AMPLIQON
PCR ENZYMES & REAGENTS

Study of Fast Cycling Real-Time PCR Conditions using RealQ Plus Master Mixes

Real-time PCR run times using RealQ Plus master mixes are reduced by 30 – 50 %, just by changing the settings of real-time PCR instruments. Run times down to 41 minutes is obtained.

ADVANTAGES OF REALQ PLUS FAST PROTOCOLS:

RealQ Plus 2x master mixes provide fast results

- High efficiency real-time PCR results
- Reliable quantification results
- Delivers results in < 50 minutes on standard real-time PCR cyclers
- Tested on StepOnePlus™, Mx3500P and LightCycler® 96
- Also compatible with fast real-time PCR Cycler such as Mic qPCR Cycler

Fast real time PCR is widely requested by DNA laboratories in order to save time, to increase throughput and also to have an increased instrument availability. Therefore, it is of interest to test the performance of RealQ Plus 2x master mixes using fast cycling conditions.

This study shows that RealQ Plus 2x Master Mix for Probe as well as RealQ Plus 2x Master Mix Green used with fast cycling real-time 2-step protocols on standard real-time PCR cyclers can reduce runtime significantly.

Fast 2-step protocols were obtained by changing the settings on the three applied standard real-time PCR cyclers; LightCycler® 96, Mx3500P and StepOnePlus™. Each instrument exhibits different requirement for ROX reference dye levels.

Two different 2-step protocols were tested on each of the three real-time PCR instruments using two targets of different length; PAH12 (203 bp) and Pthr (75 bp). All six fast protocols tested show high precision and efficiencies. The obtained R^2 and C_q values for the tested fast protocols are within the criteria normally set for the RealQ Plus master mix standard protocol. All protocols and results are shown in table 1 – 3 and figure 1 – 6.

Conclusion and remarks:

Depending on the applied real-time PCR instrument and DNA target used, total run times of as fast as ~41 – 61 minutes were obtained. This is a significantly reduction compared to the standard protocol with a total run time of approximately 83 minutes.

The present study clearly demonstrates that it is possible to shorten real-time PCR run time of RealQ Plus master mixes, severely, just by changing the standard real-time PCR protocols to 2-step protocols with short denaturation and elongation steps.

When performing fast real-time PCR, it is of high importance to consider primer design and target size. For efficient amplification during fast cycling conditions target size between 70 bp and 200 bp is recommended. The shorter target length the faster the total run time. For other targets and other primer sets, the annealing step has to be determined experimentally.

Fast real-time PCR results using RealQ Plus Master Mixes on LightCycler® 96

RealQ Plus 2x Master Mix Green, without ROX or RealQ Plus 2x Master Mix for Probe, without ROX was tested on LighCycler96 (Roche) using fast cycling real-time 2-step protocols; Fast PAH12 Green LC and Fast Pthr Probe LC, respectively.

qPCR Setup: RealQ Plus 2x Master Mix Green (without ROX) or RealQ Plus 2x Master Mix for Probe (without ROX), primers targeting either PAH12 (203 bp) or Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used. All DNA concentrations were tested in quadruple replicates. See fig. 1 and 2. The PCR reaction mix was run on LightCycler® 96 (No requirements for ROX). Instrument settings, run times and quality criteria for the applied fast protocols; Fast PAH12 Green LC and Fast Pthr Probe LC, were compared to the standard protocol; Standard Pthr LC. See table 1.

Results: The standard Fast Pthr Probe LC protocol has a run time of 80.8 minutes. Fast PAH12 Green LC and Fast Pthr Probe LC protocols, resulted in run times of 47.5 and 40.8 minutes, respectively. All protocol times are inclusive ramping time and a15 minutes hot start. The determined criteria, as shown in table 1, for Fast PAH12 Green LC and Fast Pthr Probe LC protocols were satisfying. Furthermore, the melt curve analysis using RealQ Plus Green 2x Master Mix with the Fast PAH12 Green LC protocol confirmed high specificity.

Table 1. Fast cycler real-time protocols tested on LightCycler® 96

	Standard Pthr LC	Fast PAH12 Green LC	Fast Pthr Probe LC
Primers	Probe: 0.1–1 µM Pthr Green: 0.05–0.5 µM Pthr	0.39 µM PAH12	0.5 µM Pthr
Initial heat. (95°C)	15 min	15 min	15 min
Denaturation (95°C)	30 sec	5 sec	5 sec
Annealing (60°C)	30 sec	20 sec	10 sec
Elongation (72°C)	30 sec	-	-
Cycles	40	40	40
Efficiency	1.9–2.1	1.96	1.96
R^2	≥ 0.98	1.00	1.00
C_q-value 40 ng	Probe: Cq ≤ 27 Green: Cq ≤ 25	- 23.33	25.3 -
C_q SD	≤ 0.2	0.05-0.07	0.01-0.06
Run time incl. ramping*	**80.8 min**	**47.5 min**	**40.8 min**

*Melt curve analysis is not included. The run time on LightCycler96® instrument has an accuracy of ± 2 minutes.

Fast PAH12 Green LC:

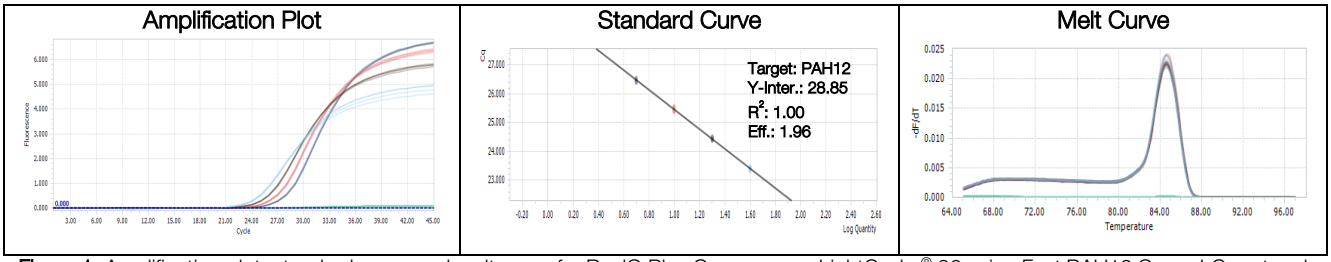
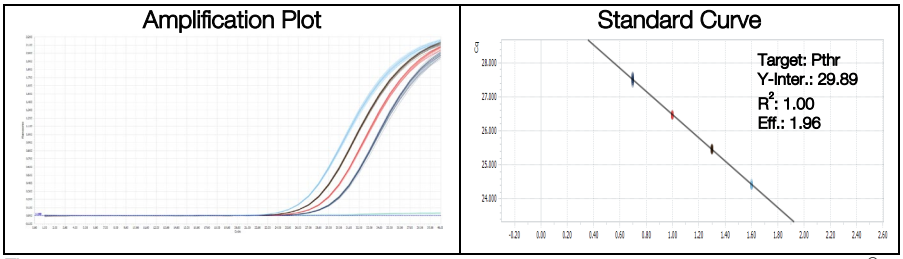


Figure 1. Amplification plot, standard curve and melt curve for RealQ Plus Green run on LightCycler® 96 using Fast PAH12 Green LC protocol. Primers targeting PAH12 (203 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used. All DNA concentrations were tested in quadruple replicates.

Fast Pthr Probe LC:



Acceptance criteria:
- R^2 ≥0.98
- Efficiency 1.9 – 2.1
- C_q ≤ 27 (RealQ Plus 2x MM for Probe)*
  or
- C_q ≤ 25 (RealQ Plus 2x MM Green)*

*C_q values for 40 ng gDNA

Figure 2. Amplification plot and standard curve for RealQ Plus for Probe run on LightCycler® 96 using Fast Pthr Probe LC protocol. Primers targeting Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used.

Fast real-time PCR results using RealQ Plus Master Mixes on Mx3005P

RealQ Plus 2x Master Mix Green, Low ROX or RealQ Plus 2x Master Mix for Probe, Low ROX was tested on Mx3005P (Agilent Technologies) using fast cycling real-time 2-step protocols; Fast Pthr Green Mx and Fast Pthr Probe Mx, respectively.

qPCR Setup: RealQ Plus 2x Master Mix Green (Low ROX) or RealQ Plus 2x Master Mix for Probe (Low ROX), primers targeting Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used. All DNA concentrations were tested in quadruple replicates. See fig. 3 and 4. The PCR reaction mix was run on MX3005P (Requiring Low ROX). Instrument settings, run times and quality criteria for the applied fast protocols; Fast Pthr Green Mx and Fast Pthr Probe Mx, were compared to the standard protocol; Standard Pthr Mx. See table 2.

Results: The standard Fast Pthr Probe Mx protocol has a run time of 83.4 minutes. Fast Pthr Green Mx and Fast Pthr Probe Mx protocols, resulted in run times of 50.1 and 46.8 minutes, respectively. All protocol times are inclusive ramping time and a15 minutes hot start. The determined criteria, as shown in table 2, for Fast Pthr Green Mx and Fast Pthr Probe Mx protocols were acceptable. Furthermore, the melt curve analysis using RealQ Plus Green 2x Master Mix with Fast Pthr Green Mx protocol, confirmed high specificity.

Table 2. Fast cycler real-time protocols tested on Mx3005P

	Standard Pthr Mx	Fast Pthr Green Mx	Fast Pthr Probe Mx
Primers	Probe: 0.1–1 µM Pthr Green: 0.05–0.5 µM Pthr	0.3 µM Pthr	0.5 µM Pthr
Initial heat. (95°C)	15 min	15 min	15 min
Denaturation (95°C)	30 sec	5 sec	5 sec
Annealing (60°C)	30 sec	20 sec	15 sec
Elongation (72°C)	30 sec	-	-
Cycles	40	40	40
Efficiency	90 - 110 %	96.5 %	91.1 %
R^2	≥ 0.98	0.995	0.991
C_q-value 40 ng	Probe: Cq ≤ 27 Green: Cq ≤ 25	- 23.92	25.18 -
C_q SD	≤ 0.2	0.03-0.14	0.07-0.14
Run time incl. ramping*	**83.4 min**	**50.1 min**	**46.8 min**

*Melt curve analysis is not included. Run time on Mx3005P instrument has an accuracy of ± 2 minutes.

Fast Pthr Green Mx:

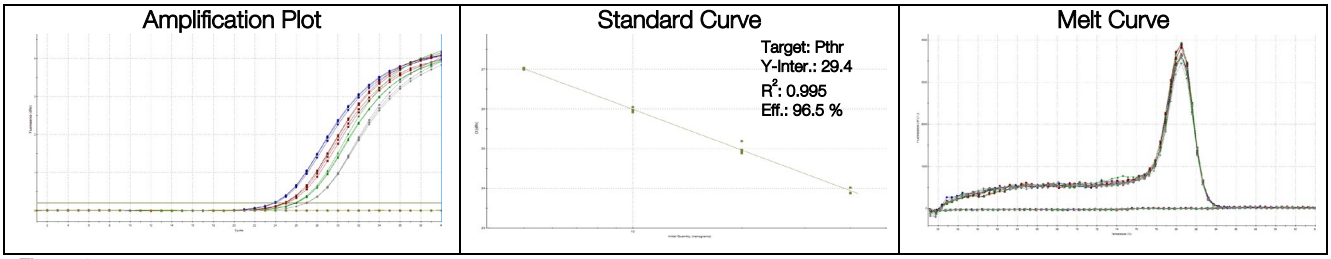
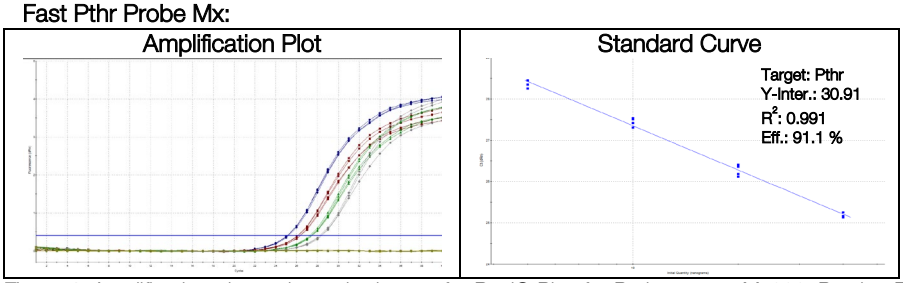


Figure 3. Amplification plot, standard curve and melt curve for RealQ Plus Green run on Mx3005P using Fast Pthr Green Mx protocol. Primers targeting Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used. All DNA concentrations were tested in quadruple replicates.

Fast Pthr Probe Mx:



Acceptance criteria:

- R^2 ≥0.98
- Efficiency 90 - 110 %
- C_q ≤ 27 (RealQ Plus 2x MM for Probe)*
  or
- C_q ≤ 25 (RealQ Plus 2x MM Green)*

*C_q values for 40 ng gDNA

Figure 4. Amplification plot and standard curve for RealQ Plus for Probe run on Mx3005P using Fast Pthr Probe Mx protocol. Primers targeting Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used.

Fast real-time PCR results using RealQ Plus Master Mixes on StepOnePlus™

RealQ Plus 2x Master Mix Green (High ROX) or RealQ Plus 2x Master Mix for Probe (High ROX) were tested on StepOnePlus™ (Life Technologies) using fast cycling real-time 2-step protocols; Fast PAH12 Green S1 and Fast Pthr Probe S1, respectively.

qPCR Setup: RealQ Plus 2x Master Mix Green (High ROX) or RealQ Plus 2x Master Mix for Probe (High ROX), primers targeting either PAH12 (203 bp) or Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used. All DNA concentrations were tested in quadruple replicates. See fig. 5 and 6. The PCR reaction mix was run on StepOnePlus™ (Requiring High ROX). Instrument settings, run times and quality criteria for the applied fast protocols; Fast PAH12 Green S1 and Fast Pthr Probe S1, were compared to the standard protocol; Standard Pthr S1. See table 3.

Results: The standard Fast Pthr Probe S1 protocol has a run time of 85.9 minutes. Fast PAH12 Green S1 and Fast Pthr Probe S1 protocols, resulted in run times of 61.3 and 45.9 minutes, respectively. All protocol run times are inclusive ramping time and a 15 minutes hot start. The determined criteria, as shown in table 3, for Fast PAH12 Green S1 and Fast Pthr Probe S1 protocols were satisfying. Furthermore, the melt curve analysis, when using RealQ Plus Green 2x Master Mix with the Fast PAH12 Green S1 protocol confirmed high specificity.

Table 3. Fast cycler real-time protocols tested on StepOnePlus™

	Standard Pthr S1	Fast PAH12 Green S1	Fast Pthr Probe S1
Primers	Probe: 0.1–1 µM Pthr Green: 0.05–0.5 µM Pthr	0.2 µM PAH12	0.5 µM Pthr
Initial heat. (95°C)	15 min	15 min	15 min
Denaturation (95°C)	15 sec	5 sec	5 sec
Annealing (60°C)*	60 sec	33 sec	10 sec
Cycles	40	40	40
Efficiency	90 - 110 %	91.1 %	93.6 %
R^2	≥ 0.98	0.999	0.995
C_q-value 40 ng	Cq ≤ 27 (Probe) Cq ≤ 25 (Green)	- 23.1	26.8 -
C_q SD	≤ 0.2	0.011-0.057	0.075-0.112
Run time incl. ramping**	**85.9 min**	**61.3 min**	**45.9 min**

*It is not possible to test protocols with shorter annealing time than 10 seconds since StepOnePlus™ is limited at this step for data acquisition.
**Melt curve analysis is not included. Run time on StepOnePlus™ instrument has an accuracy of ± 10 minutes.

Fast PAH12 Green S1:

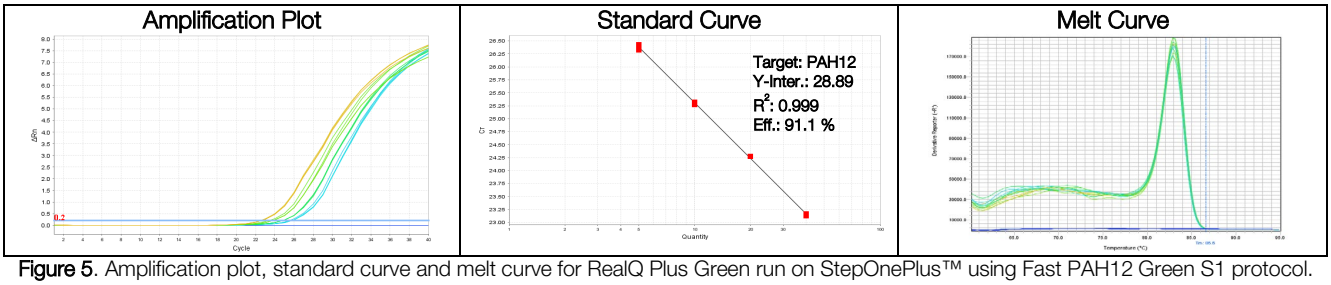
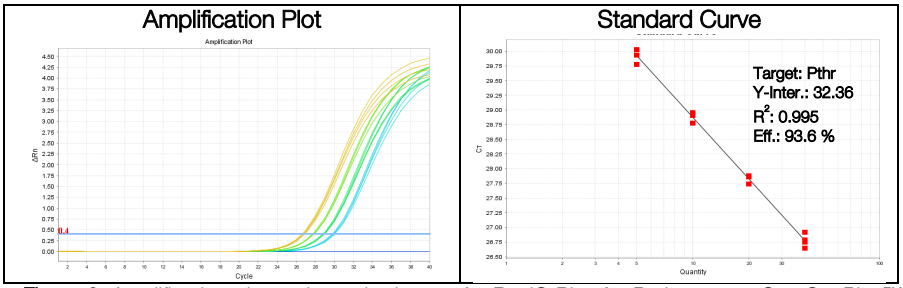


Figure 5. Amplification plot, standard curve and melt curve for RealQ Plus Green run on StepOnePlus™ using Fast PAH12 Green S1 protocol. Primers targeting PAH12 (203 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used. All DNA concentrations were tested in quadruple replicates.

Fast Pthr Probe S1:



Acceptance criteria:

- R^2 ≥0.98
- Efficiency 90 - 110 %
- C_q ≤ 27 (RealQ Plus 2x MM for Probe)*
  or
- C_q ≤ 25 (RealQ Plus 2x MM Green)*

*C_q values for 40 ng gDNA

Figure 6. Amplification plot and standard curve for RealQ Plus for Probe run on StepOnePlus™ using Fast Pthr Probe S1 protocol. Primers targeting Pthr (75 bp) and 4 concentrations of gDNA (40 ng, 20 ng, 10 ng and 5 ng) were used.