



Multiplex qPCR LightCycler Analysis

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1. Introduction

In multiplex PCR assays, more than one primer set is included in the reaction pool allowing two or more different DNA targets to be amplified in a single reaction tube.

Due to the six detection channels of the LightCycler 2.0 Instrument, up to 4 differentially labeled HybProbe Probes can be detected in one capillary. Therefore, the amplification of up to 4 different DNA templates can be measured in one capillary.

Multiple color detections in Real Time PCR is a field of growing interest in molecular biological laboratories as this technique provides the opportunity to generate several data within one approach. Multiplex Assays can be used to measure target gene and reference gene in one reaction which minimizes assay variation. Furthermore, the amount of biological material needed is reduced and the cost factor compared to separated assays is decreased.

However, designing multiplex reactions may require some optimization strategies to cope with difficulties such as cross complementarities, competition of parameters, and by products which can impair results.

The newly designed LightCycler Multiplex DNA Master HybProbe from Roche Applied Science allows easy establishment of multiplex assays. As the new kit uses a very efficient polymerase and evaluated buffer conditions, high sensitivity and the detection of small amounts of template – in particular under multiplex conditions – can be achieved.

2. Materials and Methods

Quantification:

To validate the new LightCycler Multiplex DNA Master HybProbe we have performed amplification assays of cDNA from four housekeeping genes (β 2-Microglobulin (β 2M), Porphobilinogen deaminase (PBGD), Hypoxanthine-phosphoribosyl-transferase (HPRT), and Glucose-6-phosphate dehydrogenase (G6PDH)) in two different cell lines (DAUDI and MCF-7).

Samples:

Total RNA was isolated from 10^6 cells with the MagNA Pure LC RNA Isolation Kit II. 200 ng of RNA were reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV). 1 μ l of cDNA equivalent to 10 ng of RNA were used for Multiplex PCR. As a reference, standard RNA from the LightCycler h-Housekeeping Gene Selection Set was diluted from 10^5 to 10^3 copies per μ l for each target. 4 mM $MgCl_2$ were used at final concentration.

Mutation Analysis:

For mutation detection, we performed analysis of four point mutations: one in Factor V, one in Prothrombin, and two mutations in HFE gene: HFE845, and HFE187.

Samples:

We amplified 2×10^5 copies of wildtype plasmid, mutation plasmid, and a mixture of both plasmids (heterozygote) and performed melting curve analysis in order to show appropriate detection of the genotype.

Furthermore, we performed analysis of the genotype of 200 ng (6×10^5 copies) of human DNA. As reference we used 2×10^5 copies of wildtype plasmid (Factor V) or heterozygote (Prothrombin, HFE845, and HFE187).

All measurements were performed with the LightCycler 2.0 Instrument and the LightCycler Multiplex DNA Master HybProbe.

Run protocol:

Program	Cycles	Target temperature	Hold (hh:mm:ss)	Slope	Acquisition mode
Denaturation	1	95°C	00:10:00	20°C/s	-
PCR	45	95°C	00:00:10	20°C/s	-
		55°C	00:00:15	20°C/s	Single
		72°C	00:00:15	20°C/s	-
Melting	1	95°C	00:00:00	20°C/s	-
		40°C	00:00:30	20°C/s	-
		95°C	00:00:00	0.1°C/s	cont.
Cooling	1	40°C	00:00:30	20°C/s	-

3. Results and Discussion (Quantification)

For each sample the amount of the four different housekeeping gene RNAs was analyzed in one single capillary by performing multiple color detection.

For each concentration, the four housekeeping genes were amplified in one reaction.

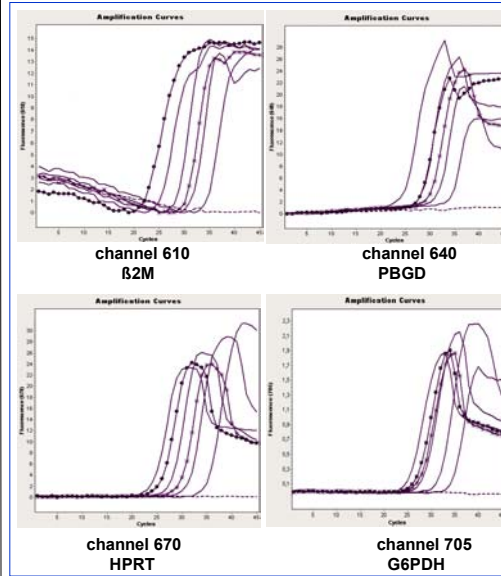


Fig. 1: Shown are the amplification curves of four housekeeping genes. Solid lines: standards (10^6 to 10^3 copies), solid line with stars: MCF-7 cell line, solid line with circles: DAUDI cell line

Figure 1 shows appropriate standard curves for each housekeeping gene.

Expression levels of the respective housekeeping genes differ between the two cell lines, except for G6PDH. Furthermore, the expression of the four different housekeeping genes varies in the same cell line (e.g., expression of β 2M in Daudi is higher than expression of the other three genes in the same cell line).

Additional, to show the dynamic range between different target concentrations in one reaction, we performed a triplex assay with β 2M, PBGD and G6PDH. For each reaction, all three targets were amplified in one capillary. β 2M and G6PDH were kept at 10^6 copies while PBGD was titrated from 10^6 to 10^2 copies.

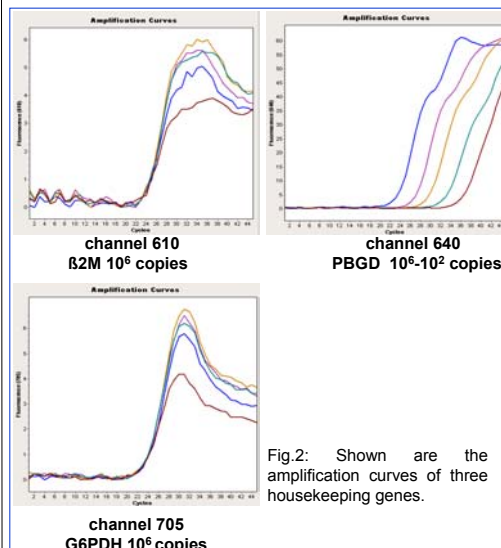


Fig. 2: Shown are the amplification curves of three housekeeping genes.

Figure 2 shows that 10^2 copies of PBGD can sensitively be measured in the same reaction with 10^6 copies of β 2M and 10^6 copies of G6PDH.

3. Results and Discussion (Genotyping)

To demonstrate mutation analysis in multiplex applications, we have performed melting curve analysis of positive controls concerning four point mutations (one mutation in Factor V, one in Prothrombin and two mutations in haemochromatosis, HFE gene: HFE187 and HFE845). In order to show appropriate genotyping, we employed wildtype, mutation, and heterozygote controls. From each sample, the genotypes of all four genes have been analysed in one capillary.

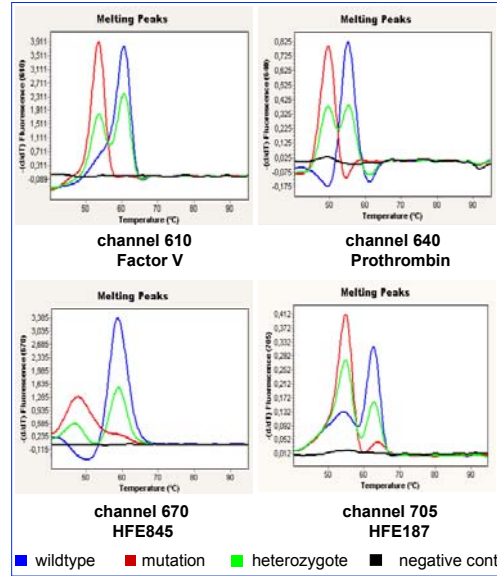


Fig. 3: Melting curve analysis of four point mutations, 10^5 copies each plasmid.

Furthermore, we performed genotype analysis in human DNA, concerning the four point mutations in Factor V, Prothrombin, and HFE gene (HFE845, and HFE187).

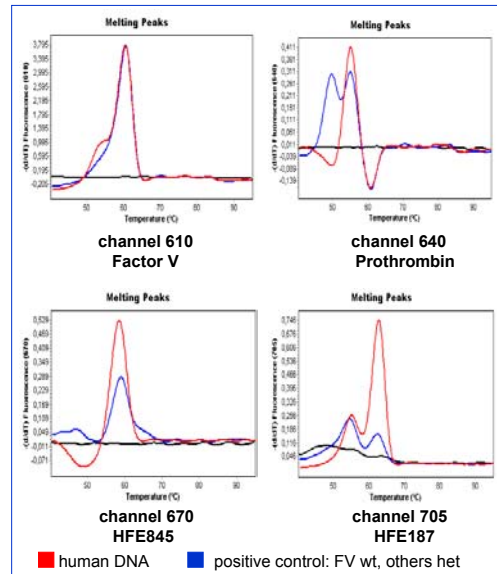


Fig. 4: Melting curve analysis of four point mutations. Control plasmid, 10^5 copies, human DNA: 200 ng (6×10^5 copies).

In Figure 4, human DNA can be identified as wildtype for Factor V, Prothrombin, and HFE845. In HFE187, we detect a heterozygote genotype. Normally, we would expect wildtype in all targets. As this DNA originates from more than one patient, and as several experiments showed the same results, it may be that one patient contains a heterozygote genotype. Another possibility is a suboptimal melting behaviour of the probes. This can be improved by testing alternative probe sequences.

4. Conclusion

These results show that using the LightCycler Multiplex DNA Master HybProbe allows the measurement of four parameters with varying expression profiles in one reaction. In first experiments, a dynamic range of five orders of magnitude was shown.

In Mutation Detection, up to four different DNA targets can be analysed in one approach. Genotypes in biological material can be detected in an appropriate way.