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AceQ® qPCR

SYBR® Green Master Mix (Without ROX)

YH Biosearch

www.yh-biosearch.com

SHANGHAI YEHUA Biological Technology Co., Ltd.

Instructions

Version 1.2

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Catalog

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1/Product Abstract

This product is the special reagent for qPCRntakeing SYBR® Green I along with the fluorescence method. AceTaq® DNA Polymerase in this product is activated over heat. Along with optimized buffer aimed at qPCR, it can efficiently resist non-specific amplifications, resulted into a higher efficiency. Therefore, this product is adaptable for qPCR of high sensitivity. SYBR® Green I, a 2× premixed reagent, of the most proper concentration to be tested for qPCR is contained in this product, which is convenient for use. A good standard curve can be achieved in a wide quantitative area if this product is used for qPCR. This product can be used for an accurate quantitative test at target genes with a good repeatability and reliability.

2/Product Composition

Product Composition	Q121-01 (50 rxn/50 µl/rxn)	Q121-02 (200 rxn/50 µl/rxn)	Q121-03 (1,000 rxn/50 µl/rxn)
AceQ® qPCR SYBR® Green Master Mix (Without ROX)[a]	1.25 ml	1.25 ml × 4	Q121-02×5

a Containing AceTaq® DNA Polymerase，dNTP Mix，Mg2+，SYBR® Green I.

3/Applicable Models

This product contains no ROX Reference Dye of low concentration to revise fluorescence signal error between holes, applicable for following fluorescence quantitative models:

Bio-Rad CFX96™, CFX384™, iCycler iQ™, iQ™5,MyiQ™; MiniOpticon™,
Opticon®, Opticon 2, Chromo4™;
Cepheid smartcycler®;
Eppendorf Mastercycler® ep realplex, realplex 2 s;
Lllumina Eco qPCR;
Qiagen/Corbett Rotor-gene®Q, Rotor-gene®3000, rotor-Gene®6000;
Roche Applied Science LightCycler™ 480;
Thermo Scientific PikoReal Cycler;

Other fluorescence quantitative PCR models requiring no ROX Reference Dye.

4/ Preservation and Validity

This product should be preserved at -20℃ avoiding light; better to avoid multigelation during experiment, and subpackages of small dose are recommended if that is necessary.

Valid within one year.

5/Experiment Announcements (Please read carefully)

1)Avoid multigelation reducing enzyme activity and subpackages of small dose are recommended if that is necessary.
2)Turn upside down to blend Mix before using it. Do not vortex, in case extra bubbles causes the misalignment of reaction system's volume and resulted in an influenced quantitative result. Blended Mix should be used after a slight centrifugation. Movement range should be small during procedures, a second centrifugation is necessary if extra bubbles occur because of inappropriate operations.
3) Light should be avoided both for preservation of Mix and preparation of its reaction system, since fluorochrome SYBR® Green I is contained in this product.
4)Given to its high testing sensitivity, reaction system should be prepared in an ultra-clean bench to avoid traces of aerosol in the air polluting this product and then leading into an experiment failure. Clean sterilized spears and reaction tubes are required during preparation. To avoid pollution, specialized pipettors used in laboratories are recommended, if conditionspermits, especially pepittor with filter element.

6/Reference Implementation

1)qPCR reaction system's preparation

AceQ® qPCR SYBR® Green Master Mix (Without ROX)	10	μl
Primer 1 (10 μM)	0.4	μl
Primer 2 (10 μM)	0.4	μl
Template DNA	x	μl
Sterilized stilled water	Up to 20	μl

Note: Volumes of different ingredients can be adjusted according to the following principles:
I.Generally speaking, primer concentration of 0.2 μM leads to better amplifications. If performances are bad, primer concentration can be adjusted from 0.1-1.0 μM.
II.If directly take cDNA stoste as the template, volume used should not be over 1/10 of the total volume of qPCR reaction.
III. qPCR has a super high sensitivity, the accuracy of templates added to build the reaction system has a great influence on the final quantitative result. Therefore, when the repetitions are not good enough between holes, the templates can be diluted to a certain concentration and be added into the reaction system with a bigger volume. Repetitions will be enhanced in return.
IV.100 bp-500 bp is the best length range for amplification production, especially between 100 bp-200 bp.

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2)qPCR reaction procedure set
Usually reactions are proceed in a two-step procedure as following chart shows:
Anneal/Extension set on 60℃, Three-step procedure is also feasible.
Note: Since the enzyme activity of AceTaq ® DNA Polymerase needs to be activated over heat, please set PCR reaction predegeneration condition at least on 95℃ for 5 minutes. If the template contains high GC, predegeneration time can be extended to 10 minutes.

Two-step standard procedure:

Stage 1 Predegeneration	Reps：1	95℃	5	min
Stage 2 Circular reaction	Reps：40	95℃	10	sec
		60℃	30	sec
Stage 3 Melt curve*	Reps：1	95℃	15	sec
		60℃	60	sec
		95℃	15	sec

*Melt curve data collection procedure can be adjusted according to the Real Time PCR used. In general condition, default program is good enough.

3)Confirm the Amplification curve (Figure one) and Melt Curve (Figure two) to make a standard cure and others. Specificity of amplification products can be confirmed with sepharose gel through electrophoresis.

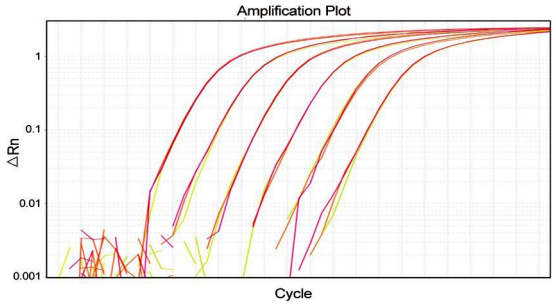

Figure one: Amplification Curve

Note: Reaction Ct between20-28 is best for the accuracy of fluorescence quantitative test. With too low a Ct, another test with diluted template is necessary; with a Ct higher than 35, Real-Time PCR test is invalid, and the target gene have no expressions; with a Ct between 32-35, three repetitions at least are needed to judge if the target gene is expressed.

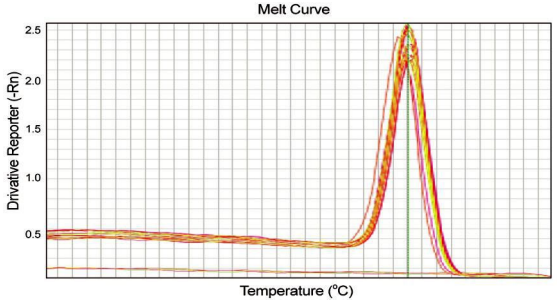

Figure two: Amplification Product Melt Curve

Note: If the specificity of the reaction is high and no primer senior structure nor non-specific amplification products occur, the Melt Curve should be an unimodal one and the quantitative result is valid; if the Melt Curve gets obvious multiple peaks, the result is invalid and another quantitative test is necessary with optimized conditions.（Unreasonable primer design would lead
to a peak of primer dimer in Melt Curve usually around 75℃, and another test is necessary with replaced primer if this peak is obvious.)

Clients operating absolute quantitative tests have to draw the standard curve by themselves; clients operating relative quantitative tests could calculate the expression of target genes according to the following formulas:

Set Ct of the target gene of Template No.1 to be CtA1and that of the reference gene of Template No.1 to be CtB1； set Ct of the target gene of Template No.2 to be CtA2 and that of the reference gene of Template No.2 to be CtB2; and the ratio of expression levels of template No 1 and Template No.2 can be roughly calculated in (2 -△△Ct) method:
△△Ct =(CtA2-CtB2) - (CtA1-CtB1) = X
And the expression level of target gene in Template No.2 is 2-X times that of Template No.1.

Note: The calculation method above is based on the assumption that the amplification efficiency is 100% (product of every cycle is two times that of the last cycle). The calculation formula should be adjusted according to the real efficiency when the amplification efficiency is below 100% because of not optimized conditions. For instance, when the amplification efficiency of target gene and reference gene is 1.90, the calculation formula should be amended to 1.90 -△△Ct.

7/Primer Design Guideline

Note: Since qPCR has a high sensitivity and a poor fault tolerance, the following content should be read carefully before designing primer to ensure a successful experiment::
1). Ideal length of amplification product is between 100 bp-150 bp.Important!
2). Ideal length of primer is between 17 bp-25 bp. Either longer or shorter primer would affect the accuracy of quantitative results.
3). The 3' end of the primer should avoid areas with high GC or AT.
4). The last basic group of 3' end of the primer is best to be G or C. Try to avoid T.
5). The gap between Tm values of the forward primer and the reverse primer should better be within 1℃. Tm is best to be arranged within 60℃-65℃ (Primer 5 is recommended to calculate the primer Tm value).
6). GC content of primer should better be controlled within 40%-60%, 45%-55% is the best.

7). Primer A、G、C、T should better be arranged balanced, and areas with high GC or TA should be avoided. The 3' end in particular should avoid areas with unbalanced GC.

8). Primer designing should better avoid continuous structures of T/C or A/G.

9). Avoid complementary sequences with more than 3 basic groups inside the primer or between primers. The 3' ends between two primers should avoid complementary sequences with more than 2 basic groups.

10). Test primer's specificity through NCBI BLAST after primer design is complished to avoid non-specific amplification.

8/FAQ & Solutions

1). Abnormal shape of amplification curve

a).Rough curve: weak signal, occurs on system's correction. A second test should be done with higher template concentration.

b).Fractured or gliding curve: high template concentration, the finishing end of base line is bigger than Ct. Reduce the finishing end of base line (Ct- 4) and analyses the data again.

c).Some curves plunge: reaction tube has gotten bubbles inside, and bubbles burst when temperature gets higher,only to give the equipment a plunging fluorescence value.centrifugation should be paid attention to when dealing with templates and reaction tubes should be carefully checked if thereie any bubble left before amplification.

2). No amplification curve occurs after reaction

a). Not enough reaction cycles: usually reaction cycles are set to be 40. Too many cycles would increase more background signal to reduce data reliability.

b).To check if signal collection procedure is set:: it is usually set at Anneal/Extension stage in two-step amplification procedure while at 72℃ Extension stage in three-step amplification procedure.

c).To confirm if primer is degraded: the integrity of primer that not used for a long time should be tested through PAGE electrophoresis to avoid the possibility of primer's degradation.

d). Template concentration is too low: to reduce the dilution and repeat the experiment, usually begin with the highest concentration if the template concentration is unknown.

e). Template degradation: to re-prepare templates and a second test is necessary.

3). Ct value occurs too late

a).Extremely low amplification efficiency: to optimize reaction conditions, try three-step amplification procedure, or re-design primer.

b).Low template concentration: to reduce the dilution and repeat the test usually begin with the highest concentration if the template concentration is unknown.

c).Template degradation: to re-prepare templates and a second test is necessary.

d).Too long PCR product: PCR products' lengths are usually designed between100 BP-150 BP.

e).PCR reaction inhibitors are existing in reaction system: usually brought in along with template, raise the dilution ratio and re-prepare templates for another test.

4). Obvious amplification in negative control

a). Reaction system or water is polluted: to replace with new Mix or water to repeat the experiment. Reaction system should be prepared on super clean bench to reduce aerosol pollution.

b). Primer dimer: amplifications in negative control are normal after 35 cycles, and they can be analyses together with Melt Curve.

5). Bad linear relation of standard curve in absolute quantitative tests

a). Sample addition error: to raise dilution ratio and the sample volume.

b). Standard sample degradation: to re-prepare standard sample and repeat tests.
c). Too high the template concentration: to raise the dilution ratio of the template.

6). Multiple peaks in Melt Curve

a).Optimization of primer is not enough: to design a new primer according to design guidelines.
b). Too high the primer concentration: to reduce properly the primer concentration.
c). Gene groups polluted in cDNA template: to re-prepare cDNA templates.

7). Bad repeatability in tests

a). Volume error in sample addition: to use pipette of better quality, expand reaction volume. Dilute the template at higher ratio and add the template dilution of big volume in reaction system .
b). Quantitative PCR equipment control different temperatures at different places: regular calibrations on the equipment.
c). Too low the template concentration: the higher the dilution ratio is, the worse the repeatability. Lower the dilution ratio or enlarge the volume of adding sample.

8). If this product can be preserved at 4℃

a). No. Preservation at 4℃ would lead to a decline in products' activity.
b). Preservation at -20℃ is recommended for it would keep the products' activity for a long term.
c). Preservation in subpackages of small dose at -20℃ are recommended, for multigelation could reduce the products' activity.

9). Predegeneration time

This product is a Taq enzyme, hot-started on AceTaqTM DNA Polymerase with chemical modification.Predegeneration should be set at 95℃ for at least 5 minutes to release enzyme activity completely. Predegeneration time can be extended to 10 minutes if GC is too high in template.

9/Standards of crisps Validity

1). Confirmation of linear relation and amplification validity:
Correlation index of standard curve (R2)＞0.98
Standard curve slope between -3～-3.5
PCR amplification validity (E) between0.9～1.2
2). Confirmation of acceptability: STD between two duplications ＜0.2
3). Confirmation of specificity:
No obvious peaks for non-specific amplification product/primer dimer in Amplification Melt Curve (confirm by agarose electrophoresisif necessary)