AMPLIQON
PCR ENZYMES & REAGENTS

15 MIN HOT START

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RealQ Plus 2x Master Mix for Probe

Low ROX

MADE IN DENMARK

Cat. No.	Reactions	RealQ Plus PCR Master Mix for Probe low ROX ID: 5000810
A314402	400	4 x 1.25 ml
A314406	4000	40 x 1.25 ml
A314499	Sample - 40	0.5 ml

Key Features

- Ready-to-use optimized 2x master mix
- Suitable for quantification
- Suitable for multiplexing
- High efficiency and sensitivity
- Wide dynamic range
- Hot start capacity for room temperature setup

Introduction

The RealQ Plus 2x Master Mix for Probe is a single-tube 2x reagent including all components necessary to perform probe based real-time DNA amplification. Just add your probes, primers and DNA. The RealQ Plus 2x Master Mix for Probe is suitable for multiplexing for up to four DNA targets in the same tube, thereby saving PCR consumables, time, workload and valuable DNA.

The RealQ Plus 2x Master Mixes promote high specificity and low background by using TEMPase Hot Start DNA Polymerase, a chemically modified Taq DNA polymerase with hot start capabilities.

The detection limit of RealQ Plus for Probe is approximately 2 copies (~0.007 ng of human gDNA, correlating to 1 diploid genome, with 2 gene copies per diploid genome). Quantification limit is approximately 24 copies (0.08 ng of human gDNA, correlating to 12 diploid genomes, with 2 gene copies per diploid genome).

Composition of RealQ Plus 2x Master Mix for Probe, Low ROX:

- Optimized buffer system including TEMPase Hot Start DNA Polymerase and dNTPs

Quality Control

TEMPase Hot Start DNA Polymerase is tested for contaminating activities, with no traces of endonuclease activity, nicking activity or exonuclease activity. The RealQ Plus 2x Master Mix for Probe is functionally tested for efficiency and absence of contaminating human genomic DNA.

Recommended Storage and stability

Long term storage at -20 °C. Product expiry at -20 °C is stated on the label.
Option: Store at +4 °C for up to 3 months.

Pre-protocol Considerations

Primers and Probes

The design of primers and probes is critical especially for successful multiplex real-time PCR

- Design primers with similar annealing temperatures.
- Analyse primer and probe sequences to avoid primer/probe hairpins, homo- or heterodimers, or any primer/probe complementarity across the targets.
- Optimization of primer and probe concentrations is highly recommended.
- Test assay efficiency by running each assay in singleplex reactions before conducting multiplex qPCR.
- Choose reporter dyes with appropriate excitation wavelengths with little to no overlap in their emission spectra. Check the instrument manual for recommendations.

Amplicon size

Recommended amplicon size is less than 200 bp.

Preventing Template Cross-Contamination

Due to the high sensitivity of quantitative PCR there is a risk of contaminating the reactions with the products of previous runs. To minimize this risk, tubes or plates containing reaction products should not be opened or analysed by gel electrophoresis in the same laboratory area used to set up reactions.

Instrument compatibility

Real-time instruments which require low ROX such: Applied Biosystems® 7500, 7500 Fast and ViiA™7, QuantStudio™ instruments, Agilent Mx3000P™, Mx3005P™, Mx4000™ and AriaMx.

Protocol

Note:

- Prior to the experiment, it is crucial to carefully optimize experimental conditions and to include controls at every stage. See pre-protocol considerations for details.
- Thaw the RealQ Plus 2x Master Mix. Following initial thawing of the master mix, store the unused portion at +4 °C. **Important:** Multiple freeze-thaw cycles should be avoided.

1. Prepare the experimental reaction by adding the components in the order shown in table 1.
2. Gently mix without creating bubbles* (do not vortex).
   * Bubbles interfere with detection of fluorescence.
3. Place the reaction in the instrument and run the appropriate program according to the manufacturer's instructions.

Table 1. Reaction components

Component	Vol./reaction	Final concentration
RealQ Plus 2x Master Mix	12.5 µl	1x
Forward primers (10 µM)	1 µl (0.25-2 µl)	0.4 µM (0.1-0.8 µM)
Reverse primers (10 µM)	1 µl (0.25-2 µl)	0.4 µM (0.1-0.8 µM)
Probes (10 µM)	0.5 µl (0.125-0.625 µl)	0.2 µM (0.05-0.25 µM)
PCR-grade H_2O	X µl	-
Template DNA	X µl	genomic DNA: 20 ng (1-100 ng) plasmid DNA: 0.5 ng (0.1-1 ng) bacterial DNA: 5 ng (1-10 ng)
TOTAL volume*	25 µl	-

* If using smaller reaction volumes, scale all components proportionally. Reaction volumes < 10 µl is not recommended. Smaller reaction volumes decrease signal intensity.

Three-step PCR Program

Cycles	Duration of cycle	Temperature
1[a]	15 minutes	95 °C
40	15-30 seconds[b]	95 °C
	30 seconds[c]	55-65 °C[d]
	30 seconds	72 °C

Two-step PCR Program (recommended)

Cycles	Duration of cycle	Temperature
1[a]	15 minutes	95 °C
40	15-30 seconds[b]	95 °C
	60 seconds[c]	55-65 °C[d]

a. For activation of the TEMPase hot start enzyme.
b. Denaturation time is varying between thermocyclers.
c. Set the qPCR instrument to detect and report fluorescence during the annealing/extension step of each cycle.
d. Choose an appropriate annealing temperature for the primer set used.

For Research Use Only. Not for use in diagnostics procedures.

Other product sizes, combinations and customized solutions are available. Please look at www.ampliqon.com or ask for our complete product list for PCR Enzymes. For customized solutions please contact us.

Made in Denmark

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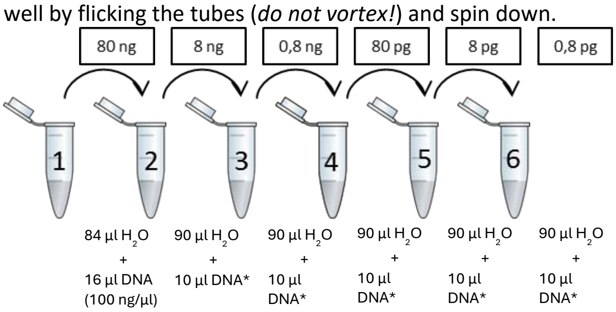
Real-Time PCR Mix Evaluation Guideline for Probe

When evaluating a new real-time PCR master mix it is important to look at the correct parameters. This guideline provides a protocol and tips on how to perform a quick evaluation of a real-time PCR master mix.

1) Prepare a DNA dilution series

Prepare a DNA dilution series according to the figure below. Mix well by flicking the tubes (*do not vortex!*) and spin down.



* Employ previous dilution.

For genomic DNA include the tubes 1-4. For other types of DNA include tubes 1-6. Initial DNA concentration is 100 ng/µl. The final DNA concentrations in each tube are noted in the boxes above the tubes.

2) Prepare a reaction mix

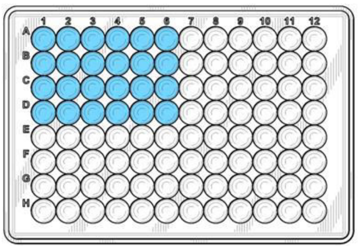
Prepare a reaction mix according to the table below. After adding all the components, vortex for 2 seconds and spin down.

	4 dilutions	6 dilutions
2x Master Mix	225 µl	325 µl
Primer F (10 µm)*	22.5 µl	32.5 µl
Primer R (10 µm)*	22.5 µl	32.5 µl
Probe (10 µm)*	11.25 µl	16.25 µl
H_2O	78.75 µl	113.75 µl

* Optimization of primers and probe concentrations is highly recommended.

3) Distribute the reaction mix

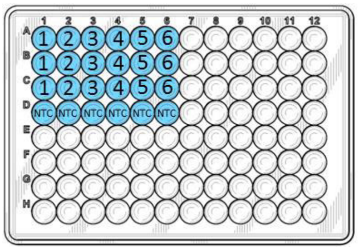
Distribute 20 µl of the reaction mix into the bottom of the blue wells according to the figure below.



4) Distribute the DNA

Distribute 5 µl of DNA from the dilution series into the wells. Make sure that the tip is submerged as little as possible into the liquid of the tube and the well. DNA should be distributed into the liquid and not onto the sides of the wells.

NB! Do not pipet up and down into the wells; the DNA will mix during the initial heating of the PCR run.



The numbers in the figure above correspond to the tube numbers in the dilution series in step 1. Add PCR Grade Water to the NTC wells instead of DNA.

5) Run the plate

Use the PCR cycling protocol below when running the plate on the real-time PCR instrument.

Phase	Time	Temperature	Cycles
Initial heating	15 min.	95 °C	1
Denaturation	30 sec.	95 °C	
Annealing	30 sec.	60 °C*	40
Elongation	30 sec.	72 °C	

* Apply the annealing temperature for the specific primer set.

6) Evaluate the results

Use the table below to evaluate the mix. The results should be within the specifications listed below in order to accept the mix.

Parameter	Specifications	Achieved?	
PCR efficiency	90-110 %	Yes □	No □
R^2	≥ 0.98	Yes □	No □
Standard deviation	≤ 0.2	Yes □	No □
NTC's	No amplification	Yes □	No □
Accept mix?	**"Yes" to all**	**Yes** □	**No** □

Notes SD;____

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Notes NTC's;____

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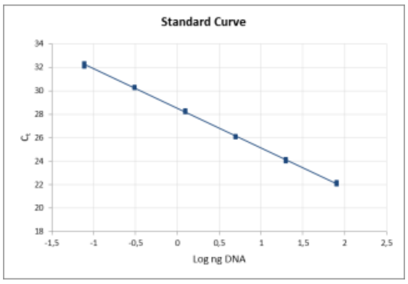

Notes

The results should be within the listed specifications in order to accept the mix. Note that the C_q-values are buffer-dependent and therefore cannot, nor should give the exact same results as experiments with a different buffer.
For a better understanding of what to look for when evaluating a mix please read the guidelines below.

Standard Curve

When looking at the standard curve, the three main parameters to focus on are: PCR efficiency, R^2-value and standard deviation between replicates.
The PCR efficiency indicates how efficient the target has been amplified and should be between 90-110 % to be acceptable. This is one of the most important parameters to look at when evaluating a mix.
The R^2-value is a statistical measure of how close the data are fitted to the regression line and should be ≥ 0.98.
The standard deviation between replicates indicates the accuracy of pipetting. The standard deviation should be ≤ 0.2.

C_q-value

C_q-values should be evaluated in relation to PCR efficiency and *not* be the only focus of interpretation, as long as the PCR efficiency is calculated and kept within the specifications (90-110 %). If the C_q-value is extremely high, it can be due to a low PCR efficiency, and thus the issue might be detected in the evaluation of efficiency. Inconsistency in C_q-values can also be due to inhibitors in the sample and may result in an efficiency of more than 110 %
Furthermore C_q-value can be influenced by ROX level and target length and can therefore vary from master mix to master mix. Small differences in C_q-values are expected when comparing two mixes.

Comparing two mixes

If two different master mixes are compared, then prepare a reaction mix with the other master mix as well and with the same primer concentrations.

If the same PCR cycling protocol is used, then distribute the reaction mix and DNA dilutions according to step 3 and 4 but in wells A7 to D12.

If the other master mix is run with a different PCR cycling protocol, then distribute the reaction mix and DNA dilutions into a second PCR plate according to step 3 and 4. Run the plates separately.

Other parameters

This guideline focuses on simplicity and keeping the required amount of work time to a limit. In order to make a thorough evaluation of a master mix other parameters should be taken into account. The most important parameters include:

- Primer and probe design
- Annealing temperature
- Primer and probe concentration
- Sample concentration
- Inhibitors contamination
- Choice of controls
- Setting threshold
- Correct fluorescence chemistry

When looking at all these parameters it is possible to make a deep and thorough evaluation and the experiment setup will then be optimal.