Real-Time Multiplex PCR of five Different DNA Targets Using the LightCycler® 480 System

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Introduction

One of the most interesting aspects of real-time PCR based on detection of fluorophoric-labeled oligonucleotides, such as Hydrolysis probes, and Molecular Beacons, is the possibility of being able to detect conveniently multiple targets in the same PCR reaction (multiplex PCR) [1]. Ideally, a real-time multiplex PCR should be able to detect, differentiate, and provide a quantitative result for many different targets without a single target influencing the detection of one of the others (cross-talk) and without loss of sensitivity. It is evident that due to the limited number of fluorophoric labels available [2] and the significant overlap in their emission spectra, quantification of multiplex reaction products is difficult and often not possible for more than two targets [3].

Recently, Roche Applied Science launched a real-time PCR platform, the LightCycler® 480 System, which should be well-suited for multiplex real-time PCR analysis. It makes use of a broad-spectrum xenon lamp and five band pass filters for excitation of fluorophores at five different fixed wavelengths combined with six band pass filters for detection of fluorophore emission at six different fixed wavelengths. Both excitation and emission wavelengths are chosen at the peak fluorescence of commonly available fluorophores and are separated by at least 30 nm. This should allow for minimal cross-talk between the different channels and provide a platform for quantitative detection of multiple targets within the same sample.

In the study presented here, the performance of the LightCycler® 480 Instrument and accompanying software was analyzed for the multiplex detection of five different (plasmid) DNA species.

Materials and Methods

Five different plasmid DNAs (A-E) containing a specific amplicon sequence were constructed by standard molecular cloning techniques [4]. Hydrolysis probe-based real-time PCRs for each DNA species were developed with specific fluorescent-labeled probes (Table 1).

Serial dilutions of plasmid DNA were made in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) supplemented with 20 ng/µl of calf thymus DNA. Typical reactions contained 10 µl of 2x LightCycler® 480 Probes Master, 900 nM of primer (each), 200 nM of probe (each), and 5 µl of a plasmid DNA dilution in a total volume of 20 µl. Cycling conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles each consisting of 15 seconds at 95°C and 1 minute at 60°C. Data were analyzed with the LightCycler® 480 Software using the Second Derivative Maximum Method. Color compensation objects were created as described in the LightCycler® 480 Operator's Manual.

Results and Discussion

The dynamic range of the five different PCRs in single-target detection format (*i.e.*, one primer pair and one probe) was analyzed by a linear range of tenfold dilutions ranging from 10^6 to 10^2 copies of target plasmid in PCR. To determine the amount of cross-talk between the five different wavelength channels we determined the contribution of all fluorophores in their non-corresponding channels. After the application of color compensation as described in the LightCycler® 480 Operator's Manual, we determined that the amount of cross-talk was less than 5% in all cases (data not shown). This demonstrates that in a multiplex experiment more than 95% of the total fluorescence will be derived from the corresponding fluorophore. All five single PCRs displayed similar characteristics and showed PCR efficiencies close to 2.0. Standard deviations were very

Table 1: Detection formats.

DNA	Amplicon length (kb)	Fluorophores	λ Excitation (nm)	λ Emission (nm)
A	95	Cyan 500/DB	450	500
B	105	6FAM/BBQ	483	533
C	146	VIC/NFQ	523	568
D	152	LightCycler® Red 610/BBQ	558	610
E	143	LightCycler® Red 670/BBQ	615	670

DB: Dabcyl, BBQ: BlackBerry Quencher, NFQ: non-fluorescent quencher.

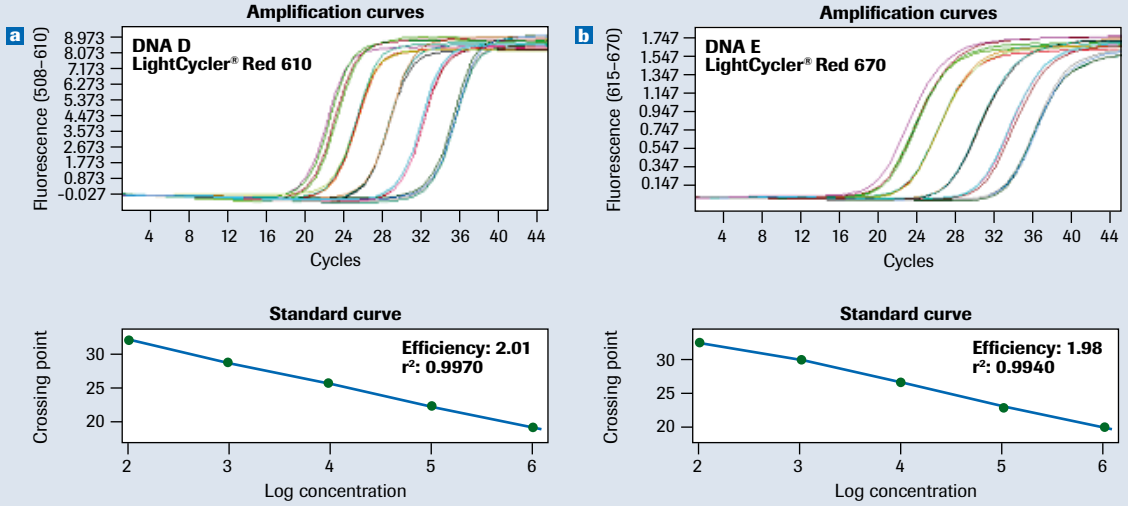

Figure 1: Examples of dynamic ranges. (a) Serial tenfold dilutions of plasmid DNAs D (LightCycler® Red 610) or **(b)** DNA E (LightCycler® Red 670) were analyzed by single-target PCR. PCR efficiencies and r^2 values are given.

small (max. 0.56; n=4) and linearity was observed in the concentration range tested (representative examples are shown in Figure 1). On the basis of these experiments standard curves were generated by linear regression analysis.

Subsequently, plasmid DNAs A–E were mixed in equimolar amounts (10^4 copies/PCR). Multiplex PCR was performed in replicates of 12 and resulting mean crossing-point (Cp) values were plotted against standard curves obtained from single-target PCRs. Table 2 shows the measured Cp-values in comparison to the expected Cp-values. Measured and expected Cp-values were in accordance with each other and the largest difference was 0.6. These data show that using a pentaplex PCR and the LightCycler® 480 System, reliable detection of five different DNA targets within the same sample is possible.

More interesting is the investigation of the effect of differential DNA concentrations on the outcome of multiplex real-time PCR for each plasmid DNA. To study this, each of target DNA plasmids C, D, and E were mixed in equimolar amounts of 10^5 (sample series 1), 10^4 (sample series 2), 10^3 (sample series 3), and 10^2 (sample series 4) copies/PCR. Subsequently, target DNA A was spiked in these mixtures at final concentrations of 10^5, 10^4, 10^3, and 10^2 copies/PCR. For instance, sample 1.1 contained 10^5 copies/PCR for DNAs A, C, D and E, whereas sample 3.4 contained 10^3 copies/PCR of DNAs C, D, E and 10^2 copies/PCR of DNA A. All samples were tested in the multiplex PCR and the results are summarized in Figure 2. Cp-values for a given concentration of DNA target A did not differ significantly in the background of various concentrations of targets C, D, and E. For example, in sample 1.4, there was a low amount (10^2 copies/PCR) of DNA A present in the background of high (10^5 copies/PCR) concentrations of targets C, D and E. In this sample DNA A was detected at a Cp of ~31. In contrast, sample 4.4 contained low amounts (10^2 copies/PCR each) of all target DNAs. DNA A was detected in this sample at a Cp of ~30. For DNA targets C, D, and E the difference in Cp-value at a given concentration did not differ significantly between each one. In addition, the Cp-values for a given concentration of targets C, D, and E were similar in the background of various concentrations of DNA target A. These data demonstrate that over a range of at least a 1000-fold difference in target concentration, reliable quantification is still possible for all targets, without loss of sensitivity.

Table 2: Measured versus expected Cp-values for DNA A-E.

	Measured[a]	Expected[b]	ΔCp
DNA A	25.7 ± 0.11	25.9	0.2
DNA B	27.0 ± 0.21	27.3	0.3
DNA C	25.9 ± 0.17	26.5	0.6
DNA D	25.3 ± 0.16	25.7	0.4
DNA E	26.0 ± 0.18	26.5	0.5

[a]Cp-values are mean of 12 replicates.
[b]Expected Cp-values were derived from standard curves.

Apart from the technical specifications of the PCR apparatus, another major difference between the LightCycler® 480 Instrument and the majority of other real-time PCR platforms lies within the software algorithms used to convert raw fluorescence data into comprehensive results. Whereas other real-time platforms use a cycle threshold that can be arbitrarily set to a certain value by the user, the LightCycler® 480 Software calculates the second

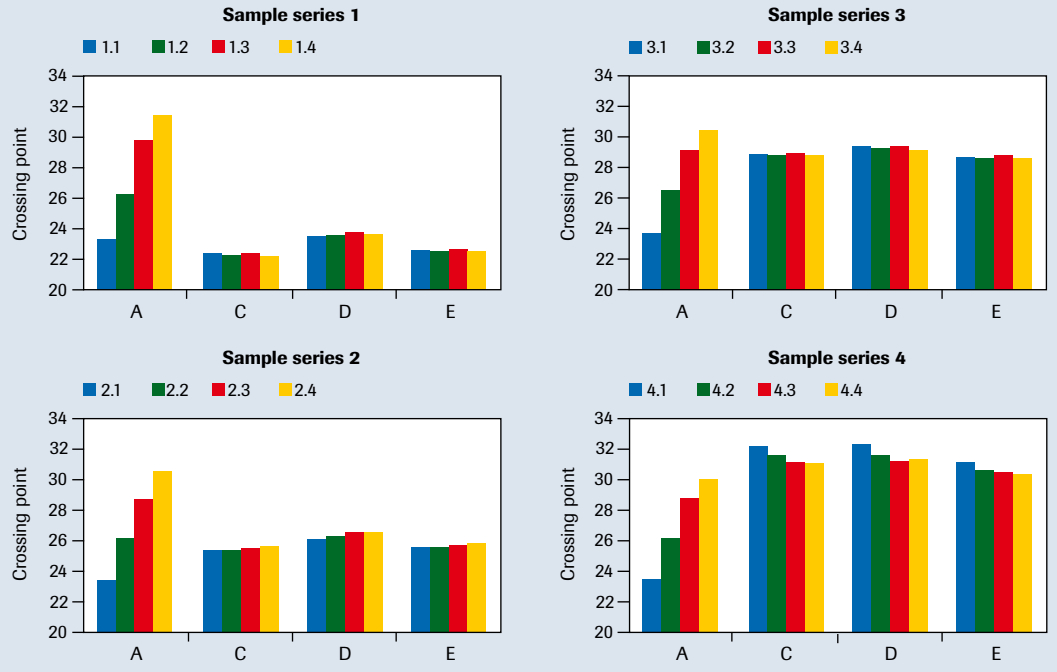

Figure 2: Effect of different target DNA concentrations in a sample on the Cp-values of the multiplex PCR. Composition of different samples is indicated. Cp-values for each target DNA (A, C, D, and E) in a sample are displayed in the figures. All Cp-values are means of duplicates.

derivatives of entire amplification curves and determines where this value is at its maximum. This value (crossing point, Cp) represents the cycle at which the increase of fluorescence is highest and where the logarithmic phase of a PCR begins. By using the second-derivative algorithm, data obtained are more reliable and reproducible, even if fluorescence is relatively low. The use of this second-derivative maximum algorithm might have contributed greatly to the excellent qualitative and quantitative results in the multiplex PCR described here.

Conclusions

We made use of the excellent properties of the recently introduced LightCycler® 480 System for the reproducible detection of five different DNA targets within one reaction. It was shown that when these DNA targets were mixed, each individual DNA could still be quantitatively detected in the background of the other DNAs. In addition, a 1,000-fold difference in DNA concentration between DNAs did not significantly influence the detection of the other DNA targets. These results show that the LightCycler® 480 Instrument is a convenient and reliable platform for (quantitative) multiplex PCR detection. ■

References

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