

Product Information

SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR, Capillary Formulation

Catalog Number **S1816**

Storage Temperature $-20\text{ }^{\circ}\text{C}$

TECHNICAL BULLETIN

Product Description

SYBR Green JumpStart Taq ReadyMix for Quantitative PCR combines the performance enhancements of JumpStart Taq antibody for hot start PCR with SYBR Green I dye in an easy-to-use ReadyMix solution that is specially formulated for use with the Roche LightCycler®. Since the ReadyMix includes a fluorescent dye and the reagents for PCR, this is the ideal solution for performing high-throughput quantitative PCR. This ready-to-use mixture of SYBR Green I dye, JumpStart Taq DNA polymerase, 99% pure deoxynucleotides, glass passivator, and reaction buffer is provided in a 2× concentrate for convenience. JumpStart Taq DNA polymerase uses JumpStart Taq antibody to inactivate the DNA polymerase at room temperature. When the temperature is raised above $70\text{ }^{\circ}\text{C}$ in the first denaturation step of the cycling process, the complex dissociates and the polymerase becomes fully active.

The double-strand DNA-specific SYBR Green I fluorescent reporter offers distinct advantages. SYBR Green I dye is inexpensive, easy to use and sensitive. The dye has excitation and emission maxima of 494 nm and 521 nm, respectively, which make it compatible for use on any quantitative PCR instrument. Well-designed primers must be used in SYBR Green quantitative PCR reactions, because SYBR Green I dye will detect nonspecific products, which results in an overestimation of the target concentration. It is not suitable for multiplex quantitative PCR reactions, because SYBR Green I dye cannot distinguish between the different targets.

Features and Benefits

- Designed for high throughput, quantitative PCR applications.
- Formulated for use on the Roche LightCycler, which provides rapid PCR amplification, detection, and analysis.
- SYBR Green I dye 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.
- 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 100 or 400 PCR reactions (20 μL reaction volume)

- SYBR Green JumpStart Taq ReadyMix, Catalog Number S1691, containing 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 7 mM MgCl_2 , 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, passivator, Taq DNA Polymerase, JumpStart Taq antibody, and SYBR Green I.
- Magnesium chloride solution, 25 mM, Catalog Number M8787 1.5 ml/vial.

Materials and Reagents Required but not Provided

- Water, PCR reagent, Catalog Number W1754
- Specific primers
- DNA template
- Dedicated pipettes
- Aerosol resistant pipette tips
- Glass capillary tubes and plastic PCR tubes that are recommended by the manufacturer of the quantitative PCR instrument
- Thermal cycler for quantitative PCR

Storage/Stability

Store SYBR Green JumpStart*Taq* ReadyMix for Quantitative PCR, Capillary Formulation at $-20\text{ }^{\circ}\text{C}$ for up to one year. Protect from light.

Precautions and Disclaimer

This product 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.

Preliminary Considerations

DNA Preparation

One of the most important steps in assuring success with PCR is high quality DNA. Integrity and purity of DNA template is essential. Quantitative PCR involves multiple rounds of enzymatic reactions and is therefore more sensitive to impurities. Contaminants can also interfere with fluorescence detection. The ratio between 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

Since SYBR Green I dye will detect all nonspecific quantitative PCR product formation, only well designed specific primers are recommended for this system to ensure the highest possible specificity. 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 SYBR Green quantitative PCR.

Magnesium Chloride Concentration

Lower MgCl_2 concentrations usually result in the formation of fewer nonspecific products. The ReadyMix solution is provided at a $2\times$ concentration of 7 mM magnesium chloride (final concentration 3.5 mM). A vial of 25 mM MgCl_2 is provided for further optimization of the final MgCl_2 concentration if necessary.

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.²

Data Analysis

Follow the recommendations of the real time instrument used to perform quantitative SYBR Green PCR. The following may help new instrument users. Generally the number of cycles is plotted against the fluorescence. Threshold cycles ($C_{T\text{S}}$) or crossing points are used to determine the template amount in each sample. Threshold cycle or crossing point is the first cycle that shows a detectable increase in fluorescence due to the formation of PCR products. The cycles before the crossing point are the baseline cycles. The baseline cycles show no detectable increase in fluorescence due to PCR products. The threshold used to determine when the first detectable increase in fluorescence occurs may also be adjusted manually. The threshold should always be done on a logarithmic amplification plot. In a logarithmic amplification plot the threshold should be set in the log-linear range and not the plateau phase.

Melting Curves

Performing a melting curve analysis at the end of the run will help to analyze only the PCR product of interest. Follow the real time instrument manufacturer's instructions for melting curve analysis. Successive runs with the same primers can be modified to remove the contribution of primer dimer formation to product signal by collecting data in an additional cycling step, the temperature of which must lie between the already determined dimer and product melting temperatures ($T_{m\text{S}}$).

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 SYBR Green PCR kit 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.³

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.

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 qPCR is most often used to measure genetic polymorphism differences, for instance, between tissues or between healthy and diseased samples. The advantage of this technique is that using an internal standard can minimize the variations in sample preparation and handling. When using SYBR systems, the target and internal reference quantification must be run in separate reactions.

The accuracy of relative quantification depends on the appropriate choice of a reference template for standards. Variability of the standard will influence the results and so it is most important that standards be appropriate.³ Some researchers choose not to run a standard curve and report target quantities as a fraction of the reference, a technique termed comparative quantitation. Alternatively, one may assume that the amplification efficiencies of target and reference are negligible and quantify target based solely on the standard curve determined for the reference sequence. Finally, in the most accurate of the relative quantification techniques, the amplification efficiencies of both the reference and target are measured, and a correction factor is determined. This process, termed normalization,³ requires a sample containing known concentrations of both target and reference and the generation of two standard curves.

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 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.

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. Lovatt, A., et al., Validation of Quantitative PCR Assays, *BioPharm.*, March 2002, p. 22-32.
3. Bustin, S. A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, *J. Mol. Endocrinol.* **29**, 23-9 (2002).
4. Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual*, Third Edition, (Cold Spring Harbor Laboratory Press, New York 2000). (Product Code M 8265)

Procedure

Note: Because SYBR Green I fluorescent dye binds to all double-stranded DNA, it is important to test primers and cycling conditions to insure the PCR product is a single band, otherwise the results may not be interpretable. PCR amplicons should not exceed 800 bp in length and work best when between 100-150 bp in length. PCR specificity may be checked on a non-quantitative thermal cycler by analyzing the results using agarose gel separation⁴ before performing quantitative PCR with SYBR Green I dye.

For best results, optimal concentrations of primers, MgCl₂, 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. 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.

A. Optimizing Primer Concentrations

1. Prepare and dispense diluted primers (Fig 1).
 - a. Prepare 60 μ L of 8 μ M working solutions of both forward (fwd) and reverse (rev) primers in the first tubes of 2 separate 8-tube strips.
 - b. Dispense 30 μ L of water into tubes 2-5.
 - c. Transfer 30 μ L of the 8 μ M 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 μ L 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 μ L 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

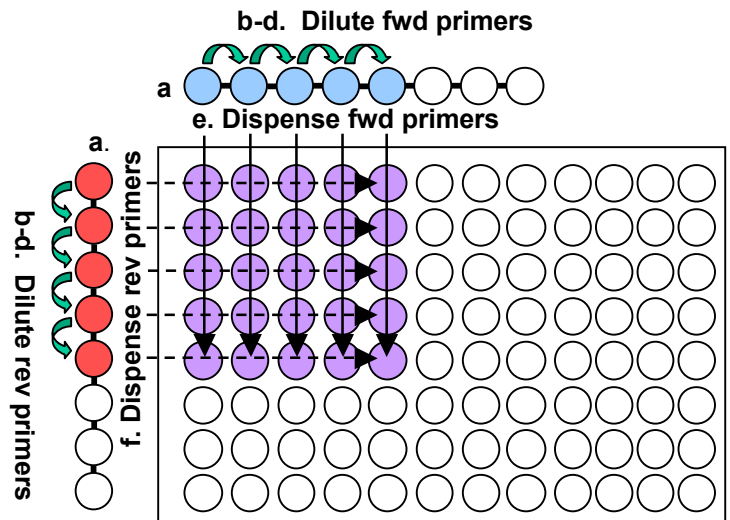


Fig 1: Follow steps 1a – 1f using diagram above

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 μ L	2x SYBR Green JumpStart <i>Taq</i> ReadyMix	1.25 units <i>Taq</i> DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM MgCl ₂ , 0.2 mM dNTP, stabilizers
q.s. to 676 μ L	Water	
676 μ L	Total Volume	

3. Aliquot 26 μ L master mix into all wells in the PCR plate that contain primers (A1-E5)
4. Mix Thoroughly and transfer 18 μ L from each of wells A1 through E5 to wells A8 through E12.
5. Add 2 μ L template DNA (10-50 ng genomic DNA or 0.1-1 ng plasmid) to one set of reactions (columns 1-5) and 2 μ L 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. If the reactions were not set up in the glass capillary transfer them to the capillary and centrifuge the capillaries with the metal adaptors provided by the instrument manufacturer according to their recommendations.
8. Perform Thermal cycling:

Optimum amplification parameters will vary depending on the primers. It may be necessary to optimize the system for individual primers and template to achieve maximum product yield and/or quality. A typical 3 step PCR cycling protocol is recommended when using SYBR Green I dye as the detection method.

The following cycling parameters are recommended for use with the Roche LightCycler. Other instruments may require optimization of amplification parameters.

Step	Temperature	Time	Temperature Transition Rate
Denaturation	94 °C	30 sec	20 °C/sec
For Cycles 1 - 40+:			
Denaturation	94 °C	0 sec	20 °C/sec
Annealing	5 °C below T_m of primers	5-10 sec	20 °C/sec
Extension	72 °C	1 sec per 25 bp of amplified product	Between 2-20 °C/sec (See Note 1 below)
Detection (Optional)	T_m of primer-dimer or misprimed product < T_m of product	5 sec	20 °C/sec

Note 1: For primers with a T_m below 55 °C, a lower ramp time is recommended.

9. Follow the qPCR instrument manufacturer's instructions for SYBR Green I dye analysis.

Data acquisition is performed during the extension step or at an optional detection step. If data acquisition is performed at the optional detection step, the temperature for detection may be derived using the melt curve analysis software.

Evaluate fluorescence plots (ΔR_n) 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. Add the following reagents to a thin walled plastic PCR tube for transfer to a glass capillary or directly to a glass capillary for thermal cycling. The following procedure serves as a reference.

Volume	Reagent	Final Concentration
10 μ L	2x SYBR Green JumpStart <i>Taq</i> ReadyMix	1.25 units <i>Taq</i> DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM MgCl ₂ , 0.2 mM dNTP, stabilizers
--- μ L	25 mM MgCl ₂	3.5 mM from ReadyMix (without addition; optimize as necessary)
--- μ L	Forward Primer	Optimal Conc. from Sec. A
--- μ L	Reverse Primer	Optimal Conc. from Sec. A
--- μ L	Template DNA	10 ng-100 ng
q.s. to 20 μ L	Water	
20 μ L	Total Volume	

The following cycling parameters are recommended for use with the Roche LightCycler. Other instruments may require optimization of amplification parameters.

Step	Temperature	Time	Temperature Transition Rate
Denaturation	94 °C	30 sec	20 °C/sec
For Cycles 1 - 40+:			
Denaturation	94 °C	0 sec	20 °C/sec
Annealing	5 °C below T_m of primers	5-10 sec	20 °C/sec
Extension	72 °C	1 sec per 25 bp of amplified product	Between 2-20 °C/sec (See Note 1 below)
Detection (Optional)	T_m of primer-dimer or misprimed product < T_m of product	5 sec	20 °C/sec

Note 1: For primers with a T_m below 55 °C, a lower ramp time is recommended.

5. Follow the qPCR instrument manufacturer's instructions for SYBR Green I dye analysis.

Note: A template-primer master mix is recommended when performing multiple PCR reactions.

2. Gently mix the reactions and centrifuge the reactions briefly.
3. If the reactions were not set up in the glass capillary transfer them to the capillary and centrifuge the capillaries with the metal adaptors provided by the instrument manufacturer according to their recommendations.
4. Optimum amplification parameters will vary depending on the primers. It may be necessary to optimize the system for individual primers and template to achieve maximum product yield and/or quality. A typical 3 step PCR cycling protocol is recommended when using SYBR Green I dye as the detection method.

Data acquisition is performed during the extension step or at an optional detection step. If data acquisition is performed at the optional detection step, the temperature for detection may be derived using the melt curve analysis software.

Troubleshooting Guide

Symptom	Possible Cause	Solution
No PCR product (signal) is observed	A PCR component is missing or degraded.	A positive control should always be run to insure components are functioning. Check concentrations and storage conditions of reagents, including primers and template RNA. A checklist is also recommended when assembling reactions.
	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 1-4 °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. If the primers are less than 27 nucleotides long, try to lengthen the primer to 27-33 nucleotides.
	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.	The best results are obtained when PCR products are between 100-150 bp and do not exceed 800 bp.
	The denaturation time is too long or too short.	The Roche LightCycler recommends a 0-second denaturation time for normal templates and should be increased in 5 second or less increments.
	Mg ²⁺ concentration is suboptimal.	Start with the magnesium concentration provided in SYBR Green JumpStart Taq ReadyMix (3.5 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 or activated at the wrong step.	Confirm that the acquisition mode is turned on for proper detection. The acquisition mode for SYBR Green detection is "single" and is collected at the extension step or optional detection step.
Primers are degraded.	Check for primer degradation on a polyacrylamide gel.	
Multiple PCR products	Primers are not optimally designed.	Confirm the accuracy of the sequence information and the specificity of primer sequence to non-target sequence. Increase the length of the primers to make them more target specific.
	Primers are degraded.	Check for primer degradation on a polyacrylamide gel.
	Mg ²⁺ concentration is not optimal.	Start with the magnesium concentration provided in SYBR Green JumpStart Taq ReadyMix (3.5 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 2-3 °C.
	Contaminating DNA	Check all reagents for possible contamination and set up reactions in a laminar flow hood to prevent contamination from other reactions.

Troubleshooting Guide (continued)

Symptom	Possible Cause	Solution
Multiple PCR products (continued)	Primer-dimers were amplified	Include the optional detection step in the cycling program to avoid detection of primer-dimers.
	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 μ M, 0.05 μ M, 0.025 μ M and 0.0125 μ M) and subject these trial reactions to PCR.
Linearity of crossing point values do not correspond to log of the template amount	Template amount too high.	Do not exceed the maximum recommended amounts of template DNA.
	Template amount too low.	Increase the amount of DNA.
	Contaminating DNA	Check all reagents for contamination and set up reactions in a laminar flow hood to prevent cross contamination.
	Primer-dimers were amplified	Include the optional detection step in the cycling program to avoid detection of primer-dimers.
Fluorescence is not detected or is variable	SYBR Green ReadyMix was not well mixed.	Mix ReadyMix thoroughly before using to insure that the SYBR Green will be added in the proper concentration to all capillaries.
	Quantitative PCR instrument is contaminated.	Decontaminate the instrument per the manufacture's instructions.

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