DESCRIPTION

TOROIVD® Probe 1-step RT-qPCR 5G kit 2.0 provides for sensitive, reproducible detection up to five RNA and DNA targets in a single multiplex reaction .Particularly useful for virus detection with TaqMan®probe assays , the kit includes UNG, dsDNase, thermostable MMLV reverse transcriptase, RNase inhibitor , TOROIVD® 5G DNA polymerase and reaction buffer. The improved enzymes also enables a high resistance to PCR inhibitors and high stability in room temperature. The 1-step system is suitable for high-throughput analysis because of its simple reaction setup. In addition, this system can reduce the risk of cross-contamination with Uracil-N-glycoslyase （UNG） and dsDNase. The kit is suitable for high-speed RT-qPCR and enables accurate detection and quantification of targets, making it possible to obtain highly reproducible and reliable realtime PCR results over a wide dynamic range.

FEATURES

-Rapid and highly sensitive

This kit can achieve the rapid and highly sensitive quantification of a low-copy targets by a 1-step RT-qPCR method with probes and be suitable for the quantification of RNA/DNA viruses or mRNA expressed at a low level.

-Optimized for multiplexing

This kit has been validated for multiplexing up to five targets simultaneously, allowing for additional targets and/or controls to be run simultaneously for efficiency or quality control purposes.

-Inhibitor tolerant

The unique proprietary formulation of this kit allows robust performance even in the presence of substances that can normally inhibit PCR, such as heparin, hematin, or EDTA, increasing your confidence when working with a variety of complex clinical samples.

-Wide dynamic range compatible with RNA and DNA

This kit has been optimized to provide high specificity and dynamic range for use with both RNA and DNA targets. This input flexibility can help streamline the number of different workflows in your lab to improve efficiency.

-Broad instrument compatibility

This kit can be run in either fast or standard cycling conditions with equivalent performance across a wide variety of real-time cyclers. The 50×ROX Reference dye （not supplied） is added can be applied to the real-time cyclers that require a passive reference dye.

-Avoid Contamination

This kit contains dUTP in the reaction buffer and UNG and small amounts of dsDNase in the enzyme mix. The crossed contamination caused by PCR product or dsDNA can be removed so that the rate of false-positive detection can be reduced.

COMPONENTS

The kit includes the following reagents, and QPR-303UD can be used for 200 reactions for a total 25μl reaction volume. All reagents should be stored at −20°C.

Cat NO.	Components	Size
QPR303UD13	RT-qPCR Enzyme Mix UD	260μl/tube×1
ASF104BB	2×5G qPCR Buffer BB	1.25ml/tube×2

Notes:

-RT-qPCR Enzyme Mix UD contains UNG, dsDNase, TOROIVD®III reverse transcriptase, RNase inhibitor and TOROIVD®5G DNA polymerase. Bulk package of 1.3ml or 26ml per tube can be supplied .

-2×5G qPCR Buffer BB contains 0.4mM dA/C/G/T/UTP, 5mM Mg^{2+}, reaction buffer and stabilizer, etc.). Bulk package of 12.5 ml or 40ml per tube can be supplied .

NOT SUPPLIED

In some experimental applications, the following reagents may be used with QPR-303UD,which are not supplied in this kit. Please contact us to order.

Cat NO.	Components	Size
ASF101GB	2×5G qPCR Buffer GB	1.25ml/tube； 12.5ml/tube； 40ml/tube.
END-UD13	Enzyme Mix Dilution Buffer	1.25ml/tube， 12.5ml/tube； 40ml/tube.
ROX-050	50×ROX Reference dye	100μl/tube， 1ml/tube， 10ml/tube.
RDB-100	RNA Dilution&Storage Solution	100ml/pcs,10L/pcs

Notes:

-2×5G qPCR Buffer GB can also be used instead of 2×5G qPCR Buffer BB in the kit for some experimental applications with higher sensitivity and specificity.2×5G qPCR Buffer BB is more suitable for the amplification of short amplicon（≦100bp）and direct RT-qPCR.

-Enzyme Mix Dilution Buffer (END-UD13) is used to dilute the RT-qPCR Enzyme Mix UD without changing the performance and stability of the Enzyme Mix. And the Maximum added volume is 1ml for per ml RT-qPCR Enzyme Mix UD.

-The 50× ROX reference dyes are used for analyses with instruments that correct for cross-talk between wells, such as the real- time PCR instruments by Applied Biosystems and Agilent Technologies. 0.5μl 50×ROX Reference Dye was added for a total 25μl reaction volume in when using the following instruments,Applied Biosystems 7300/ 7700/7900HT, StepOnePlus, etc. And 0.05 μl was added for using the following instruments,Applied Biosystems 7500/7500Fast Step OnePlus ,Agilent Technologies AriaMx,etc.No ROX Reference Dye is required when using other brand instruments,such as LightCycler 96/LightCycler 480 system (Roche), CFX96 Real-Time PCR Detection System (Bio-Rad), Smart Cycler System (Cepheid) ,etc.

-RNA Dilution&Storage Solution is a buffer that provides greater RNA stability than TE Buffer， RNase-free water and RNA Virus VTM medium. RNA Dilution&Storage Solution Solution is compatible with direct 1- step RT-qPCR.

PRIMER/PROBE DESIGN

-Design of primers

Primer length: 18–25bp； Tm of primer: 60–65°C； GC content: 40–60%；

Target length: 70–200 bp；Larger targets (>200 bp) tend to reduce the efficiency and specificity of amplification.

Purification grade: OPC or HPLC grade ；

-Design of probes

Probe length: 20–30bp； Tm of probe: 65–70°C； GC content: 40–60%； Purification grade: HPLC.

-Checking the performance of primers and probes:

-Prepare a dilution series with five or more dilutions of template RNA/ssDNA. Perform RT-qPCR assay using the diluted RNA/DNA with the newly designed primers and probe, and draw a standard curve.

- Confirm that the PCR efficiency is between 90% and 110% and R^2 is equal to or greater than 0.99. if the PCR efficiency or R^2 are outside of these ranges, the reaction conditions should be optimized. If this does not improve the result, the primers and/or probe should be redesigned.

PROTOCOL

1. This kit should be fully thawed before use. Gently vortexed and briefly centrifuged.
2. Purified or crude template RNA/DNA can be may be used directly or after dilution。
3. Prepare the following reaction mixture in a thin-walled qPCR tube or plate.

Components	25μL reaction	
2×5G qPCR Buffer BB	12.5μL	Premix
RT-qPCR Enzyme Mix UD	1.3μL	
10μM Forward primer	1μL	
10μM Reverse primer	1μL	
10μM TaqMan® probe	0.4μL	
50×ROX	0/0.05/0.5μL	
DNase/Rnase Free Water	XuL	
Template RNA/DNA solution	5μL	

4. Gently mix the reaction solutions and spin down in microcentrifuge.

Notes:

- For the direct RT-qPCR to crude template RNA, the $MgCl_2$ concentration may need to be optimized between 2.5-8mM of final concentration. BSA and Triton X-100 may need to be added to improve the performance of direct RT-qPCR.

-The primer concentration should be optimized between 0.2-0.8 μM and TaqMan® probe optimized between 0.1-0.4 μM with 10-50 copies templates /reaction . So the best primers-probe concentration sets was selected by orthogonal design of experiments .

CYCLING CONDITIONS

The recommended 2-step PCR protocol is described below:

For ABI 7500/7300 etc.				
Steps		Temperature	Time	Cycles
1	UDG enzyme action	37°C	2 min	1
2	Reverse transcription	52°C	5min	1
3	Prenaturation	95℃	1min	1
4	Denaturation	95°C	3 sec	40-45
	Annealing/ Extension	60°C	30 sec	

For Bio-Rad CFX96,ABI StepOne Plus,etc.				
Steps		Temperature	Time	Cycles
1	UDG enzyme action	37°C	2 min	1
2	Reverse transcription	52°C	5min	1
3	Prenaturation	95℃	1min	1
4	Denaturation	98°C	3 sec	40-45
	Annealing/ Extension	60°C	10 sec	

For Bioer LineGene 9600 Plus, Roche LightCycler 96 /LightCycler 480 systems,etc.				
Steps		Temperature	Time	Cycles
1	UDG enzyme action	37°C	2 min	1
2	Reverse transcription	52°C	5min	1
3	Prenaturation	95℃	1min	1
4	Denaturation	95°C	10 sec	40-45
	Annealing/ Extension	60°C	20 sec	

Notes:

-Use this protocol first and optimize PCR conditions when necessary. Perform 3-step PCR when using primers

with low Tm values or when 2-step PCR is not feasible.

-The indicated UNG treatment temperature can be optimized 25-37°C, and time between 0-5min.

-The indicated RT temperature can be optimized between 50-60°C and time between 2-15min.

-The indicated Pre-denaturation temperature can be optimized 95-98°C, and time between 2min-5min.

-The indicated denaturation temperature can be optimized 95-98°C, and time between 3sec-10sec.

-The indicated Extension /Annealing temperature can be optimized 60-65°C, and time between 5sec-30sec.

Fluorescence signal gathering should be set up at this step.

APPLICATION DATA

Example 1. High sensitivity detection of 2-3 copies SARS-CoV-2 reference RNA.

Template RNA:

SARS-CoV-2 reference RNA (GBW (E) 091111, China);

N Gene: 2.4×10^{5}copies/ul; E Gene: 1.8×10^{5}copies/ul;ORF1ab Gene: 0.70×105copies/ul; Dilution factor is 10^3, 10^4, 10^{5}和10^6.

Primer and Probe: From China CDC

ORF1ab-F: 5`- CCCTGTGGGTTTTACACTTAA-3` 300nM

ORF1ab-R: 5`- ACGATTGTGCATCAGCTGA-3` 300nM

ORF1ab-P: 5`-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-3`BHQ1 200nM

N-F: 5`- GGGGAACTTCTCCTGCTAGAAT-3` 600nM

N-R: 5`- CAGACATTTTGCTCTCAAGCTG-3` 600nM

N-P: 5`-FAM-TTGCTGCTGCTTGACAGATT-3`TAMRA 200nM

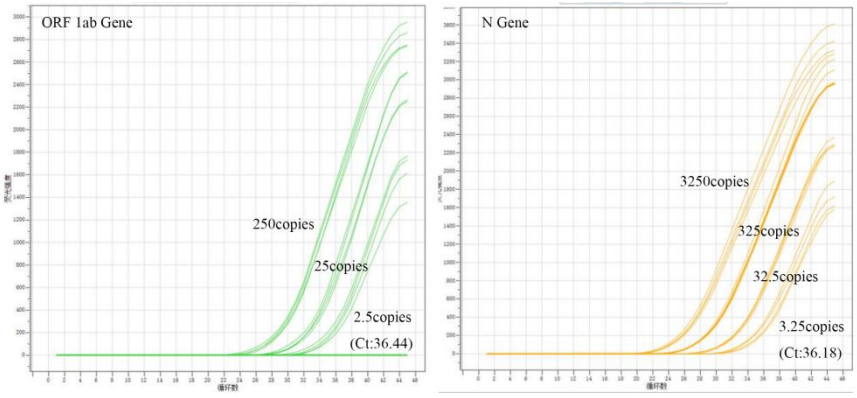
Instrument:

Line Gene 9600 Plus Bioer.

Reagent:

2×5G qPCR Buffer GB (ASF101GB) & RT-qPCR Enzyme Mix UD (QPR303UD13)

Results:



Example 2. LOD comparison with Company N

Template RNA:

2.25copies /reaction of ORF1ab Gene,20 replicates

2.75copies /reaction of N Gene,20 replicates

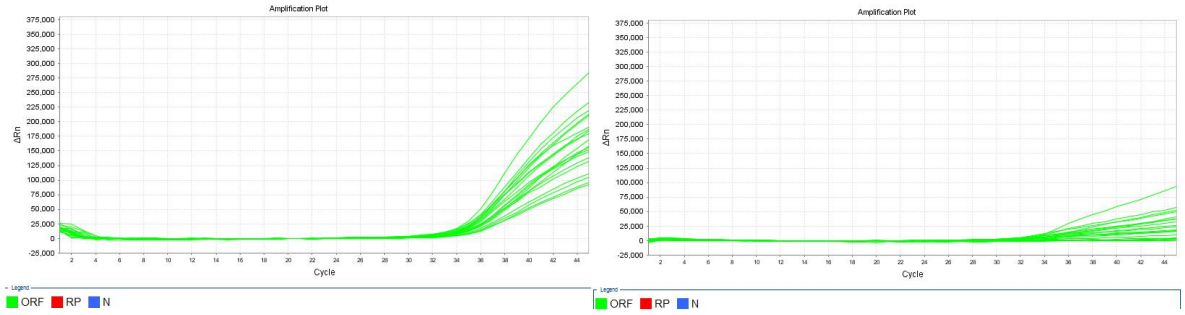
Instrument:

ABI 7500

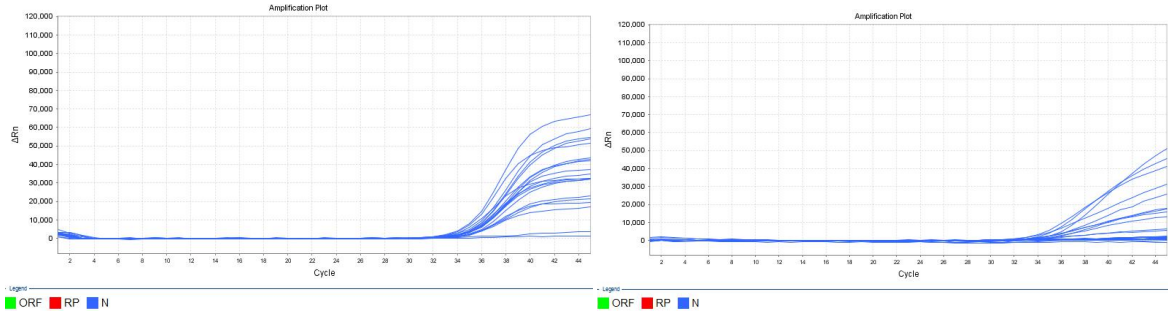
Reagents:

2×5G qPCR Buffer GB (ASF101GB) & RT-qPCR Enzyme Mix UD (QPR303UD13)

Results:



ORF -QPR-303UD （20/20）　　ORF -Company N （14/20）



N -QPR-303UD （20/20）　　N -Company N （13/20）

Example 3. Contamination removal capacity of QPR-303UD

Template:

Purified PCR product by MS2RNA and QPR-303UD

Primer and Probe:

Forward primer:GCCTTAGCAGTGCCCTGTCT 400nM

Reverse primer:AACATGCTCGAGGGCCTTA 400nM

Taqman Probe:FAM-CCCGTGGGATGCTCCTACATGTCA-TAMRA 200nM

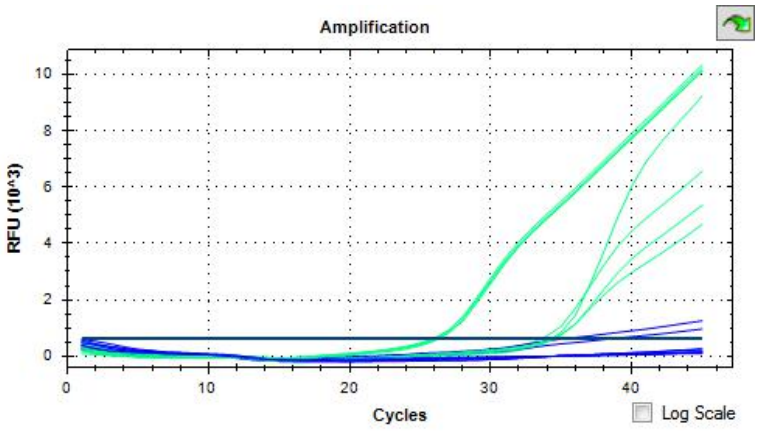
Instrument:

CFX 96

Reagents:

2×5G qPCR Buffer BB （ASF104BB）& RT-qPCR Enzyme Mix UD （QPR303UD13）

Results:



Green: without UNG　Blue: QPR-303UD

Example 4. Thermal stability of QPR303UD13 Enzyme Mix

Template:

MS2RNA from Roche

Primer and Probe:

Forward primer:GCCTTAGCAGTGCCCTGTCT 400nM

Reverse primer:AACATGCTCGAGGGCCTTA 400nM

Taqman Probe:FAM-CCCGTGGGATGCTCCTACATGTCA-TAMRA 200nM

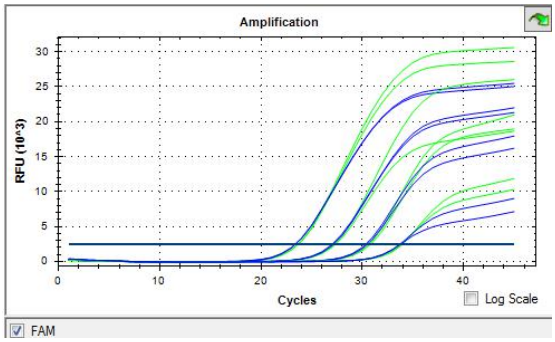
Instrument:

CFX 96

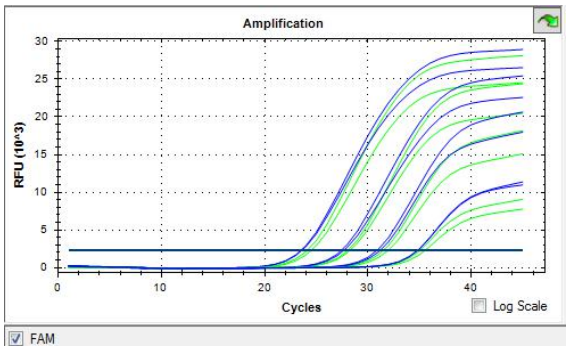
Reagents:

2×5G qPCR Buffer BB (ASF104BB) & RT-qPCR Enzyme Mix UD (QPR303UD13)

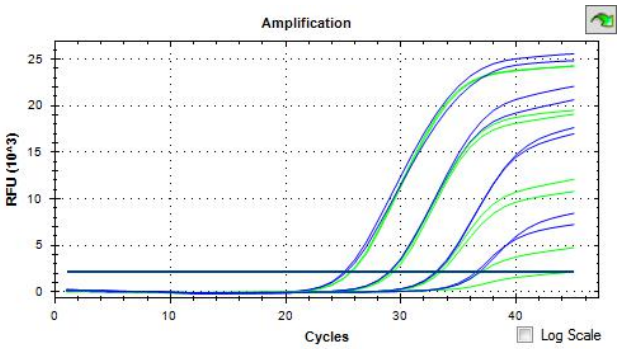
Results: *Green：-20℃ Blue：7d at 37℃*



1.3μl QPR303UD13/25μlreaction



1.5μl Enzyme Mix /25μlreaction (0.2μl END-UD13 added)



2.5μl Enzyme Mix /25μlreaction (1.2μl END-UD13 added)

STORAGE

This reagent can be stored at 4°C for 2 months. For longer storage, this reagent should be kept at -20°C for 2 years .