LiQuant™ Probe qPCR Master Mix

Cat. #: M0027   Size: 5 ml/25 ml

Introduction

LiQuant™ Probe qPCR master mix is a special reagent designed for probe based qPCR. Utilizing hot-start LiQuant™ DNA Polymerase, alone with optimal buffer specially optimized for qPCR, it is capable of significant restraining non-specific amplification as well as enhancing the amplification efficiency, thus is applicable for high-sensitivity qPCR reactions. It is a 2× premixed reagent which is convenient to handle. When using this product for qPCR reactions, reliable standard curve can be obtained in a wide range and accurate qualification of target genes can be achieved with high repeatability and reliability.

Package Information

Components	M0027-05 (500 rxn/ 20 µl/ rxn)	M0027-25 (2,500 rxn/ 20 µl/ rxn)
LiQuant™ Probe 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 LiQuant™ DNA Polymerase, dNTPs, Mg^{2+}

** Be used to rectify the error of fluorescence signals between different wells. When using ABI 7900HT/ 7300 Real-Time PCR System and StepOnePlus™, use 50 × ROX Reference Dye 1; use 50 × ROX Reference Dye 2 for ABI 7500, 7500 Fast Real-Time PCR System, and Stratagene Mx3000P. No ROX is needed for Roche and Bio-Rad Real Time PCR instruments.

Storage

This product should be stored at -20°C; Try to avoid repetitive freeze-thaw cycles while using. If only a small amount is needed in each time, it is recommended to be divided and kept in smaller batches. The quality guarantee period is one year.

Attention Points in Operation (Please Read Carefully)

1) Try to avoid repetitive freeze-thaw cycles of the product to keep the activity of enzyme from decreasing. If only a small amount is used each time, it is recommended to divide it into smaller batches.

2) Invert the tube upside down for several times to mix thoroughly before use. Do not vortex for fear that excessive bubbles will be produced which may result in inaccurate reaction volume, thereby influencing the quantitative result. Mix can be used after blended and centrifuged slightly. Blow and beat gently during operation. If Mix foams, centrifuge before use.

3) Due to the extremely high sensitivity, this product can be contaminated by trace aerosol in the air, resulting in experimental failure. Therefore, please set up the reaction system in the clean bench and use clean sterilized tips and reaction tube. If possible, it is better to use special pipettes to avoid cross-contamination. Tips with filter cartridges are recommended.

Protocol

Take qPCR reaction on ABI StepOnePlus™ Real-Time PCR System for example. Quantify human GAPDH gene by using LiQuant™ qPCR Probe Master Mix or qPCR premixed solution containing ROX Reference Dye provided by Q Company. Use 10-folddiluted Hela genomic DNA ($10^{4\sim10}$ copies) as template.

Note: Please set up at least 3 replications (the statistical analysis of quantitative results requires at least 3 replications).

1. Setup of qPCR Reaction System

LiQuant™ Probe qPCR Master Mix	10µl
Template	Variable
Primer 1 (10 µM)	0.4µl
Primer 2 (10µM)	0.4µl
TaqMan Probe (10 µM)	0.2µl
ROX Reference Dye	0.4µl
Distilled Water (dH_2O)	Add to 20µl

Note: The amount of each component in the reaction can be adjusted according to the following principles:

· Generally, 0.2µM of each primer in the 20µl reaction is applicable for most cases. When the result is not satisfactory, the concentration can be adjusted within 0.1-1.0µM.

· The final concentration of the probe may be adjusted between 50nM and 250nM.

· qPCR is of high sensitivity. Accuracy of template amount added has great impact on the final quantitative result. Therefore, we recommend you to add the templates after dilution (say diluted to 5µl/sample) to increase the repeatability between the replications.

· The volume of cDNA template should not exceed 1/10 of the reaction volume.

2. Cycling Conditions

Generally, two-step approach shown in the diagram below is adopted, i.e. combining the annealing/extension step and setting at 60°C. Three-step approach may also be adopted.

Note: LiQuant™ DNA Polymerase need thermal activation to recover its enzyme activity, so please set the pre-denaturation condition as 95°C and 5 min. If the amplicon is GC-rich, the time for pre-denaturation can be prolonged to 10 min.

Melting curve is a default program preinstalled in the qPCR instrument, can be added at the end of the amplification.

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![LifeSct.com Life Scientists' Service Center]

Email: support@lifesct.com
Tel: 240-715-2985   Fax: 240-252-7376
14323 Woodcrest Dr., Rockville, MD 20853

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Standard procedures of two-step approach:

Stage	1	2	
	Pre-denaturation	Cycling reaction	
Reps	1	40	
Temp.	95°C	95°C	60°C
Time	5 min	10 sec	30 sec*

* Please adjust the extension time by yourselves according to the instruments you use. Set the time at least as 30 sec when using ABI 7700 and 7900HT, 31 sec when using ABI 7000and 7300, 34 sec when using ABI 7500 and 10 sec when using ABI StepOnePlus™.

2. Confirm the amplification plot (Diagram on the left) and make standard curve (Diagram on the right), etc. The specificity of amplification can also be verified by agarose gel electrophoresis.
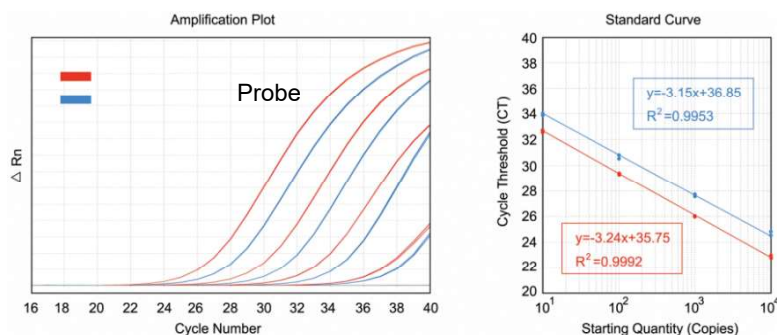
Carry out qPCR detection of human GAPDH gene by using LiQuant™ Probe qPCR Master Mix (red line) or qPCR premixed solution provided by Q Company (blue line). Amplification Plot shows that AceQ® qPCR Probe Master Mix possesses higher amplification efficiency and lower detection limit (left), and better linear relation (right).

Notes on Primer Design

1) The optimal length of primers is 17bp-25bp. Too short primers will result in decrease of amplification efficiency; too long primers will result in higher probability to form advanced structures. Both will interfere with the accuracy of quantitative result.

2) GC content of primers should be within 40%-60%. It will be best if it is within 45%-55%.

3) The holistic distribution of primer A, G, C and T should be as even as possible. Avoid using GC- or TA-rich areas. Especially for 3' end, areas where GC content is not even should be avoided.

4) Avoid continuous structures of T/C or A/G while designing primers.

5) The last five bases at 3' end of the primers should not contain over 2 G or C.

6）The forward or reverse primers should be as close to the probe sequence as possible, but should not overlap with it.

Notes on Primer Design

The probe sequence should be as close to the forward or reverse primers as possible, but should not overlap with them.

1) Generally the length of probe is 18～40bp.

2) Avoid the choosing continuous identical bases, especially GGGG or more.

3) The 5' end of the probe should avoid using G.

4) The annealing temperature of probe should be 65～67°C. If there is polymorphic locus in the sequence, make it lie in the middle of the probe.

8. Trouble Shooting

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.

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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.

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).

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