR reaction, contamination of air or aerosols may lead to reaction failure or result inaccuracy. Please set up the qPCR reaction in a clean environment using filtered tips, and sterilized tubes and pipette sets.

Protocol

1) To obtain reliable quantitative PCR reaction results, it is recommended to run three replicates for each sample with reaction volume of 50µl/ well. The suggested template amount is 10 to 100ng for genomic DNA or 1 to 10ng for cDNA template. The following loading table is designed for reaction volume of both 50µl and 20µl.

Components	Amount per reaction (µl)	Amount per reaction (µl)	Final concentration
LiQuant™ Green Mix	25	10	1×
Template	Variable	Variable	1 to 100ng
Forward Primer (5 µM)	0.5 - 9	0.2 - 3.6	50 to 900nM
Reverse Primer (5 µM)	0.5 - 9	0.2 - 3.6	50 to 900nM
ROX Reference Dye	1	0.4	1×
Distilled Water (dH_2O)	Add to 50	Add to 20	-
Total Reaction Volume	50	20	-

Note:

· 200 nM of primer final concentration is applicable for most cases. The concentration can be adjusted within 50-900 nM when amplification efficiency is not satisfactory.

· Too much or too little template used may lead to inaccuracy of quantitative result. A range of 1-100 ng is recommended to result in a good Ct value (15< Ct< 35). If template is stocked at high concentration, dilute it prior to loading to prevent possible loading errors.

2) Perform quantitative PCR using optimized cycling conditions. Provided below is a standard two-step program shown in which annealing/ extension step is combined at 60°C (step 2). Please note that the hot-start polymerase in this system needs to be activated at 95°C for 5 minutes prior to amplification (step 1). If the amplicon sequence is GC-rich, the time for pre-denaturation/enzyme activation (step 1) can be prolonged to 10 minutes.

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Standard two-step amplification program:

Step	1	2	
	Hot-Start DNA Polymerase Activation	PCR	
	Hold	CYCLE(40 cycles)	
		Denature	Anneal/Extend
Temp.	95°C	95°C	60°C
Time	5-10 min	15 sec	30-60 sec
Volume	50 µl		

IMPORTANT! The 5-10 mins, 95°C step is required to activate the Hot-start DNA Polymerase.

* Extension time may be adjusted according to the qPCR instruments used. For example, the extension time should be set to no less than 30 seconds when using ABI 7700 and 7900HT, 31 seconds when using ABI 7000 and 7300, 34 seconds when using ABI 7500, or 10 seconds when using ABI StepOne Plus™.

Data Analysis

Two types of quantification methods are commonly applied to quantify target gene expression when using Liquant™ Green qPCR master mix.

1) **Relative Quantitation:** target gene expression is measured against an internal standard.Gene expression can be measured by the quantitation of cDNA converted from a messenger RNA corresponding to this gene relative to a calibrator sample serving as a physiological reference. In a typical experiment, gene expression levels are studied as a function of either a treatment of cells in culture, of patients, or of tissue type. The calibrator sample in each case is the cDNA from either the untreated cells or patients, or a specific tissue type. All quantitations are also normalized to an endogenous control such as 18S rRNA to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction.

Set Ct^{A1} as the Ct value of the target gene of sample 1, and Ct^{BI} as the Ct value of the internal control gene of sample 1; set Ct^{A2} as the Ct value of the target gene of sample 2, and Ct^{B2} as the Ct value of the internal control gene of sample 2. The expression difference (in folder) of the target gene in sample 1 and in sample 2 can be calculated this way($2^{-\triangle\triangle Ct}$ approach): $\triangle\triangle Ct=(Ct^{A2}-Ct^{B2})-(Ct^{A1}-Ct^{BI})$

The expression level of the target gene in sample 2 is $2^{-\triangle\triangle Ct}$ times that of sample 1.

Note:
This calculation method is based on the assumption that the amplification efficiency is 100 %(the amount of products after each cycle is doubled). If the amplification efficiency is not 100%, the calculation formula needs to be amended according to actual reaction efficiency. For example: if the amplification efficiency of the target gene and internal control genes is 1.90, then the formula should be amended into $1.90^{-\triangle\triangle Ct}$.

2) **Absolute quantitation:** compares the Ct of an unknown sample against a standard curve with known copy numbers. Absolute quantitation is applicable only if isolation procedure and sample contents do not effect PCR amplification. The quantitation of genomic DNA may lend itself for absolute quantitation against a standard curve.

3) **Passive reference ROX:** is a dye molecule included in the Liquant™ Green PCR Master Mix that does not participate in the PCR amplification. On applied Biosystems real-time PCR system, the passive reference provides an internal reference to which the SYBR Green/ dsDNA complex signal can be normalized during data analysis. Normalization is necessary to correct for well-to-well fluorescent fluctuation.

4) **Determination of baseline and threshold:** please refer to the real-time PCR system software used to calculate baseline and threshold values for a detector or manually set it up according amplification curves (Fig 1).

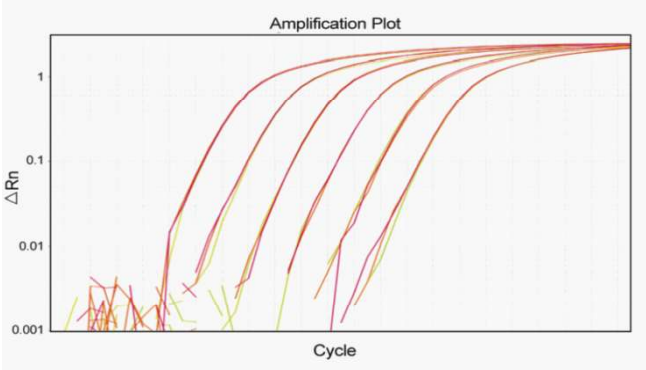

Figure 1: Amplification curve

Notes:
Quantitation is most accurate when Ct value is located between 20 and 28. If Ct value is too small (< 15), please dilute the template and repeat the quantitation; if Ct value is higher than 35, the amplification is invalid; if Ct value is between 32 and 35, t 3 replications are required to validate the results.

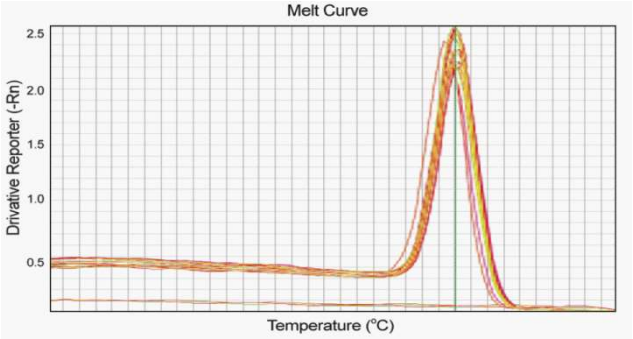

Figure2: Unimodal melt curve

Notes:
A typical valid dissociation curve should be unimodal due to highly specific amplification and no primer dimer formation (Fig 2). If the dissociation curve is apparently multimoda, it often suggests primer dimer formation, non-specific amplification, or other contamination.Alternatively, confirmation of non-specific amplification can be analyzed by agarose gel electrophoresis

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Notes on Primer Design

Forward and reverse primers for target gene quantification should be unique or as specific as possible to avoid non-specific amplification. Primer sequence design and specificity check can be conducted using NCBI's "BLAST" (an open source tool and free) or other primer design softwares. Additionally, the following general principals may be considered when designing primers.

1) The amplicon length is recommended to be within the range of 100 bp-200bp.

2) The optimal length of primers is 17bp-25bp.

3) The 3' end of primers should avoid GC-rich or AT-rich areas.

4) The last base at 3' end of the primers should be G or C and avoid T if possible.

5) The Tm value difference between forward and reverse primer may not exceed 1°C. The Tm value should be between 60°C and 65°C.

6) GC content of primers should be within 40%-60% with 45%-55% preferred.

7) The holistic distribution of A, G, C and T within primers should be as even as possible. Avoid using GC- or TA-rich areas.

8) Avoid continuous structures of T/C or A/G.

9) Avoid complementary sequence of 3 bases or more within primer itself or between forward and reverse primers. Avoid complementary sequence of 2 bases or more at the 3'end between forward and reverse primers.

Notes on Primer Design

1) Abnormal shape of amplification plot

a) Rough amplification plot: It is caused by system rectification due to weak signal. Elevate the template concentration and repeat in the reaction.

b) Broken or downward amplification plot: The concentration of templates is too high. The end value of the baseline is bigger than Ct value. Decrease the end of the baseline (Ct value -4), and re-analyze the data.

c) Amplification plot goes downward suddenly: There are bubbles left in the reaction tube, which break up when the temperature rises, thus the instrument detects the sudden decrease of the fluorescence value. Spin briefly and check closely if there are bubbles left before reaction.

2) No amplification plot

a) Cycling number is insufficient: Generally the cycling number is set to be 40. But notice that too many cycles will result in excessive background, thereby reducing the reliability of the data.

b) Check if there is signal collection procedure during cycling: in two-step program, signal collection is usually positioned at annealing and extension stage; for three-step program, signal collection should be positioned at 72°C extension stage.

c) Check if the primers are degraded: Test the integrity of primers after long-term storage through PAGE electrophoresis to confirm the presence of primers in solution.

d) The concentration of templates is too low: Reduce the dilution fold and retry. For target gene with unknown expression level, begin without template dilution.

e) Degradation of templates: Prepare new templates and retry.

3) Ct value appears too late (high)

a) Low amplification efficiency: Optimize the reaction. Try three-step program or re-design primers.

b) The concentration of templates is too low: Reduce the dilution fold and retry. For target gene with unknown expression level, begin without template dilution.

c) Degradation of templates: Prepare new templates and retry.

d) The amplicon is too long: The length of the amplicon is recommended to be within 100 bp-200 bp.

e) There are PCR inhibitors in the reaction: They are usually brought in when adding templates. Increase the dilution folds or prepare new templates and retry.

4) Apparent amplification can be observed in negative control.

a) The reagents or water used is contaminated: Change new reagents or water and retry. The reaction should be set up in a clean bench to minimize contamination from the air.

b) Appearance of primer dimer: it is normal that amplification occurs in negative control after 35 cycles. Analysis can be performed combining with the melt curve.

5) The linear relation of the standard curve is not satisfactory when performing absolute qualification

a) Pipetting error: Dilute the templates to increase the pipetting volume.

b) Degradation of standards: Prepare new standards and retry.

c) Too high template concentration: Increase the dilution fold.

6) Multimodal dissociation curve

a) The primers are not optimal: Design new primers according the design principles.

b) Too high concentration of primers: Appropriately decrease the concentration of primers.

c) cDNA template is contaminated by genomic DNA: Prepare new cDNA templates.

7) Experiment has low reproducibility

a) Inaccurate pipetting volume: Use a more accurate pipettor, elevate the reaction volume, and dilute the templates to increase the pipetting volume.

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b) Difference in temperature control in different wells of qPCR instrument: Regularly maintain the instruments.

c) Too low template concentration: The lower the template concentration, the worse the repeatability. Decrease the dilution fold or increase the volume of template used.

8) If this product can be stored at 4°C.

a) NO. Storage at 4°C will accelerate the deactivation of this product.

b) This product can keep its activity for a long time if stored at -20°C.

c) Repetitive freeze-thaw cycles may result in the decrease of product activity. Therefore, if only a small amount is needed each time, it is recommended to aliquot it into small batches and store them at -20°C.

9) Pre-denaturation/enzyme activation time

This mix is based on hot-start DNA polymerase, the pre-denaturation/ enzyme activation condition should be set to 95°C for 5 minutes to thoroughly activate the enzyme. If the template is GC-rich, the pre-denaturation/enzyme should be prolonged to 10 minutes.

Validation Parameters of qPCR Results

1) Confirmation of linear relation and amplification efficiency:

Correlation coefficient of standard curve (R^2) > 0. 98
Slope of standard curve within -3~-3. 5
PCR amplification efficiency (E) within 0.9~1.2

2) Confirmation of repeatability

STD of CT values (among replications) ＜0. 2

3) Confirmation of specificity

There are no apparent miscellaneous peaks of non-specific amplicons/primer dimers on dissociation curve (Confirm by electrophoresis if necessary).