AMPLIQON
PCR ENZYMES & REAGENTS

15 MIN HOT START

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# RealQ Plus 2x Master Mix Green

**Without ROX**

MADE IN **DENMARK**

| Cat. No. | Reactions | RealQ Plus Master Mix Green Without ROX ID: 5000850 |
|---|---|---|
| A323402 | 400 | 4 x 1.25 ml |
| A323406 | 4000 | 40 x 1.25 ml |
| A323420 | 100 | 1 x 1.25 ml |
| A323499 | Sample - 40 | 0.5 ml |

## Introduction

The RealQ Plus 2x Master Mix Green without ROX is a single-tube 2x reagent including all components necessary to perform real-time DNA amplification for DNA-binding dye based PCR. Just add your primers and DNA. No ROX internal reference dye level is included.

Detection limit of RealQ Plus Green without ROX is approximately 1 copy. Quantification limit is approximately 24 copies (~0.08 ng of human gDNA, correlating to 12 diploid genomes, with 2 gene copy per diploid genome)

### Composition of RealQ Plus 2x Master Mix Green, without ROX:

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

### 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.

### 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 Green is functionally tested for efficiency and absence of contaminating human genomic DNA.

## Pre-protocol Considerations

### PCR Primers

It is important - especially in fluorescent DNA dye based quantitative PCR applications - to minimize the formation of non-specific amplification products. Particularly at low target concentration it is important to use the lowest possible primer concentration without compromising the efficiency of the PCR. The optimal concentration of primer pairs is the lowest concentration that results in the lowest $C_q$ and an adequate fluorescence for a given target concentration with minimal or no formation of primer-dimers. The optimal concentrations of upstream and downstream primers are not always of equal molarity. Optimal concentrations of primers are in the range of 100-800 nM.

### 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 does not require ROX internal reference dye as for example: Bio-Rad CFX96 Touch™, CFX384 Touch™, CFX Connect™, DNA Engine Opticon® 2, Chromo4™, iCycler iQ™, and My iQ™, Roche LightCycler® 480, LightCycler® 1536, LightCycler® Nano, and LightCycler® 96, Thermo Scientific™ PikoReal™, Cepheid SmartCycler®, Bio Molecular Systems Mic qPCR cycler, Qiagen Rotor Gene Q, Rotor Gene 6000, MyGo Mini, and MyGo Pro.

## 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. Solutions containing fluorescent green DNA dye should be protected from light whenever possible.

1. Prepare the experimental reaction by adding the components in the order shown in table 1.

**Table 1. Reaction components (reaction mix and template DNA)**

| Component | Vol./reaction* | Final concentration* |
|---|---|---|
| RealQ Plus 2x Master Mix | 12.5 µl | 1x |
| Primer A (10 µM) | 0.5 µl (0.25-2 µl) | 0.2 µM (0.1-0.8 µM)** |
| Primer B (10 µM) | 0.5 µl (0.25-2 µl) | 0.2 µM (0.1-0.8 µM)** |
| PCR-grade $H_2O$ | X µl | - |
| Template DNA | X µl | genomic DNA: 20 ng (1-100 ng)<br>plasmid DNA: 0.5 ng (0.1-1 ng)<br>bacterial DNA: 5 ng (1-10 ng) |
| **TOTAL volume**** | 25 µl | - |

* Suggested starting conditions; theoretically used conditions in brackets.
** Optimization of primer concentrations is highly recommended.
*** If using smaller reaction volumes, scale all components proportionally. Reaction volumes < 10 µl is not recommended. Smaller reaction volumes decrease signal intensity.

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.

### Three-step PCR Program

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

### Two-step PCR Program

| Cycles | Duration of cycle | Temperature |
|---|---|---|
| 1[a] | 15 minutes | 95 °C |
| 40 | 15-30 seconds[b]<br>60 seconds[c] | 95 °C<br>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**

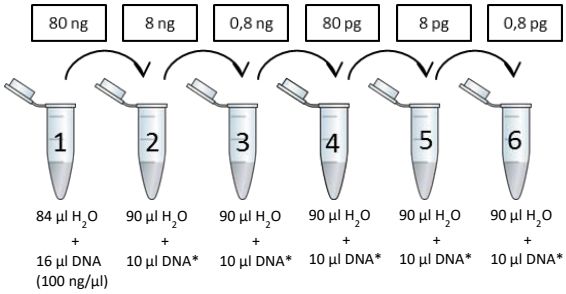
Issued 05/2025

# Real-Time PCR Mix Evaluation Guideline

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 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

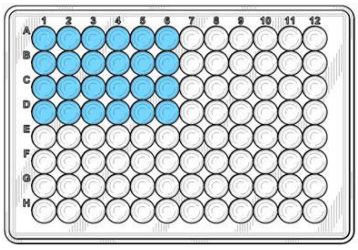
Prepare a reaction mix according to the table below. After adding all the components, vortex for 2 seconds and spin down.

| | 4 dilutions | 6 dilutions |
|---|---|---|
| 2x Master Mix | 225 µl | 325 µl |
| Primer F (10µm)* | 11.25 µl | 16.25 µl |
| Primer R (10µm)* | 11.25 µl | 16.25 µl |
| $H_2O$ | 112.5 µl | 162.5 µl |

* Optimization of primer concentrations is highly recommended.

## 3) Distribute the reaction mix

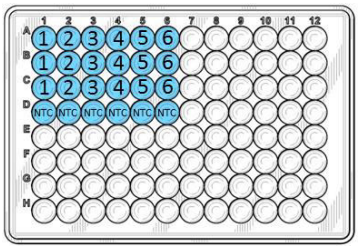
Distribute 20 µl of the reaction mix into the bottom of the blue wells according to the figure below.



## 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. Run the melt curve according to instrument default settings.

| Phase | Time | Temperature | Cycles |
|---|---|---|---|
| Initial heating | 15 min. | 95 °C | 1 |
| Denaturation | 30 sec. | 95 °C | 40 |
| Annealing | 30 sec. | 60 °C* | |
| Elongation | 30 sec. | 72 °C | |
| Melt Curve | Apply instrument default settings | | |

* 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 □ |
| Melt curve | One peak | Yes □ | No □ |
| **Accept mix?** | **"Yes" to all** | **Yes** □ | **No** □ |

Notes SD;_____

_____

Notes NTC's;_____

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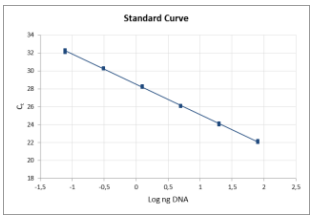


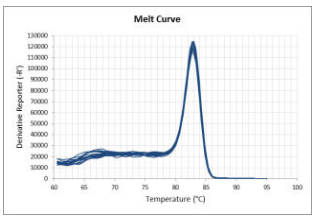

# Notes

The results should be within the listed specifications in order to accept the mix. Note that the parameters: melt point and $C_q$-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.

**Melt curve:**

When looking at the melt curve there should be one distinct peak at the intended melt temperature of the product. It is important to note that the melt temperature is pH- and buffer dependent. Therefore, it cannot be expected that PCR products from two different mixes have the exact same melt temperature. A difference of a few degrees in melt temperature is not important for how well the mix performs.

The NTC's should not have a peak at the same melt temperature as the intended product. If this is the case, then there is contamination, which can give a fake contribution to the results.

Other peaks than the peaks of the intended product can appear in the melt curve plot and is an indication of unspecific amplification. As long as the peaks are really small or as long as the NTC wells appear on the amplification plot ≥ 40 cycles or ≥ 10 cycles after the lowest DNA concentration, then the unspecific amplification can be neglected.

**$C_q$-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 SYBR level, ROX level and target length (when running SYBR experiments) and can therefore vary from master mix to master mix. Small differences in $C_q$-values are expected when comparing two mixes.

**SYBR Level**

It is less relevant to look at SYBR levels when evaluating a master mix. This can be manipulated by simply adding more SYBR to the buffer and has not necessarily anything to do with how well the mix performs. Too much SYBR can inhibit PCR amplification, but if this is an issue, then it will be detected when evaluating the PCR efficiency.

Furthermore, the SYBR level can be influenced by ROX if the amplification plot is plotted with Rn or ΔRn on the y-axis. Only in the multicomponent view of an amplification does the ROX not influence the $C_q$-value.

**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. Run the melt curve according to instrument default settings.

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. Run the melt curve according to instrument default settings.

**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.