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ProductInformation

Quantitative Reverse Transcriptase PCR (qRT-PCR) using Enhanced Avian Reverse Transcriptase (Product Code A 4464) and SYBR Green Jumpstart™ *Taq* ReadyMix without Magnesium Chloride (Product Code S 5193)

TECHNICAL BULLETIN

Product Description

Quantitative RT-PCR (qRT-PCR) allows direct measurement of gene expression. To obtain accurate data, it is often necessary to optimize PCR conditions. One suggestion is to optimize $MgCl_2$ concentration. To ease $MgCl_2$ optimization, it is essential to use reagents that package $MgCl_2$ separately. SYBR Green Jumpstart *Taq* ReadyMix without $MgCl_2$ (Product Code S 5193) and Enhanced Avian Reverse Transcriptase (eAMV RT, Product No A 4464) have been successfully used in qRT-PCR applications.

SYBR Green Jumpstart *Taq* ReadyMix without $MgCl_2$ combines the performance enhancements of JumpStart *Taq* with SYBR Green I dye in a convenient 2X concentrate ReadyMix solution that also contains 99% pure deoxynucleotides, glass passivator, and reaction buffer. When used in conjunction with eAMV RT, both have been determined suitable for qRT-PCR. eAMV RT is an exceptionally robust enzyme with an enhanced ability to transcribe through difficult secondary structures at elevated temperatures (65 °C). Additionally, no special preparation or protocol changes are required to activate the hot start mediated JumpStart Taq enzyme. During the first denaturation step of the cycling process, the Taq directed antibody dissociates from the Taq polymerase rendering it fully active.

Using SYBR Green JumpStart Taq ReadyMix and eAMV RT provides the following benefits:

- JumpStart Taq decreases unwanted amplification products while enhancing target yield
- Highly purified, exceptionally robust eAMV-RT provides superior performance. With an enhanced ability to transcribe through difficult secondary structure at elevated temperatures (up to 65 °C), eAMV-RT is the ideal enzyme for producing high quality full-length cDNA from total RNA or poly(A)$^+$ RNA with difficult secondary structure.
- SYBR Green I dye is ideal for quantifying any DNA sequence. The dye binds to double-stranded DNA and detection is monitored by measuring the increase in fluorescence throughout cycling.
- Internal Reference Dye is provided for reaction normalization. Maximum excitation of this dye is 586 nm and maximum emission is 605 nm.
- Magnesium Chloride provided in a separate vial for ease of optimization.

Quantitative RT-PCR using SYBR Green Jumpstart™ *Taq* ReadyMix without $MgCl_2$ (Product Code S 5193) and Enhanced Avian Reverse Transcriptase (Product Code A 4464) includes the following:

Reagents

SYBR Green Jumpstart™ *Taq* ReadyMix without $MgCl_2$ (Product Code S 5193) includes:

- SYBR Green JumpStart *Taq* ReadyMix, Product Code S 3443
  20 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), glass passivator, stabilizers, 0.05 unit/µl *Taq* DNA Polymerase, JumpStart *Taq* antibody, and SYBR Green I dye.
- Internal Reference Dye, Product Code R 4526, 100X dye, provided in a 0.3 mL package size.
- Magnesium Chloride, 25 mM, Product Code M 8787
  1 vial of 1.5 ml

Enhanced Avian Reverse Transcriptase (Product Code A 4464) includes:

- Enhanced Avian Reverse Transcriptase, Product Code A 4714,
  500 units/1,000 units, 200 mM KH_2PO_4 , pH 7.2,
  2 mM DTT, 0.2% Triton, 50% glycerol, 20 units/µl

- 10x Buffer for AMV Reverse Transcriptase, Product Code B 1175
  500 mM Tris-HCl, pH 8.3, 400 mM KCl,
  80 mM $MgCl_2$, 10 mM DTT provided in 1.5 mL vial.

Additional Reagents and Equipment required but not provided

- RNA to be reverse transcribed and amplified
- Specific primers
- RNase-free pipettes
- Aerosol resistant pipette tips (eliminate cross-over contamination)
- Plastic PCR tubes, plastic PCR plates or glass capillary tubes that are recommended by the thermal cycler manufacturer
- Thermal cycler
- PCR grade water, Product Code W 1754.

Precautions and Disclaimer

SYBR Green ReadyMix without $MgCl_2$ (S 5193) and Enhanced Avian Reverse Transcriptase (A 4464) are for R&D use only. Consult the MSDS for information regarding hazards and safe handling practices

Storage/Stability

All components should be stored at –20 °C and are stable for at least one year. The internal reference dye (R 4526) should be protected from light.

Preliminary Considerations

RNA Preparation

The single most important step in assuring success with RT-PCR is preparation of high quality RNA. Integrity and purity of RNA template is essential. SYBR Green RT-PCR involves multiple cycles 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 between absorbance values at 260 nm and 280 nm gives an estimate of RNA purity. Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1. Lower ratios indicate the presence of contaminants such as proteins. Either total or poly(A)$^+$ RNA can be used as template for the reverse transcription reaction. To ensure all RNA preparations are DNA-free, it is recommended to treat the RNA preparation with DNase I (Product Code AMP-D1) prior to SYBR Green quantitative RT-PCR[1]. Purified RNA should be stored at –20 °C or –70 °C.

Primer Design

Since SYBR Green I dye will detect all nonspecific quantitative RT-PCR product, only well designed specific primers are recommended for this system to ensure the highest possible specificity. To eliminate the complications introduced with primer-dimers and secondary structures, specific primers for RT and PCR should be designed with the aid of primer design software. Lower primer concentrations decrease the accumulation of primer-dimer formation and nonspecific product formation, which is critical in SYBR Green quantitative RT-PCR. If genomic DNA contamination is possible, primers spanning an intron will reduce the possibility of amplifying DNA and overestimating RNA message.

Controls

A positive control is recommended to ensure all of the kit components are working properly. Two negative controls, no template and no reverse transcriptase, are necessary to determine if contamination is present. A signal in the “no template control” demonstrates the presence of DNA contamination or primer dimer formation. A signal in the “no reverse transcriptase” reaction demonstrates the presence of significant DNA that can be amplified. See Lovatt et al. for a discussion of qRT-PCR controls.[2]

Procedure

Note: An initial 1:10 dilution of eAMV (Product Code A 4464) is recommended in 1x PCR buffer (e.g. add 2 µl of 10x PCR buffer, 2 µl of eAMV and 16 µl of PCR grade water to a microcentrifuge tube to prepare a total volume of 20 µl). Add 1 µl of the 1:10 eAMV dilution per SYBR Green RT-PCR reaction. If using less than 50 ng of total RNA per reaction, the eAMV may need to be further diluted up to 60 fold. Diluted eAMV is more sensitive to less abundant RNA in one-step SYBR Green RT-PCR while maintaining the ability to reverse transcribe more abundant RNA template.

For Real-Time Instruments using Tubes or Plates (50 µl reaction volume)

Volume	Reagent	Final Concentration
25 µl	SYBR Green JumpStart Taq Ready Mix no $MgCl_2$	1×
--- µl	25 mM $MgCl_2$	3.0 mM is recommended; optimize as required
x µl	RNA template	Up to 10 ng/µl total RNA
1 µl	Specific primers	50-400 nM each primer
1 µl	Dilute eAMV RT	See Note above for dilution protocol
--- µl	Water, PCR reagent	Add water until a total reaction volume of 50 µl is obtained
50 µl	Total Volume	

The following cycling parameters are recommended for use with the ABI 7700. Other instruments may require optimization of amplification parameters.

Step	Temperature	Time
First Strand Synthesis	Between 42-65 °C (See Note below)	30 min
Denaturation/ RT Inactivation	94 °C	2 min
For Cycles 1 - 40+:		
Denaturation	94 °C	15 sec
Annealing	5 °C below T_m of primers	30 sec
Extension	72 °C	1 min
Detection (Optional)	Approx. 3 °C below T_m of RT-PCR amplicon	5-15 sec

Note: Reverse transcription at 48 °C is initially recommended, but an optimal first strand synthesis temperature may vary from 42–65 °C and will need to be experimentally determined.

For Real-Time Instruments using Capillary Tubes (20 µl reaction volume)

Volume	Reagent	Final Concentration
10 µl	SYBR Green Jumpstart Taq Ready Mix no $MgCl_2$	1×
--- µl	25 mM $MgCl_2$	3.0 mM is recommended; optimize as required
X µl	RNA template	Up to 10 ng/µl total RNA
1 µl	Specific primers	0.3-1 µM each primer
1 µl	Diluted eAMV-RT	See Note
---	Water, PCR reagent	Add water until a total reaction volume of 20 µl is obtained
20 µl	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
First Strand Synthesis	48 °C (See Note 1 below)	30 min	20 °C/sec
Denaturation/ RT Inactivation	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 2 below)
Detection (Optional)	Approx. 3 °C below T_m of RT-PCR amplicon	5 sec	20 °C/sec

Note 1: Reverse transcription at 48 °C is initially recommended, but an optimal first strand synthesis temperature between 42–65 °C may need to be experimentally determined.

Note 2: For primers with a T_m below 55 °C, a lower ramp time is recommended.

Data acquisition is performed during the extension step or at a detection step after the extension step. If data acquisition is performed at a detection step after the extension step, the temperature of this detection step may be derived using melting curve analysis software.[3]

References

1. Bustin, S. A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol. **29**, 23-29 (2002).
2. Lovatt, A., et al. Validation of Quantitative PCR Assays –Addressing Virus Contamination Concerns*, BioPharm,* March 2002, p. 22-32.
3. Morrison, T. B., et al., Quantification of Low-Copy Transcripts by Continuous SYBR Green I Monitoring during Amplification. BioTechniques **24,** 954-962 (1998).

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Troubleshooting Guide for Quantitative RT-PCR using SYBR Green Jumpstart *Taq* ReadyMix without $MgCl_2$ (Product Code S 5193) and Enhanced Avian Reverse Transcriptase (Product Code A 4464)

Problem	Possible Cause	Solution
No RT-PCR product (signal) is observed, RT-PCR product is detected late in PCR or only primer-dimers are detected.	The RNA is degraded.	Check the RNA by denaturing agarose gel electrophoresis. Poly(A)$^+$ RNA should appear as a smear between 0.5 kb and 2 kb. The total RNA should have two sharp ribosomal RNA bands without notable degradation. For purifying RNA, use TRI Reagent or GenElute RNA isolation kits
	There is not enough RNA template.	After increasing the number of cycles has shown no success, repeat the reaction with a higher concentration of template.
	There is incomplete removal of guanidinium during RNA isolation.	For any procedure using guanidinium-based lysis solution, remove as much of the residual liquid as possible after the first precipitation and then wash once with 70% alcohol.
	There is incomplete removal of the protease (such as Proteinase K) during RNA isolation.	Proteases used during RNA isolation may be removed by phenol/chloroform extraction and alcohol precipitation.
	Reverse transcription reaction temperature is incorrect.	An optimal reverse transcription reaction temperature between 42-65 °C needs to be experimentally determined.
	Concentration of RT is too high or too low.	Add the recommended amount of eAMV reverse transcriptase to the SYBR Green RT-PCR reaction. eAMV may need to be further diluted for reactions below 50 ng of total RNA. Diluted eAMV still retains the ability to reverse transcribe higher amounts of template.
	The ratio of SYBR Green RT-PCR master mix to eAMV RT is not correct.	If the reaction volume is reduced or increased then the ratio of SYBR Green master mix to eAMV has to be adjusted accordingly.
Multiple RT-PCR products	Reactions set up at room temperature.	Set up RT-PCR reactions on ice to avoid premature cDNA synthesis from nonspecific primer annealing.
No linearity in ratio of C_T value to log of the template amount.	Genomic DNA is contaminating the RNA template in the reverse transcriptase reaction.	Digest the RNA with RNase-free DNase (Product Code AMP-D1).
	Template amount is too high.	Do not exceed the maximum recommended amounts of template RNA.
	Template amount is too low.	Increase amount of template RNA.
	Concentration of eAMV is too high or too low.	Add the recommended amount of eAMV to the SYBR Green RT-PCR reaction. eAMV may need to be further diluted for templates below 50 ng of total RNA. Diluted eAMV still retains the ability to reverse transcribe higher amounts of template.
	Primer-dimers were co-amplified.	Include an additional detection step in the cycling program to avoid detection of primer-dimers.

Additionally, if genomic DNA has contaminated the RNA template in the reverse transcriptase reaction, digest the RNA with RNase-free DNase (Product Code AMP-D1). It is also recommended to use primers that span an intron so amplification from genomic DNA is minimized.

If the reverse transcription reaction temperature is too low, start the reaction at a temperature between 42-50 °C. The reverse transcription reaction temperature may be increased if mispriming is detected.

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