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Product Information

SYBR® Green JumpStart™ *Taq* ReadyMix™ for High Throughput Quantitative PCR

Catalog Number S9194
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

SYBR Green JumpStart *Taq* ReadyMix for High Throughput Quantitative PCR combines the performance enhancements of JumpStart Taq and SYBR Green I in an easy-to-use ReadyMix solution that incorporates ROX dye for ABI and other real time instrument applications. The ReadyMix includes a detection fluor, internal standard and reagents for PCR making it the ideal solution for performing high-throughput quantitative PCR. The ReadyMix contains SYBR Green I, JumpStart Taq DNA polymerase, 99% pure deoxynucleotides, and reaction buffer. It is provided in a 2× concentrate. Simply add an equal volume of the ReadyMix to a 2× mixture of DNA template and primers.

JumpStart Taq antibody inactivates the DNA polymerase at room temperature, thereby preventing non-specific product formation. When the temperature is raised above 70 °C in the first denaturation step of the cycling process, the complex dissociates and the polymerase becomes fully active. Antibody-mediated hot start requires no special preparations or protocol changes.

Sigma's Reference Dye for Quantitative PCR is incorporated in the mix to normalize reaction data for real-time detection. Maximum excitation and emission of the dye is 586 nm and 605 nm, respectively. Instrument settings for ROX reference dye are satisfactory for the measurement of Reference Dye for Quantitative PCR.

Features and Benefits

- SYBR Green ReadyMix for High Throughput Quantitative PCR is the perfect ReadyMix for high throughput, quantitative PCR applications.
- SYBR Green I is ideal for quantifying any DNA sequence.[1] The dye binds to double-stranded DNA and detection is monitored by measuring the increase in fluorescence throughout cycling.
- The hot start mechanism, using JumpStart Taq antibody, prevents non-specific product formation and allows assembled PCR reactions to be placed at room temperature up to 2 hours without compromising performance.
- Internal Reference Dye is provided for reaction normalization. Maximum excitation and emission of the dye is 586 nm and 605 nm, respectively.
- When performing large numbers of PCR reactions, the SYBR Green JumpStart Taq ReadyMix can save a significant amount of preparation time, reduce the risk of contamination from multiple pipetting steps, and provide consistent batch-to-batch and reaction-to-reaction performance.

Reagents

Sufficient for 400 or 2000 PCR reactions (50 µL reaction volume)

SYBR Green JumpStart Taq ReadyMix, Catalog Number S9194, containing 20 mM Tris-HCl, pH 8.3, 25 °C, 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.05 unit/µl Taq DNA Polymerase, JumpStart Taq antibody, 2× internal reference dye and SYBR Green I.

Reagents and Equipment to be Supplied by User

Catalog Numbers are provided as appropriate.

- Water, PCR Reagent, Catalog Number W1754
- Primers
- DNA template
- Dedicated pipettes
- PCR pipette tips
- 0.2 ml or 0.5 ml thin-walled PCR microcentrifuge tubes or plates for specific thermal cycler
- Real time instrument for quantitative PCR

Precautions and Disclaimer
SYBR Green JumpStart *Taq* ReadyMix for High Throughput Quantitative PCR is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
SYBR Green JumpStart *Taq* ReadyMix for High Throughput Quantitative PCR can be stored at 2–8 °C for up to 3 months or at –20 °C for up to one year. There was no detectable loss of performance after 10 freeze-thaw cycles.

Preliminary Considerations
DNA Preparation
The single most important step in assuring success with PCR is high quality DNA preparation. Integrity and purity of DNA template is essential. Quantitative PCR involves multiple rounds 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 of absorbance values at 260 nm and 280 nm gives an estimate of DNA purity. Pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0. Lower ratios indicate the presence of contaminants such as proteins.

Primer Design
Specific primers for PCR should be designed with the aid of primer design software 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 in using SYBR Green I dye in quantitative PCR.

Magnesium Concentration
Lower magnesium chloride concentrations usually result in the formation of fewer nonspecific products. The ReadyMix solution is provided at a 2× concentration of 7 mM magnesium chloride (final concentration 3.5 mM). A vial of a 25 mM magnesium chloride solution is provided for further optimization of the final magnesium chloride concentration if necessary.

Internal Reference Dye
An internal reference dye is included in the ReadyMix solution for reaction normalization. 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.

Controls
A positive control is always helpful to make sure all of the kit components are working properly. A negative control is necessary to determine if contamination is present. A signal in the no template control demonstrates the presence of DNA contamination or primer dimer formation. See Lovatt et al. for a thorough discussion of qPCR controls.[3]

Data Analysis
Follow the recommendations of the real time instrument manufacturer to perform quantitative PCR using SYBR Green I dye. Generally, the log of relative fluorescence is plotted against the number of cycles to determine the threshold cycle (C_t) or crossing point. The C_t value is used to determine the amount of template in each sample. Consider the following points when determining the C_t:

- C_t is the first detectable increase in fluorescence due to PCR product formation
- Cycles before the C_t are the baseline cycles
- The threshold can be adjusted manually
- Threshold should always be set using a logarithmic amplification plot
- Threshold should be set in the most exponential phase of the reaction, not after reaching the plateau.

Melting Curves
Performing a melting curve analysis at the end of the run will help analyze only the PCR product. Follow the real time instrument manufacturer's instructions for melting curve analysis. After running a melting curve, any additional runs involving the same PCR product can be done with data collected in an additional detection step to eliminate primer-dimer and other misprimed product signal.

Methods of Quantification

Standard Curves

Standard curves are necessary for both absolute and relative quantification. When generating standard curves, different concentrations of DNA (typically five) 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 product may be used to quantify target DNA using either absolute or relative quantification. Absolute quantification techniques are used to determine the amount of target DNA in the initial sample, while relative quantification determines the ratio between the amount of target DNA and a reference amplicon. The ideal reference amplicon would have invariant, constitutive expression. In practice, a housekeeping gene is chosen for this function, but there are other reference choices which better adhere to the above requirements.[4]

Absolute quantification uses external standards to determine the absolute amount of target nucleic acid. These external standards contain sequences that are the same as the target sequence or which vary only slightly from the target sequence. The primer binding sites of the external standards are always identical to the target sequence. The similarity between the external standard sequence and the target sequence is necessary for amplification efficiencies between the two to be essentially equivalent. Equivalent amplification efficiencies between the target and external standard are necessary for absolute quantification. A standard curve of external standard dilutions is generated and used to determine the concentrations of unknown target samples.

Relative quantification calculates the ratio between the amount of target template and a reference template in a sample. The relative amount of gene expression is a common application for relative quantification. The reference gene, usually a housekeeping gene, must not vary in concentration in different experimental conditions or tissue states for relative quantification to be possible. Amplification of the target and reference template dilutions in the sample should be performed in separate tubes. SYBR Green PCR quantification does not allow for multiplexing. If the reference template and the target template have different amplification efficiencies, then two standard curves need to be generated. The ratio of the resulting amounts of target and reference in the sample of interest can then be determined from these two standard curves. If the reference template and the target template have very similar amplification efficiencies, then only one standard curve for the reference template needs to be generated to determine the ratio of the amounts of target and reference in the sample.

Determination of PCR Reaction Efficiencies

The PCR efficiency between a reference sample and a target sample is determined by preparing a dilution series for each target. The C_t values from either the reference or target is then subtracted from the other. The difference in C_t values is then plotted against the log of the template amount. If the resulting slope of the straight line is less than 0.1, the amplification efficiencies are similar.

References

1. Morrison, T. B., *et al*., Quantification of Low-Copy Transcripts by Continuous SYBR® Green I Monitoring during Amplification. *BioTechniques*, **24,** 954-962 (1998).
2. Sambrook, J., and Russell, D. W., *Molecular Cloning: A. Laboratory Manual*, Third Edition, (Cold Spring Harbor Laboratory Press, New York, 2001). (Product Code M8265)
3. Lovatt, A., et al., Validation of Quantitative PCR Assays, BioPharm., March 2002, p.22-32.
4. Bustin, S. A., Quantification of mRNA using realtime reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol. 29, 23-9 (2002).

Procedure

Note: Because SYBR Green I binds to all double-stranded DNA, it is important to test primers and cycling conditions to ensure that the PCR product is a single band. It is best to ensure PCR specificity by checking the reaction on a normal (non-quantitative) thermal cycler and analyzing the result using agarose gel separation.[2]

For best results, optimal concentrations of primers, $MgCl_2$, KCl and PCR adjuncts need to be determined. Testing various combinations of primer concentrations (50-1000 nM) is most efficient for primer optimization. If maximum sensitivity is not required and your PCR target is abundant, satisfactory results for SYBR Green based qPCR are often obtained with final concentrations of both primers 200-400 nM.

The following procedure serves as a guideline to establish optimal primer concentrations. Further optimization may be necessary due to primer specificity. For more optimization information, please read the qPCR user guide available online at www.sigmaaldrich.com.

Note: The use of up to 5% (v/v) dimethyl sulfoxide (DMSO) will not disturb the enzyme-antibody complex, but will affect SYBR signal and background fluorescence. Other cosolvents, 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.

A. Optimizing Primer Concentrations

1. Prepare and dispense diluted primers (Fig 1).

a. Prepare 60 µL of 8 µM working solutions of both forward (fwd) and reverse (rev) primers in the first tubes of 2 separate 8-tube strips.
b. Dispense 30 µL of water into tubes 2-5.
c. Transfer 30 µL of the 8 µM primer solution from tube 1 into tube 2. Mix thoroughly by pipetting up and down at least 5 times.
d. Repeat transfer and mixing from tube 2 to 3, 3 to 4, and 4 to 5.
e. Using a multichannel pipettor, transfer 5 µL from the strip-tubes containing diluted fwd primer into the first 5 wells down columns 1-5 of a 96-well PCR plate. After adding fwd primer, PCR mix and template, final concentrations of fwd primer will be 1000, 500, 250, 125, 62.5 nM.
f. Similarly transfer 5 µL from the strip-tubes containing diluted rev primer into the first 5 wells across rows A-E. After adding PCR mix and template, final concentrations of rev primer will be 1000, 500, 250, 125 and 62.5 nM

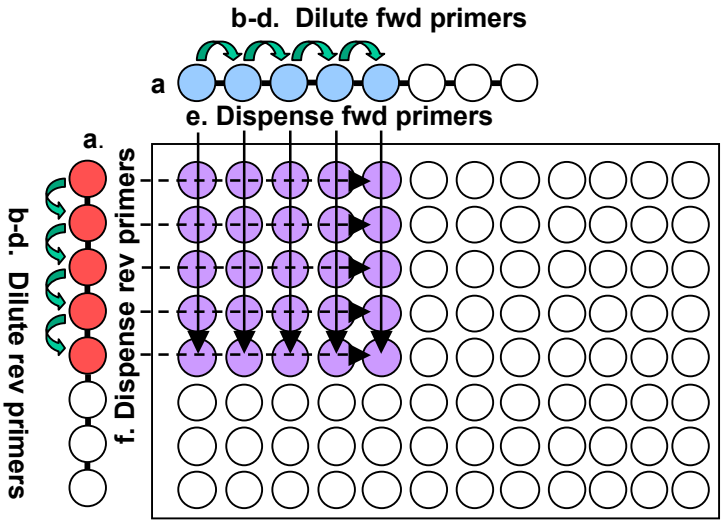

Fig 1: Follow steps 1a – 1f using diagram above

2. Prepare qPCR master mix:

Add reagents below in an appropriate sized DNase-free tube. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.

Volume	Reagent	Final Concentration
520 µL	2x SYBR Green JumpStart *Taq* ReadyMix	1.25 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTP, reference dye, stabilizers
156 µL	Water	
676 µL	Total Volume	

3. Aliquot 26 µL master mix into all wells in the PCR plate that contain primers (A1-E5)

4. Mix Thoroughly and transfer 18 µL from each of wells A1 through E5 to wells A8 through E12.

5. Add 2 µL template DNA (10-50 ng genomic DNA or 0.1-1 ng plasmid) to one set of reactions (columns 1-5) and 2 µL of water to the other columns (8-12).

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

7. Perform Thermal cycling:

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.

Typical cycling parameters for 100 bp – 600 bp fragments:

This protocol has been successfully tested on the following thermal cyclers: Stratagene MX 3000P, BioRad iCycler, MJ Opticon and ABI 7700.

Initial denaturation	94 °C	2 min
40 cycles:		
Denaturation	94 C	15 sec
Annealing, extension, & read fluorescence	60 °C or 5 °C below lowest primer T_M	1 min
(Optional) Hold	4 °C - only if products will be run out on a gel	

8. Evaluate fluorescence plots (∆Rn) for reactions containing target nucleic acid (columns 1-5). Primer combinations with the lowest C_t and the highest fluorescence will give the most sensitive and reproducible assays.

B. 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. Once template is diluted into the reaction vessel, master mix is aliquoted into the proper tube or plate for thermocycling.

Volume*	**Reagent**	**Final Concentration**
25 µL	2× SYBR Green JumpStart *Taq* ReadyMix	1.25 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTP, reference dye, stabilizers
--- µL	Forward Primer	Optimal Conc. from Sec. A
--- µL	Reverse Primer	Optimal Conc. from Sec. A
--- µL	Template DNA	10 ng-100 ng
q.s. to 50 µL	Water	
50 µL	Total Volume	

* Volume for 50 µL reaction, however component volumes may be scaled to give the desired reaction volumes.

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

3. Perform Thermal cycling

Typical cycling parameters for 100 bp – 600 bp fragments:

Initial denaturation	94 °C	2 min
40 cycles:		
Denaturation	94 °C	15 sec
Annealing, extension, & read fluorescence	60 °C or 5 °C below lowest primer T_M	1 min
(Optional) Hold	4 °C - only if products will be run out on a gel	

Troubleshooting Guide

Symptom	Possible Cause	Solution
No PCR product (signal) is observed	A PCR primer is missing or degraded.	A positive control should always be run to ensure components are functioning. A checklist is also recommended when assembling reactions.
	Too few cycles are performed.	Increase the number of cycles.
	The annealing temperature is too high.	Decrease the annealing temperature in 2–4 °C increments.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primer to 27–33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primer with a GC content of 45–60%.
	There is not enough template.	After increasing the number of cycles has shown no success, repeat the reaction with a 10-fold higher concentration of the template.
	The template is of poor quality.	Evaluate the template integrity by agarose gel electrophoresis. It may be necessary to repurify template using methods that minimize shearing and nicking.
	PCR product is too long.	For optimal results, qPCR products should be 100–500 bp.
	Target template is difficult.	In most cases, inherently difficult targets are due to unusually high GC content and/or secondary structure. Betaine has been reported to help amplification of high GC content templates at a concentration of 0.8–1.3 M. [Rees, W., *et al*., Biochemistry, **32**, 137-144 (1993).]
Signal is independent of template dilution (multiple products or smeared products)	The annealing temperature is too low.	Increase the annealing temperature in increments of 2–3 °C.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primers to 27–33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primers with a GC content of 45–60%.
	The template concentration is too high.	Reduce the concentration of the template in the PCR reaction.
	The primer concentration is too high.	Reduce the primer concentrations in a series of two-fold dilutions (i.e. 0.1 μM, 0.05 μM, 0.025 μM, and 0.0125 μM) and subject these trial reactions to PCR.
Large variability within samples and/or duplicates.	Reactions not well mixed.	Gently vortex and centrifuge reactions.
	Wells not tightly capped or covered.	Tightly cap or cover all wells, even the empty ones. Loose caps can compromise the seal of adjacent wells.
	Initial denaturation is too long.	Decrease initial denaturation to not exceed two minutes.
No PCR product (signal) is observed.	Wrong dye layer chosen.	Ensure the reporter being used is activated in the setup view of the Sequence Detection Software.
	Incorrect values on Y-axis	Change the values on the y-axis. By doubling clicking on ΔRn, the values of the y-axis can be changed.

Troubleshooting Guide (cont.)

Symptom	Possible Cause	Solution
Varying fluorescent intensity	Amplification curves reach a maximum and then decrease at high template amounts	Reduce the number of cycles used for baseline calculation. Baseline correction is overcompensating, and negating signal.
	Improper exposure time	Change the exposure time appropriately if using caps (25) or optical adhesive covers (10).

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