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Real-Time Mastermix Evaluation Guidelines

Guidelines for the Genaxxon Green- and Probe 2-time Mastermixes

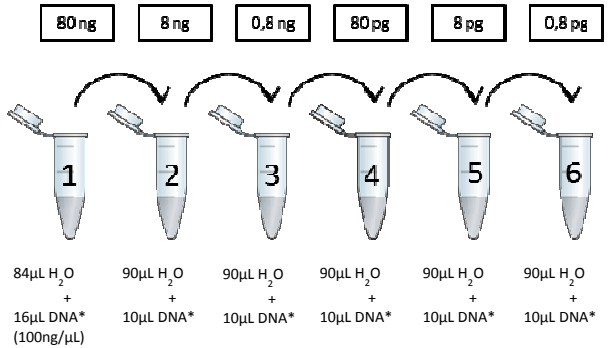
M3011, M3023, M3052
M3010, M3031, M3045

Version: 200116

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

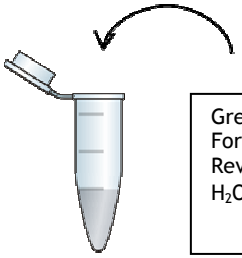
Prepare a DNA dilution series according to the figure below. Mix well by flicking the tubes (do *not vortex!*) and spin down. For genomic DNA include tubes 1-4. For other DNA include tubes 1-6. Final DNA amount per well is noted in the boxes above the tubes.



* 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.



Green-/Probe 2x Mastermix:	300µL
Forward primer (10µM):	15µL
Reverse primer (10µM):	15µL
H_2O:	170µL

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.

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.

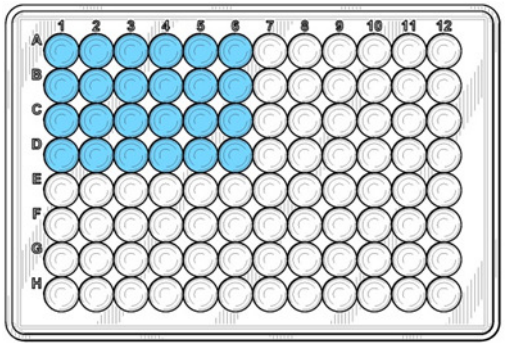
SYBR Level

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 the 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.

3) Distribute the reaction mix

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

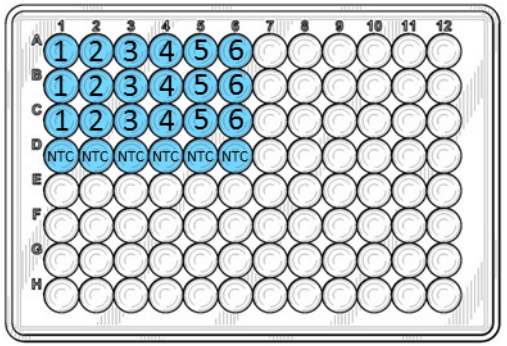


4) Distribute the DNA

Distribute 5µL 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. Run the melt curve according to instrument default settings. Melt curve is only applicable for Green 2x Mastermix evaluation.

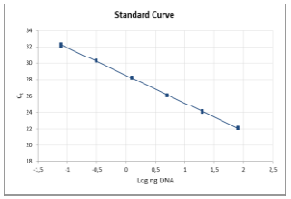
Phase	Time	Temperature	Cycles
Initial heating	15 min.	95°C	1
Denaturation	30 sec.	95°C	
Annealing	30 sec.	60°C*	40
Elongation	30 sec.	72°C	
Melt Curve	Apply instrument default settings		

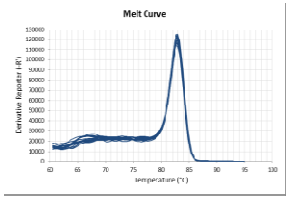
* Apply the annealing temperature for the specific primer set.

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 □	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

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 ≥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 a contamination, which can give a fake contribution to the results.

Other peaks than the peaks from 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.