KAPABIOSYSTEMS

Technical Data Sheet

KAPA PROBE FORCE qPCR

Master Mix (2X) Universal Kit

KR1140 – v1.15

This Technical Data Sheet provides product information and a detailed protocol for the KAPA PROBE FORCE qPCR Master Mix (2X) Universal Kit.

Product Description

The KAPA PROBE FORCE qPCR Master Mix Universal Kit is designed for real-time PCR using sequence-specific fluorogenic probes. This master mix is compatible with many fluorogenic probe-based technologies, including hydrolysis probes (e.g., TaqMan®) and displacement probes (e.g., molecular beacons).

KAPA PROBE FORCE qPCR Master Mix is a ready-to-use cocktail containing all components except primers, probe(s), and template for probe-based real-time PCR. The 2X Master Mix contains KAPA3G HotStart DNA Polymerase, dNTPs (including dUTP), $MgCl_2$, ROX reference dye, and stabilizers.

The KAPA PROBE FORCE qPCR Master Mix contains the novel KAPA3G DNA Polymerase, which was engineered via a process of directed evolution for improved tolerance to common PCR inhibitors, carry-over inhibitors from crude DNA extraction methods, and high concentration of salts. The unique characteristics of this evolved enzyme result in robust amplification across a wide range of sample types, extraction and purification methods. The KAPA PROBE FORCE qPCR Master Mix is also very well suited to amplification of templates directly from cDNA synthesis reactions

Product Applications

The KAPA PROBE FORCE qPCR Master Mix is ideally suited for:

- Direct qPCR analysis of challenging sample types without purification (e.g., crude DNA extractions from blood, tissue, and plant)
- Gene expression analysis (cDNA templates and direct carry-over from cDNA synthesis reactions)
- High-throughput workflows, such as mouse genotyping, GMO testing, and SNP detection

Kit Codes and Components*	
KK4300 100 x 20 µL reactions	qPCR Master Mix (2X) Universal 1 x 1 mL
KK4301 500 x 20 µL reactions	qPCR Master Mix (2X) Universal 1 x 5 mL
KK4302 1000 x 20 µL reactions	qPCR Master Mix (2X) Universal 2 x 5 mL
KK4303 5000 x 20 µL reactions	qPCR Master Mix (2X) Universal 1 x 50 mL

* These kits are suitable for all ABI instruments, regardless of ROX requirements, as well as for all instruments that do not require ROX as a passive reference dye. Refer to your instrument manual to determine whether ROX normalization is required.

Quick Notes

- This kit contains the KAPA3G HotStart DNA Polymerase enzyme, enabling probe-based qPCR for both routine and challenging sample types.
- Initial denaturation of 3 min at 98°C is recommended to ensure complete denaturation of complex target DNA. A 5-min denaturation time may be required for some crude samples.
- For two-step cycling, use a 20-sec combined annealing/extension/data acquisition at 60°C as a first approach.
- A 10-sec annealing/extension/data acquisition time may be used with most assays, but this must be determined empirically.
- For crude samples, the amount of sample in the reaction may be reduced to improve performance, but this must be determined empirically.

Product Specifications

Shipping and storage

KAPA PROBE FORCE qPCR Master Mix Universal is shipped on ice packs. Upon arrival, store the master mix at -20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the master mix will retain full activity until the expiry date indicated on the kit label.

Handling

ROX reference dye is sensitive to exposure to light. Avoid repeated freezing and thawing. Always ensure that the product has been fully thawed and mixed before use.

Quality control

KAPA PROBE FORCE qPCR Master Mix Univeral is subjected to stringent functional quality control, free of detectable contaminating exo- and endonuclease activities, and meets strict requirements with respect to DNA contamination. Contact support@kapabiosystems.com for more information.

Important Parameters

Primers and Probes

The use of previously validated assays or dedicated qPCR design software such as Beacon Designer 7 (http://www.premierbiosoft.com/molecular_beacons/index.html) when designing probe-based assays.

Lyophilized primers and probes should be resuspended in 10 mM Tris-HCl (pH 8.0 – 8.5). A freezer stock should be prepared at an initial concentration of 100 µM, which should be thawed only as necessary. Primers and probes should be resuspended and diluted in a nuclease-free buffer, and stored at -20°C in Low-bind tubes. Stored in this manner, they should be stable for years.

Optimal primer concentration should be determined empirically. A primer/probe concentration of 0.2 – 0.3 µM is recommended as a first approach. The typical concentration range is 0.1 – 0.5 µM for both primers and probes. To maximize the sensitivity of the assay without compromising reaction efficiency, use the lowest concentration of primers and probe(s) possible.

Purified DNA Samples

Purified DNA prepared with commercially-available extraction kits or other methods may be used at high concentrations in each KAPA PROBE FORCE qPCR reaction (efficiency and linearity have been maintained up to 200 ng DNA/20 µL reaction).

Crude-extract and Crude Samples

Crude-extract or crude samples are generally better suited for qualitative, rather than quantitative analyses, because of variations in DNA extraction efficiency, PCR inhibition, etc. Always include a positive control reaction with purified DNA in each experiment to benchmark performance.

Some crude extracts or crude samples may lead to cleavage of the probe; these samples are not suitable for further probe-based PCR.

Crude DNA extraction methods may be used in conjunction with KAPA PROBE FORCE qPCR Master Mix, including:

- NaOH-based extractions for plant and mammalian DNA[1]
- KAPA3G Plant Extraction Buffer[2]:
  - 50 mM Tris-HCl (pH 8.0 – 8.5)
  - 0.1 mM EDTA
  - 2% ß-mercaptoethanol

[1] The KAPA PROBE FORCE qPCR Master Mix can tolerate up to 50 mM final NaOH in the reaction allowing some samples to be added directly without neutralization.

[2] Dithiothreitol (DTT) may be used at a final concentration of 10 mM as an alternative should ß-mercaptoethanol not be available. The stability of an extract prepared from a particular sample type should be determined empirically. Extracts prepared with this protocol using Extraction Buffer with ß-mercaptoethanol are typically stable for 3 – 5 days at 4°C. Extracts prepared using Extraction Buffer with DTT are generally less stable, so DTT should only be used if ß-mercaptoethanol is not an option.

Sample Volume

The optimal volume of either crude DNA extract or crude sample per reaction should be determined empirically.

For crude DNA extracts, inhibition should be assessed by performing a titration of crude extract spiked into a reaction with purified DNA. Inhibition is determined by the measurement of later Cq scores compared to reactions containing only purified DNA.

For crudes samples, the least amount that can be sampled reproducibly, will work best. Optimization of reaction volume may also be considered to improve performance.

For leaf tissue, a disc made by a 0.35 mm diameter punch is suggested. Other sampling devices, such as needles, have also been used with success.

FTA and other blood cards work well with the use of one or two 0.5 mm punch(es) added directly to a 20 µL reaction.

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 Agilent Mx3000P™, Mx3005P™ and Mx4000™ cyclers. Bio-Rad/MJ Research, Cepheid, Corbett Research, 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.

Magnesium Chloride

The concentration of $MgCl_2$ affects the binding dynamics of primers and probes with regard to the template DNA. A higher final $MgCl_2$ concentration in the PCR reaction increases the binding affinity of the primers and probes for target DNA, but too high a $MgCl_2$ concentration may result in non-specific interactions.

KAPA PROBE FORCE qPCR Master Mix provides $MgCl_2$ at a final concentration of 4.5 mM. This is sufficient for the majority of assays, but there may be assays where increasing the $MgCl_2$ concentration will increase performance, and this should be kept in mind during any assay optimisation or troubleshooting.

Some sample types may contain Mg-chelating compounds. Depending on the concentration of Mg-chelating compounds and amount of sample added to a reaction, this may significantly decrease the available concentration of magnesium ions in the PCR reaction. In this case, additional $MgCl_2$ should be added to optimize performance (titrate in 0.5 mM increments).

KAPA PROBE FORCE qPCR Protocol

Any existing qPCR assay performed efficiently using standard cycling conditions may be converted to faster qPCR assays with the KAPA PROBE FORCE qPCR Master Mix. Typically, minimal re-optimization of reaction parameters is required.

1. Step 1: Set up the qPCR reaction

1.1 Ensure that all reaction components are properly thawed and mixed.

1.2 Calculate the required volumes of each component based on the following table:

Components	Final conc.	20 µL rxn[1]
PCR-grade water	–	Up to 20 µL
KAPA PROBE FORCE qPCR Master Mix (2X) Universal[2]	1X	10 µL
Forward Primer (10 µM)	0.2 µM[3]	0.4 µL
Reverse Primer (10 µM)	0.2 µM[3]	0.4 µL
Probe (10 µM)	0.2 µM[3]	0.4 µL
Template DNA	As required	As required

[1] Reaction volumes may be adjusted between 5 – 25 µL. Reaction volumes >25 µL are not recommended.

[2] Final $MgCl_2$ concentration at 1X is 4.5 mM. A higher concentration should not be required for the majority of assays. See **Important Parameters: Magnesium Chloride** for further details.

1.3 Use the lowest primer/probe concentration that will still give optimal reaction efficiency. See **Important Parameters: Primer and Probes** for further details.

Prepare a PCR master mix consisting of the appropriate volumes of KAPA PROBE FORCE qPCR Master Mix, PCR-grade water and any other component (e.g., primers or probes) that is common to all or a subset of the reactions to be performed. Ensure that first the reaction components, and then the PCR master mix, are mixed properly.

2. Step 2: Set up the plate

2.1 Transfer the appropriate volumes of PCR master mix and template to each well of a PCR tube/plate.

2.2 Cap or seal the reaction tube/plate and centrifuge briefly.

3. Step 3: Perform the PCR reaction

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	Cycle
Enzyme activation/ template denaturation[1]	98°C	3 min	Hold
Denaturation[2]	95°C	10 sec	30 – 45
Annealing/ extension/ acquisition[3]	55 – 65°C	10 – 30 sec	

[1]20 sec at 98°C is sufficient for enzyme activation; however, optimal denaturation of complex targets may require up to 3-min denaturation. Some crude-sample templates may require 5 – 10 min initial denaturation for optimal results.

[2]Start with a 10-sec denaturation time, but 3 – 5 sec is sufficient for most amplicons in a 20 µL reaction.

[3]A 10-sec annealing/extension time is sufficient for most assays with PCR products below 200 bp. For 3-step cycling protocols, anneal at optimal annealing temperature for 20 sec followed by the minimum time required for data acquisition at 72°C, according to instrument guidelines.

4. Step 4: Analyze the results

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

Troubleshooting

The recommendations suggested below may first be tried individually, then in combination, as appropriate:

Symptom	Possible cause	Solution
Late Cq or no amplification during cycling	Too much crude sample or crude extract in the reaction	Use less crude sample, or a smaller volume of crude extract.
	Too short an initial denaturation time for crude samples	An initial denaturation time of 10 min will improve results with many crude sample types. Some crude sample types may require an even longer denaturation time.
	Incorrect reaction setup	Verify that all the components have been added at the correct concentrations.
	Effective magnesium concentration too low	Increase magnesium concentration in 0.5 mM increments.
	Non-specific products may be amplified	Increase annealing temperature in 2°C increments.
	Incorrect cycling protocol	Verify that the correct recommended cycling conditions were used.
	Incorrect annealing temperature	Decrease annealing temperature in 2°C increments.
	Incorrect detection filter/channel	Check that the correct filters have been selected for data acquisition.
	Degraded template DNA, primers, or probe	Always store and dilute primers and DNA in 10 mM Tris-HCl (pH 8.0 – 8.5), not in PCR-grade water.
	Sub-optimal primer/probe design	We recommend using prevalidated assays or designing assays using dedicated software.
Positive signal in no-template control (NTC)	Contamination of reagents	Discard all reagents and repeat experiment with new components.
	Contamination during setup	Review setup procedure and ensure that aerosol-barrier pipette tips are used.
	Degradation of primers and probe	Always store and dilute primers and DNA in 10 mM Tris-HCl (pH 8.0 – 8.5), not in PCR-grade water. Use new stocks of primers and probe or redesign the assay.
Extremely high ΔRn or Rn values	ROX was not selected as the passive reference dye at setup	Select ROX as the passive reference dye when setting up the plate.
High variability across replicates	Insufficient mixing of reaction master mix	Ensure that the reaction master mix is properly mixed by vortexing or inverting the tube a sufficient number of times, followed by brief centrifugation.
	Insufficient mixing of template samples	As above.
	Faulty pipettes or poor pipetting technique	Calibrate pipets, and verify pipetting technique
	Evaporation	Ensure that lids are closed properly, or sealing film is properly attached.

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