

## Product Information

### JumpStart™ Taq ReadyMix™ For Quantitative PCR

Catalog Number **D7440**  
Storage Temperature  $-20\text{ }^{\circ}\text{C}$

## TECHNICAL BULLETIN

### Product Description

JumpStart Taq ReadyMix for Quantitative PCR combines the performance enhancements of our JumpStart Taq Antibody for hot start<sup>1,2</sup> PCR with the convenience of an easy-to-use reaction mixture. Since it has no added dyes, this is the ideal solution for performing high-throughput, quantitative PCR methods that rely on a fluorescent probe. This ready-to-use mixture of JumpStart Taq DNA polymerase, 99% pure deoxynucleotides and reaction buffer is provided in a 2× concentrate for ease of use. Simply add 25 μL of the 2× mix to the DNA template, primers, fluorescent probe, and water. At room temperature, the JumpStart Taq antibody inactivates the Taq DNA polymerase. 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.

- 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.
- For a typical qPCR reaction, mix 25 μL of JumpStart Taq ReadyMix, 0.5 μL reference dye, a fluorescent probe, and the desired amount of magnesium chloride, template DNA, primers, and water in a final volume of 50 μL. Reaction volumes can be scaled down, if desired. A 25 mM MgCl<sub>2</sub> solution is provided for concentration optimization.
- The hot start mechanism using JumpStart Taq antibody, which prevents non-specific product formation, allows assembled PCR reactions to be placed at room temperature for up to 2 hours without compromising the performance.
- Sigma's Reference Dye for Quantitative PCR is included to normalize reaction data for real-time detection. Maximum excitation of this dye is 586 nm and maximum emission is 605 nm. Instrument settings for ABI ROX reference dye are satisfactory for the measurement of Reference Dye for Quantitative PCR.

### Reagents Provided

- JumpStart Taq ReadyMix, Catalog Number P2893  
20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM MgCl<sub>2</sub>, 0.002% gelatin, 0.4 mM of each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.06 unit/μL Taq DNA Polymerase, JumpStart Taq antibody. Provided as 100 reactions and 400 reactions (50 μL reaction volume).
- Reference Dye for Quantitative PCR, Catalog Number R4526, 100x. A vial containing 0.3 ml is provided.
- Magnesium chloride solution, 25 mM, PCR Reagent, Catalog Number M8787. A vial containing 1.5 ml is provided.

### Reagents and equipment required but not provided

- 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
- Thermal cycler for quantitative PCR

### 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.

### Storage/Stability

JumpStart Taq ReadyMix for Quantitative PCR can be stored at 2-8 °C for up to 6 months so there is no waiting for the reaction components to thaw. It can also be stored at  $-20\text{ }^{\circ}\text{C}$  for up to 18 months. Store the Reference dye for quantitative PCR and magnesium chloride 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 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. This internal reference dye is necessary for ABI Sequence Detection Systems. 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.<sup>3</sup>

### 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.<sup>4</sup>

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.<sup>4</sup> 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,<sup>4</sup> 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 Catalog Number Z364118.
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 real-time reverse transcription PCR (RT-PCR): trends and problems, *J. Mol. Endocrinol.* **29**, 23-9 (2002).
5. Rees, W., et al., *Biochemistry*, **32**, 137-144 (1993).

## Procedure

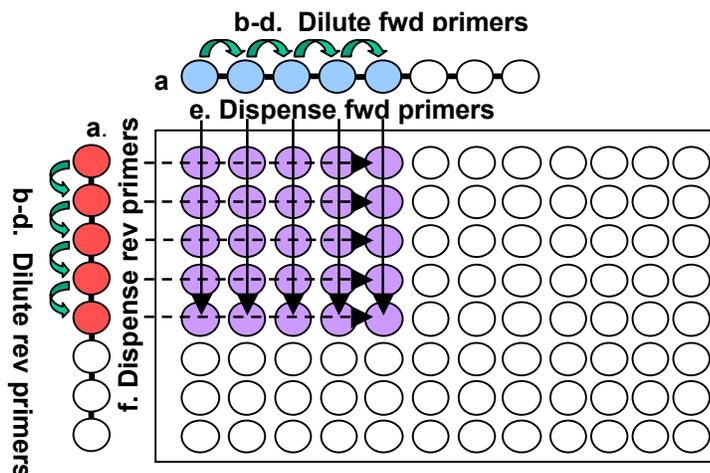
For best results, optimal concentrations of primers, probes, MgCl<sub>2</sub>, KCl and PCR enhancers need to be determined. Testing various combinations of primer concentrations (50-1000 nM) while keeping the probe concentration constant (250 nM) is most efficient for primer optimization. The same method 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](http://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  $\mu$ L of 8  $\mu$ M working solutions of both forward (fwd) and reverse (rev) primers in the first tubes of 2 separate 8-tube strips.
  - b. Dispense 30  $\mu$ L of water into tubes 2-5.
  - c. Transfer 30  $\mu$ L of the 8  $\mu$ 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  $\mu$ 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  $\mu$ 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 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 $\mu$ L	2x JumpStart <i>Taq</i> ReadyMix	1.5 units <i>Taq</i> DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl <sub>2</sub> , 0.001% gelatin, 0.2 mM dNTP, stabilizers
(7 $\mu$ L)	Reference Dye* (optional)	1 $\times$
--- $\mu$ L	25 mM MgCl <sub>2</sub> (optional)	See Note below
--- $\mu$ L	Fluorescent Probe	250 nM
q.s. to 676 $\mu$ L	Water	
676 $\mu$ L	Total Volume	

\* Use 0.1 $\times$  for ABI 7500 and Stratagene instruments; replace with FITC for BioRad iCycler.

**Note:** Many probes are designed to work optimally at higher magnesium concentrations than the 1.5 mM MgCl<sub>2</sub> 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  $\mu\text{L}$  master mix into all wells in the PCR plate that contain primers (A1-E5)
4. Mix Thoroughly and transfer 18  $\mu\text{L}$  from each of wells A1 through E5 to wells A8 through E12.
5. Add 2  $\mu\text{L}$  template DNA (10-50 ng genomic DNA or 0.1-1 ng plasmid) to one set of reactions (columns 1-5) and 2  $\mu\text{L}$  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. 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 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.

**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  $T_m$  ~ 60 °C). Use the cycling conditions for which your probes were designed.

<b>Initial denaturation</b>	94 °C	2 min.*
<b>40 cycles:</b>		
Denaturation	94 °C	15 sec
Annealing/Extension	60 °C or 5 °C below lowest primer $T_M$	1 min **
<b>(Optional) Hold</b>	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 ( $\Delta R_n$ ) 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. 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 $\mu$ L	2x JumpStart <i>Taq</i> ReadyMix	1.5 units <i>Taq</i> DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl <sub>2</sub> , 0.001% gelatin, 0.2 mM dNTP, stabilizers
(0.5 $\mu$ L)	Reference Dye** (optional)	1x
--- $\mu$ L	Forward Primer	Optimal Conc. from Sec. A
--- $\mu$ L	Reverse Primer	Optimal Conc. from Sec. A
--- $\mu$ L	Template DNA	10 ng-100 ng
q.s. to 50 $\mu$ L	Water	
50 $\mu$ L	Total Volume	

\* Volume for 50  $\mu$ L reaction, however component volumes may be scaled to give the desired reaction volumes.

\*\* Use 0.1x for ABI 7500 and Stratagene instruments; replace with FITC for BioRad iCycler.

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 bp – 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  $T_m \sim 60$  °C). Use the cycling conditions for which your probes were designed.

<b>Initial denaturation</b>	94 °C	2 min.*
<b>40 cycles:</b>		
Denaturation	94 °C	15 sec
Annealing/Extension	60 °C or 5 °C below lowest primer $T_M$	1 min **
<b>(Optional) Hold</b>	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.
	Too little magnesium in the reaction.	Supplement up from the 1.5mM base level TaqMan and Epoch primers require 5-6 mM magnesium Scorpion primers bind best at 2-3.5 mM magnesium Beacons give optimal results at 3.5 – 5.5 mM magnesium.
	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. <sup>5</sup>
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 (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.
	Initial denaturation is too long.	Decrease initial denaturation to not exceed two minutes long.

## Troubleshooting Guide Specific to ABI Sequence Detection Systems

Problem	Possible Cause	Solution
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 $\Delta R_n$ , the values of the y-axis can be changed.
	Too little magnesium	TaqMan probes require 5-6 mM total magnesium for optimal performance.
Varying fluorescent intensity	Wavy amplification curves at high template amounts	Reduce the number of cycles used for baseline calculation.
	Improper exposure time	Change the exposure time appropriately if using caps (25) or optical adhesive covers (10).
	Wrong quencher activated.	Ensure that the proper quencher is activated in the setup view.
	Reference dye was not included	Dye is absolutely required for ABI machines. Add recommended value (1% volume) for laser excitation systems, 0.3% volume for tungsten bulb instruments

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