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Product Information

JumpStart™ *Taq* ReadyMix™ For High Throughput Quantitative PCR

Catalog Number **D6442**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

JumpStart *Taq* ReadyMix For High Throughput Quantitative PCR combines the performance enhancements of JumpStart *Taq* Antibody for hot start[1,2] PCR with the convenience of an easy-to-use reaction mixture that incorporates ROX dye for ABI and other real time instrument applications. This is the ideal solution for performing high-throughput, quantitative PCR methods using fluorescent probe detection. The ReadyMix contains JumpStart *Taq* DNA polymerase, 99% pure deoxynucleotides and reaction buffer. It is provided in a 2× concentrate. Simply add an equal volume of the 2× ReadyMix to a 2× mixture of DNA template, primers and fluorescent probe.

JumpStart *Taq* antibody inactivates the *Taq* 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 included 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. A separate vial of 25 mM Magnesium Chloride is also provided for concentration optimization in the PCR reaction.

Features and Benefits

- JumpStart *Taq* ReadyMix For High Throughput Quantitative PCR is the ideal ReadyMix for high throughput, quantitative PCR applications.
- For a typical qPCR reaction, mix 25 µL of JumpStart *Taq* ReadyMix, a fluorescent probe, the desired amount of magnesium chloride (above 1.5 mM final concentration), template DNA, and primers in a final volume of 50 µL. Reaction volumes can be scaled down, if desired.
- JumpStart *Taq* antibody prevents non-specific product formation, and allows assembled PCR reactions to be placed at room temperature for up to 2 hours without compromising the performance.
- Internal Reference Dye is provided for reaction normalization.
- When performing large numbers of PCR reactions, 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.
- JumpStart *Taq* ReadyMix for Quantitative PCR can be stored at 2-8 °C for up to six months without compromising PCR. As such, there is no need to wait for the mixture to thaw when performing daily or weekly reactions.

Reagents

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

- JumpStart *Taq* ReadyMix, Catalog Number T4950, 20 mM Tris-HCl, pH 8.3, 25 °C, 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM of each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 2× internal reference dye, 0.1 unit/µL *Taq* DNA Polymerase, JumpStart *Taq* antibody
- Magnesium chloride solution, 25 mM, PCR Reagent, Catalog Number M8787,.Each vial contains 1.5 ml or 5.0 mL.

Reagents and equipment required, not provided

- Water, PCR Reagent, Catalog Number W1754
- Primers
- Fluorescent probe for detection of amplicon
- 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

JumpStart *Taq* ReadyMix 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

JumpStart Taq ReadyMix for Quantitative PCR can be stored at 2-8 C for up to 6 months or at –20 °C for up to a year and a half. Store the 25 mM $MgCl_2$ solution at 2-8 °C.

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 and Probe Design

Specific primers and probes 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.

Magnesium Chloride Concentration

Lower $MgCl_2$ concentrations usually result in the formation of fewer nonspecific products. The ReadyMix solution is provided at a 2× concentration of 3 mM magnesium chloride (final concentration 1.5 mM). A vial of 25 mM $MgCl_2$ is provided for further optimization of the final $MgCl_2$ 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. Check the fluorescent properties of your probe to ensure it is compatible with the 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 Probe Based PCR. 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.

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 quantitative 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.[4]

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.

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.[4] 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 is 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, [4] 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 $\pm$ 0.1 the amplification efficiencies are judged to be similar.

References

1. Dieffenbach, C., and Dveksler, G., (Eds). *PCR Primer: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995). (Product No. Z36,411-8).
2. Kellogg, D. E., et al., TaqStart Antibody: "hot start" PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. *BioTechniques* **16**,1134-1137 (1994).
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

For best results, optimal concentrations of primers, probes, $MgCl_2$, KCL and PCR enhancers need to be determined. Testing various combinations of primer concentrations (50-1000 nM) while keeping the probe concentration constant (250 nM) is most efficient for primer optimization. The same method could be used to optimize probe concentrations by varying probe concentrations (50-250 nM) and keeping optimal primer concentrations constant. If maximum sensitivity is not required and your PCR target is abundant, satisfactory results for probe-based qPCR are often obtained with final concentrations of both primers at 500 nM and probe at 250 nM.

The following procedure serves as a guideline to establish optimal primer and probe 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 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.

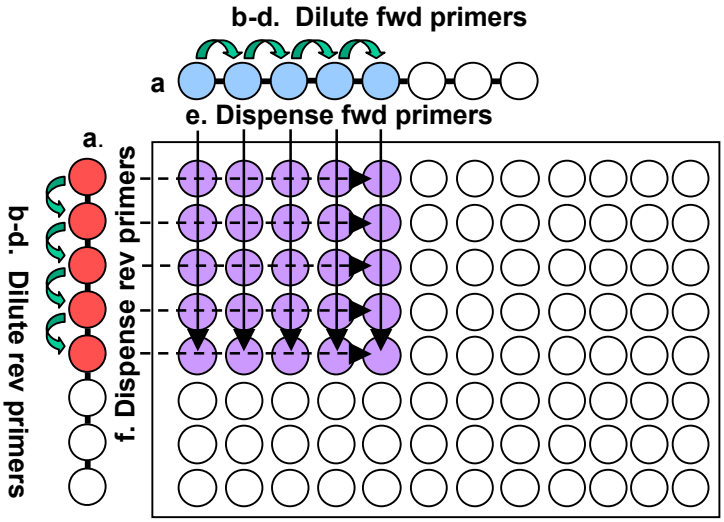

Fig 1: Follow step 1a – 1f using diagram above

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	2× JumpStart *Taq* ReadyMix	2.5 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, reference dye, stabilizers
--- µL	25 mM $MgCl_2$ (optional)	See Note below
--- µL	Fluorescent Probe	250 nM
q.s. to 676 µL	Water	
676 µL	Total Volume	

Note: Many probes are designed to work optimally at higher magnesium concentrations than the 1.5 mM $MgCl_2$ provided in this basic mix, thus adding magnesium is recommended. See troubleshooting guide for general supplementing guidelines for several types of probes.

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/probe 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 100–600 bp fragments:

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

Note: Dual labeled probes are usually designed for two step 94°C/60 °C cycling conditions. Other probes are designed for a three-step regimen, with an annealing temperature of 55 °C (probe Tm ~ 60 °C). Use the cycling conditions for which your probes were designed.

Initial denaturation	94 °C	2 min.*****
40 cycles:		
Denaturation	94 °C	15 sec
Annealing/Extension	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	

***** Initial denaturation of greater than two minutes is not recommended, and is unnecessary (see Troubleshooting Guide, Initial Denaturation).
****** Detection is usually accomplished at this step.

8. Evaluate fluorescence plots (ΔRn) 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. Optimizing Probe Concentrations

For Dual labeled probe assays, 250 nM may be used in all assays. However if maximum sensitivity is not required, lower levels of probe may suffice, thereby reducing assay cost. To optimize probe concentration, test the probe at 50-250 nM final concentration in PCR with the optimized levels of primer from Part A. The lowest level of probe that allows acceptable detection ($C_t \leq 30$) may be used.

C. 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× JumpStart *Taq* ReadyMix	2.5 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 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 -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

Optimal cycling parameters vary with probe design and thermal cycler. Check 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 100–600 bp fragments:

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

Note: Dual labeled probes are usually designed for two step 94 °C/60 °C cycling conditions. Other probes are designed for a three-step regimen, with an annealing temperature of 55 °C (probe Tm ~ 60 C). Use the cycling conditions for which your probes were designed.

Initial denaturation	94 °C	2 min.*
40 cycles:		
Denaturation	94 °C	15 sec
Annealing/Extension	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	

* Initial denaturation of greater than two minutes is not recommended, and is unnecessary (see Troubleshooting Guide, Initial Denaturation).
****** Detection is usually accomplished at this step.

Troubleshooting Guide

Problem	Possible Cause	Solution
No PCR product (signal) is observed.	A PCR primer is missing or degraded.	A positive control should always be run to insure 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 optimally designed.	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.
	Wrong detection step	Check data collection steps and ensure the fluorescence data is collected during the extension step of the PCR program.
	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 (Gel analysis reveals 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 (0.1 μM, 0.05 μM, 0.025 μM, and 0.0125 μM) and subject these trial reactions to PCR.

Troubleshooting Guide Specific to ABI Sequence Detection Systems

Problem	Possible Cause	Solution
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, as loose caps affect adjacent wells.
	Initial denaturation is too long.	Decrease initial denaturation to not exceed two minutes. Long denaturation creates abasic sites in template, hydrolyzes dNTPs and can denature the polymerase, all affecting yield.
No PCR product (signal) is observed.	Wrong dye layer chosen, see real time instrument manual.	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.
Varying fluorescent intensity	Amplification curves increase, then decrease at high template amounts	Reduce the number of cycles used for baseline calculation, as correction is overcompensating and suppressing signal.
	Improper exposure time	Change the exposure time appropriately if using caps (25) or optical adhesive covers (10).
	Bad choice of detection fluor	The probe has an emission that overlaps the internal standard. Apparent amplicon concentration will decrease precipitously as the real time instrument normalizes output signal to an increasing normalization signal.
	Wrong quencher activated.	Ensure that the proper quencher is activated in the setup view.

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