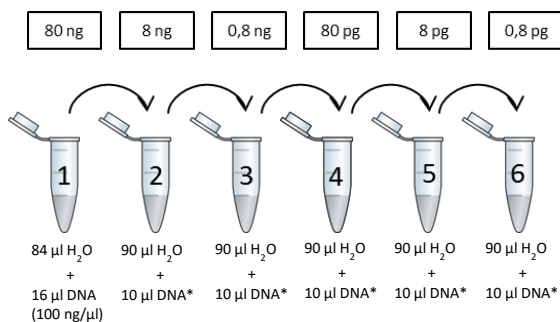


## Real-Time Mix Evaluation Guideline - for Probe

When evaluating a new real-time 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 master mix.

### 1) Prepare a DNA dilution series

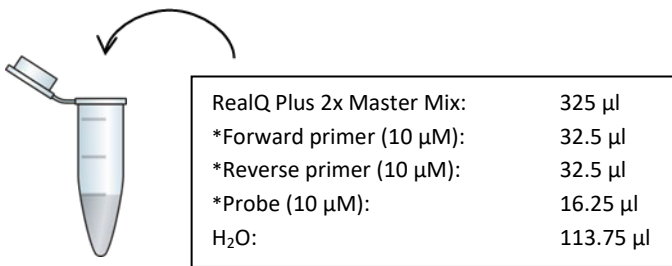
Prepare a DNA dilution series according to the figure below. Mix well by flicking the tubes (do not vortex!) and spin down. For genomic DNA include tubes 1-4. For other DNA include tubes 1-6. Final DNA amount per well is noted in the boxes above the tubes.



\* Employ previous dilution.

### 2) Prepare a reaction mix

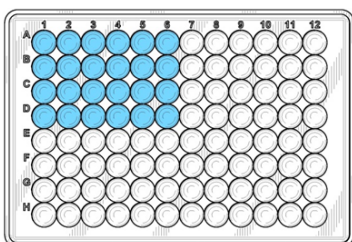
Prepare a reaction mix according to the box below. After adding all the components, vortex for 2 seconds and spin down.



\* Optimization of primers and probe concentrations is highly recommended.

### 3) Distribute the reaction mix

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

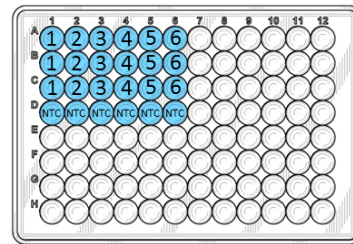


### 4) Distribute the DNA

Distribute 5 µl of DNA from the dilution series, into the wells. Make sure that the tip is dipped 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.

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

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



### 5) Run the plate

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

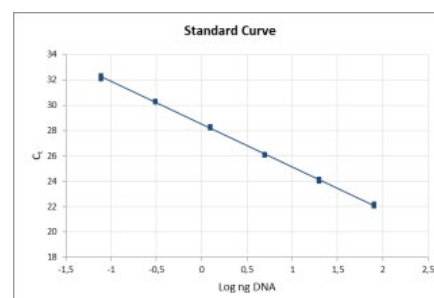
Phase	Time	Temperature	Cycles
Initial heating	15 min.	95 °C	1
Denaturation	30 sec.	95 °C	
Annealing	30 sec.	60 °C*	40
Elongation	30 sec.	72 °C	

\* Apply the annealing temperature for the specific primer set.

### 6) Evaluate the results

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

Parameter	Specifications	Achieved?	
PCR efficiency	90-110 %	Yes <input type="checkbox"/>	No <input type="checkbox"/>
R <sup>2</sup>	≥ 0.98	Yes <input type="checkbox"/>	No <input type="checkbox"/>
Standard deviation	≤ 0.2	Yes <input type="checkbox"/>	No <input type="checkbox"/>
NTC's	No amplification	Yes <input type="checkbox"/>	No <input type="checkbox"/>
Accept mix?	"Yes" to all	Yes <input type="checkbox"/>	No <input type="checkbox"/>



## 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 look for is: 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  $\geq 0.98$ .

The standard deviation between replicates indicates the accuracy of pipetting. The standard deviation should be  $\leq 0.2$ .

### $C_q$ -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 can cause the efficiency to be over 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. A small difference in  $C_q$ -value is 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. Distribute the dilutions according to step 3 and 4 but in wells A7 to D12.

### 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.