KAPABIOSYSTEMS

Technical Data Sheet

KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix (2X) Kit

KR1282_S – v3.20

Product Description

KAPA PROBE FAST One-Step qRT-PCR Master Mix (2X) Kit is a sensitive and convenient solution for real-time PCR using RNA as template. The kit is designed for high-throughput, fast-cycling, one-step RNA quantification using sequence-specific fluorogenic probes. It is compatible with all fluorogenic probe-based technologies, including hybridization probes (e.g., FRET), hydrolysis probes (e.g., TaqMan®) and displacement probes (e.g., molecular beacons). The kit comprises KAPA PROBE FAST qPCR Master Mix (2X), ROX High (50X), ROX Low (50X), KAPA RT Mix (50X), and dUTP (10 mM).

KAPA PROBE FAST One-Step qRT-PCR Master Mix (2X) Universal is a ready-to-use cocktail containing all components except primers, probe(s) and template for fast-cycling, probe-based real-time PCR. The 2X Master Mix contains KAPA Taq HotStart DNA Polymerase, KAPA PROBE FAST qPCR Buffer, dNTPs, $MgCl_2$ and stabilizers. The KAPA RT Mix (50X) comprises wild-type M-MuLV Reverse Transcriptase and RNase Inhibitor, and is optimized for rapid one-step qRT-PCR. ROX reference dye is not included in the 2X Master Mix, but is supplied separately.

KAPA Taq HotStart DNA Polymerase is an antibody-mediated hotstart formulation of KAPA Taq DNA Polymerase. In the HotStart formulation, the enzyme is combined with a proprietary antibody that inactivates the enzyme until the first denaturation step. This eliminates spurious amplification products resulting from non-specific priming events during reaction setup and initiation, and increases overall reaction efficiency.

The use of dUTP at the recommended final concentration results in amplicons that can be degraded using Uracil-DNA Glycosylase (UDG). UDG treatment is performed in subsequent reactions in order to minimize carryover PCR contamination downstream. Use of dUTP in this system is optional, and UDG is not supplied in the kit.

Product Applications

KAPA PROBE FAST qPCR Kits are ideally suited for:

- gene expression analysis;
- SNP/mutation analysis; and
- gene knockdown validation.

Kit Codes and Components	
KAPA PROBE FAST One-Step qRT-PCR Master Mix (2X) Universal	
qPCR Master Mix (2X) KAPA RT Mix (50X) dUTP (10 mM) ROX High Reference Dye (50X) ROX Low Reference Dye (50X)	**KK4752** (500 x 20 µL reactions)

Quick Notes

- The kit is suitable for all fluorogenic probe-based technologies, including hybridization probes (e.g., FRET), hydrolysis probes (e.g., TaqMan) and displacement probes (e.g., molecular beacons).
- Use only gene-specific primers for one-step qRT-PCR.
- Optimal cDNA synthesis is achieved at 42°C for 5 min.
- 3 min at 95°C is sufficient for RT inactivation and DNA polymerase activation.
- Do not exceed 25 µL reaction volumes.

Product Specifications

Shipping and storage

KAPA PROBE FAST One-Step qRT-PCR Master Mix (2X) Kits are shipped on dry ice or ice packs, depending on the destination country. Upon receipt, immediately store all components at -15°C to -25°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Handling

Always ensure that components have been fully thawed and thoroughly mixed before use. The KAPA PROBE FAST qPCR Master Mix may not freeze solidly, even when stored at -15°C to -25°C. The KAPA RT Mix is temperature-sensitive, and should be stored at -15°C to -25°C and kept on ice during use. ROX reference dye is light-sensitive. Exposure to direct light for an extended period of time will result in loss of fluorescent signal intensity.

KAPA PROBE FAST qPCR Master Mix is stable through 30 freeze-thaw cycles. Ensure that all reagents are stored protected from light at -15°C to -25°C when not in use. When protected from light, reagents are stable in the dark at 4°C for at least one week, and may be stored at this temperature for short-term use, provided that they do not become contaminated with microbes and/or nucleases.

Quality control

All kit components are subjected to stringent functional quality control, are free of detectable contaminating exo- and endonuclease activity, and meet strict requirements with respect to DNA contamination.

Safety Information

Precautions

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material can vary, the operator must optimize pathogen inactivation and follow the appropriate measures according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online at www.sigmaaldrich.com, or upon request from www.sigma-aldrich.com/techservice.

Important Parameters

Template RNA

Starting template of purified total RNA can range between 1 pg – 100 ng per 20 µL reaction. Using greater amounts of template may increase the background fluorescence, which reduces linearity of standard curves after background subtraction. Digest purified RNA with RNase-free DNase I to remove contaminating genomic DNA which can act as template during PCR. DNase treatment should be performed according to manufacturer's instructions. Treated RNA should be stored at -15°C to -25°C or -80°C in RNase-free water. Multiple freeze-thaws of RNA should be avoided.

Primers

Careful primer design and purification (HPLC-purified primers are recommended) will minimize loss in sensitivity due to non-specific amplification. This effect becomes more prominent at low target concentrations. To maximize the sensitivity of the assay, use the lowest primer concentration determined not to compromise reaction efficiency (50 – 400 nM of each primer). For optimal results, design primers that amplify PCR products 70 – 200 bp in length. Use appropriate primer design software to design primers with a melting temperature (Tm) of approximately 60°C, to take advantage of two-step cycling. Primers must be designed carefully to avoid detection and amplification of genomic DNA, which would lead to inaccurate mRNA quantification. To prevent gDNA amplification, design forward and reverse primers from different exons, or to span exon-intron boundaries.

KAPA RT Mix

KAPA RT Mix (50X) contains an optimized blend of wild-type M-MuLV Reverse Transcriptase and RNase Inhibitor. M-MuLV RT has a high affinity for RNA and is optimized for cDNA synthesis at 42°C. The RNase Inhibitor safeguards against degradation of RNA targets due to RNase contamination. KAPA RT Mix (50X) must be stored at -20°C as the enzymes are not thermostable.

dUTP

Use of dUTP allows treatment with uracil-DNA glycosylase (UDG), if required, to prevent carry-over contamination in subsequent reactions. dUTP can be used at final concentrations that range from 0.2 – 0.4 mM. Do not use UDG in one-step qRT-PCR, as the UDG will degrade the cDNA upon synthesis.

Magnesium Chloride

The concentration of $MgCl_2$ affects the binding dynamics of primers and probes to template DNA. The higher the final $MgCl_2$ concentration in the PCR reaction, the greater the binding affinity of the primers and probe for target DNA. KAPA PROBE FAST qPCR Master Mix (2X) provides $MgCl_2$ at a final (1X) concentration of 5.0 mM. It is highly unlikely that additional $MgCl_2$ will improve reaction efficiency or specificity.

ROX Reference Dye

For certain real-time cyclers, the presence of the passive reference dye, ROX, compensates for non-PCR related variations in fluorescence detection. Fluorescence from the ROX reference dye does not change during the course of real-time PCR, but provides a stable baseline against which PCR-related fluorescent signals are normalized. Thus, the ROX dye compensates for differences in fluorescence detection between wells due to slight variations in reaction volume or differences in well position. The use of ROX Reference Dye is necessary for all Applied Biosystems instruments and is optional for the Stratagene Mx3000P™, Mx3005P™, and Mx4000™ cyclers. Bio-Rad/MJ Research, Cepheid, Corbett Research/QIAGEN, Eppendorf, and Roche instruments do not require ROX. The presence of the ROX reference dye in the master mix does not interfere with real-time PCR on any instrument, since the dye is not involved in the reaction and has an emission spectrum different from that of the fluorophore with which the probe is labelled.

Table 1. Instrument Compatibility*

Instrument	Reference Dye
Applied Biosystems® 5700, 7000, 7300, 7700, 7900HT, StepOne™, and StepOnePlus™	ROX High 500 nM
Applied Biosystems 7500, ViiA™7, QuantStudio™ instruments, Agilent Mx3000P™, Mx3005P™ and Mx4000™	ROX Low 50 nM
Rotor-Gene™ instruments, DNA Engine Opticon™, Opticon™ 2, Chromo 4™ Real-Time Detector, Mastercycler® ep realplex, Smart Cycler®, Roche LightCycler® 480, 96, Nano, 1.5/2.0**, Bio-Rad CFX96, Illumina® Eco™	No ROX

*For instruments not listed here, please contact Technical Support at **sigma-aldrich.com/techservice** for more information.

**The Roche LightCycler 1.5/2.0 capillary instruments require the addition of unacetylated BSA to the qPCR reaction at a final concentration of 250 ng/µL in order to prevent the DNA polymerase and template from binding to the glass capillaries.

One-step qRT-PCR Protocol

Any existing one-step qRT-PCR assay performed efficiently using standard cycling conditions may be used with the KAPA PROBE FAST One-Step qRT-PCR Kit. Typically, no re-optimization of reaction parameters is required.

1. Prepare qPCR master mix

1.1 Ensure all reaction components are properly thawed and mixed.

1.2 Keep the KAPA RT Mix on ice during use, and assemble reactions on ice to avoid premature cDNA synthesis.

1.3 Prepare a PCR master mix containing the appropriate volume of all reaction components common to all or a subset of reactions to be performed.

1.4 Include a No Template Control (NTC) and No RT Control (NRT) when necessary. The NTC will enable detection of contamination in the reaction components, while the NRT will enable detection of contaminating gDNA.

1.5 Refer to the table in **Important Parameters: ROX Reference Dye** to determine reference dye requirements for your instrument.

1.6 Calculate the required volume of each component based on the following tables:

Component	ROX	No ROX	Final conc. (20 µL reaction[1])
PCR-grade water	Up to 20 µL	Up to 20 µL	N/A
KAPA PROBE FAST qPCR Master Mix[2]	10 µL	10 µL	1X
10 mM dUTP (optional)	As required	As required	200 – 400 µM
10 µM forward primer	0.2 – 0.8 µL	0.2 – 0.8 µL	100 – 400 nM
10 µM reverse primer	0.2 – 0.8 µL	0.2 – 0.8 µL	100 – 400 nM
10 µM probe	0.2 – 1.0 µL	0.2 – 1.0 µL	100 – 400 nM
Template RNA[3]	≤5 µL	≤5 µL	≤100 ng
50X ROX High/Low (as required)[4]	0.4 µL	—	1X

[1]Reaction volumes may be adjusted from 5 – 25 µL, depending on the block type and instrument used. Reaction volumes >25 µL are not recommended.

[2]KAPA PROBE FAST qPCR Master Mix (2X) contains $MgCl_2$ at a final concentration of 5 mM.

[3]Template RNA input of 1 pg – 100 ng total RNA is recommended. For more information, refer to **Important Parameters: Template**.

[4]The use of ROX dye is necessary for all Applied Biosystems® instruments and is optional for the Agilent Mx3000P™, Mx3005P™, and Mx4000™ cyclers. Bio-Rad/MJ Research, Cepheid, Corbett/QIAGEN, Eppendorf, Illumina®, and Roche instruments do not require ROX dye.

2. Set up individual reactions

2.1 Transfer the appropriate volumes of qPCR master mix, template, and primers to each well of a PCR plate/tube(s).

2.2 Volumes may be scaled down from 20 µL to 10 µL if low-volume tubes/plates are used.

2.3 Cap or seal the reaction plate/tube(s) and centrifuge briefly.

3. Perform one-step qRT-PCR

3.1 If applicable, select fast mode on the instrument.

3.2 Confirm that the qPCR protocol to be used conforms to the following parameters:

Step	Temp.	Duration	Cycles
Reverse transcription[1]	42°C	5 min	Hold
Enzyme activation	95°C	2 – 5 min[2]	Hold
Denaturation	95°C	1 – 3 sec	40
Annealing/ extension/ data acquisition[3]	60°C[4]	≥20 sec[5]	

[1]5 min at 42°C is sufficient for cDNA synthesis for most assays. For difficult assays, this may be increased to 10 min.

[2]20 sec at 95°C is sufficient time for enzyme activation; however, optimal denaturation of complex targets may require up to 3 min denaturation.

[3]For 3-step cycling protocols, anneal at optimal annealing temperature for 20 sec followed by 1 sec extension and data acquisition at 72ºC according to instrument guidelines.

[4]Annealing temperature is dependent on the specific primer/probe combination.

[5]Use 20 sec at 60ºC as a starting point. The minimum programmable annealing/extension time will vary for different instruments.

4. Data Analysis

4.1 Data analysis is dependent on experimental design. Refer to the instrument guidelines for more information on how to perform the appropriate data analysis.

Troubleshooting

Symptoms	Possible Causes	Solutions
Positive signal in no-template control (NTC) or no-RT control (NRT)	RNA template contaminated with genomic DNA	Take standard precautions to avoid contamination during reaction setup. Treat RNA sample with RNase-free DNase I.
	Primer-dimer formation	Resynthesize or redesign primers. HPLC purification of primers and probes greatly reduces dimer formation and increases sensitivity. Adjust primer/probe concentration and annealing temperature to prevent dimer formation.
Low fluorescence intensity	Incorrect handling	Ensure probe is stored at -15°C to -25°C in the dark and returned to the freezer immediately after reaction setup.
No product detected during qPCR or agarose gel electrophoresis	Reactions not set up on ice	Repeat setup on ice and run qRT-PCR reaction immediately after setup.
	Incorrect cycling protocol was used	Ensure that the cycling protocol contains the cDNA synthesis step, and that the correct fluorescence detection channel is selected.
	Pipetting error or missing reagent	Ensure that correct reagents have been used.
	Template RNA contains inhibitors, or is degraded	Re-purify or re-isolate template RNA, or dilute RNA in 10 mM Tris-HCl, pH 8.0 – 8.5.
	Incorrect primer design or annealing temperature	Verify primer design. Increase or decrease annealing temperature in 2°C increments.
Product detected later than expected	Amplicon is too long	Optimal results are obtained with amplicons of 70 – 200 bp.
	PCR annealing/extension time is too short	This kit requires a minimum of 20 sec annealing followed by the minimum time required for data acquisition at 72°C, according to instrument guidelines.
	Template RNA contains inhibitors or is degraded	Re-purify or re-isolate template RNA, or dilute RNA in 10 mM Tris-HCl, pH 8.0 – 8.5.
Poor low copy number sensitivity	Sub-optimal primer design or annealing temperature	Redesign primers.
		HPLC purification of primers greatly reduces primer-dimer problems and increases sensitivity.
		Adjust primer concentration and melting temperature.
		Ensure that the correct cycling parameters were used.
High baseline fluorescence	Starting amount of template is too high	Reduce the amount of template in the reaction.

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