LightCycler® 480 System: High-throughput Gene Expression and Genotyping Analysis – A Performance Study

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Introduction

The LightCycler® 480 System is a novel platform for rapid gene expression and melting curve-based genotyping for medium- and high-throughput.

In order to assess the performance and ease-of-use of the complete LightCycler® 480 System (instrument, software, and reagents) in an academic laboratory setting we scheduled a high-throughput project including gene expression (39 targets in 88 samples = 3,400 expression values) as well as genotyping applications (3,808 genotypes; 14 SNPs in 288 samples). The project, involving five technicians and two graduate students, was accomplished within 15 working days.

Therefore, this performance study evaluated not only data quality and accurateness of the LightCycler® 480 System but also comfort and robustness of the hardware and software in respect to different users.

Material and Methods

Gene expression analysis

To validate results derived from in-house microarray analysis, relative expression of 33 target genes was determined. Six different reference genes were measured and the three best suited were used for normalization.

Total RNA was extracted from blood of donors with known genotype (n=77) and control samples (n=11) using the BioRobot 8000 Workstation (Qiagen GmbH) and the PAXgene 96 Blood RNA Kit (Qiagen GmbH).

RNA was reversely transcribed using Oligo (dT) primers and the Transcriptor First Strand cDNA Synthesis Kit according to the instructions. For each sample an RT^--control (master mix without RT enzyme) was performed.

Real-Time PCR was accomplished for 33 target genes and 6 reference genes. Primer sets for SYBR Green I assays were designed using the MassArray Assay Design software (Sequenom, Hamburg) or the Primer3 online tool. To optimize the PCR amplification efficiency the best performing conditions (*e.g.*, various annealing temperatures) were evaluated for each target and reference gene.

Amplification and melting curve analysis were performed with the LightCycler® 480 System (384-well format) in combination with the LightCycler® 480 SYBR Green I Master (10 µl PCR reaction: 2 µl template and 8 µl mastermix; primer concentration: 240 µM; PCR profile: Table 1).

PCR reactions were set up using the BioRobot 8000 Workstation. For each cDNA sample three replicates and one corresponding RT^--control were performed, as well

Table 1: General PCR protocol for gene expression analysis.

Program	Cycles	Analysis Mode	Temperature [°C]	Hold	Ramp [°C/s]	Acquisition
Denaturation	1	None	95	10:00	4.6	None
Amplification	45	Quantification	95 58/60 72	00:15 00:10 00:20	4.6 2.4 4.6	None None Single
Melting Curve	1	Melting Curve	95 60 95	00:10 00:20 NA	4.6 2.4 Auto	None None Continuous (15/s)
Cooling	1	None	40	00:10	4.6	None

as three non-template controls (NTC). To estimate PCR efficiency fivefold target-specific dilution series (triplicates) were determined (Figure 1a).

The general quality assessment of the PCR results was based on the amplification and melting curve profile of the samples in relation to the assay controls (RT^--control, NTC; Figure 1). Melting curve analysis was performed to confirm the specific amplicon and to identify putative unspecific PCR products (*e.g.*, primer dimers). The quality of the PCR amplification was checked, based on the assay's negative controls (RT^--control, NTC) as well as on the efficiency of the serial dilutions of the positive control cDNA. Sporadically observed positive amplification signals for negative controls were accepted if the ΔCp sample – negative control was >10.

Fifty 384-well plates were analyzed in a short time frame for this gene expression study in parallel with the described comprehensive genotyping project (Figure 2).

Genotyping analysis

Blood samples from 272 donors who had given informed consent were taken for analysis of target genes presumably involved in nicotine addiction. DNA was prepared using either a manual high-salt method (236 samples) or the MagNA Pure Compact Instrument with the Nucleic Acid Isolation Kit II (36 samples).

PCR primers and HybProbe probes for the analysis of 14 different SNPs were designed by TIB MOLBIOL. A BioRobot 8000 platform was programmed to perform PCR setups including the LightCycler® 480 Genotyping Master. Amplification was generally done in 20 µl reaction volume and 55 cycles, using 10 pmol of each primer and an annealing temperature of 52°C; 3 pmol of each HybProbe probe (anchor and sensor) were used for detection. In a second series of experiments, the established assays were also performed in a reaction volume of 10 µl.

Results and Discussion

Gene expression analysis

To prove the offered flexibility, accuracy and speed as well as the ease of use of the LightCycler® 480 System 39 genes (33 target genes, 6 reference genes) were analyzed in this study. None of the assays had been established in previous experiments. Thus, all assays were subjected to a straightforward and manageable optimization matrix, which allows assay optimization in a high-throughput environment (data not shown).

25 assays (64.0%) of the complete panel showed a good PCR result initially, represented by an optimal PCR effi-

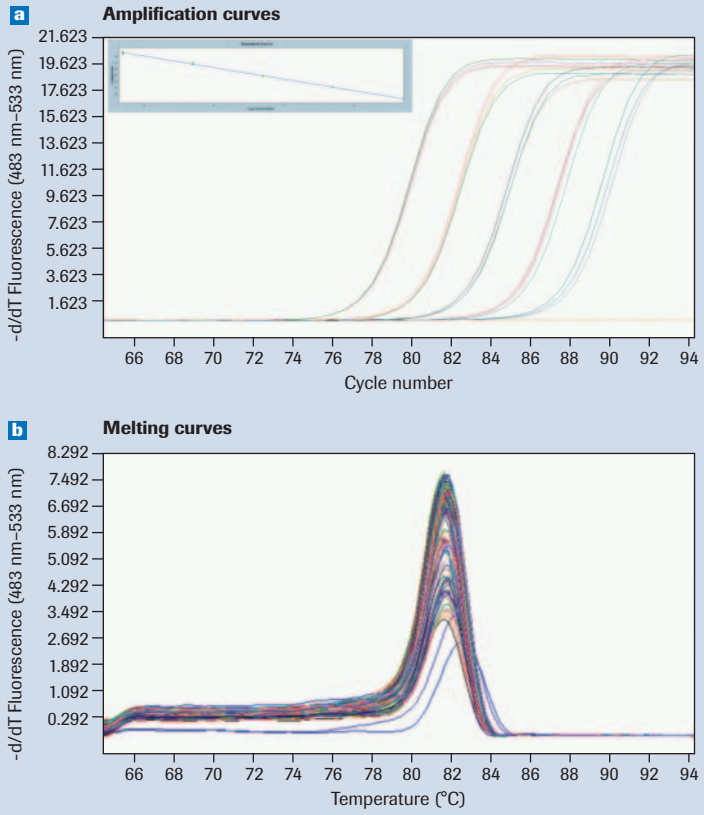

Figure 1: Gene expression analysis for representative target gene. **(a)** Amplification curves of serial dilutions (fivefold) of standard DNA was used for PCR efficiency calculation. Cp values were used to generate regression curve (small inset). Efficiency was 1.869 (r^2=0.998). **(b)** Melting curve analysis for all cDNA samples.

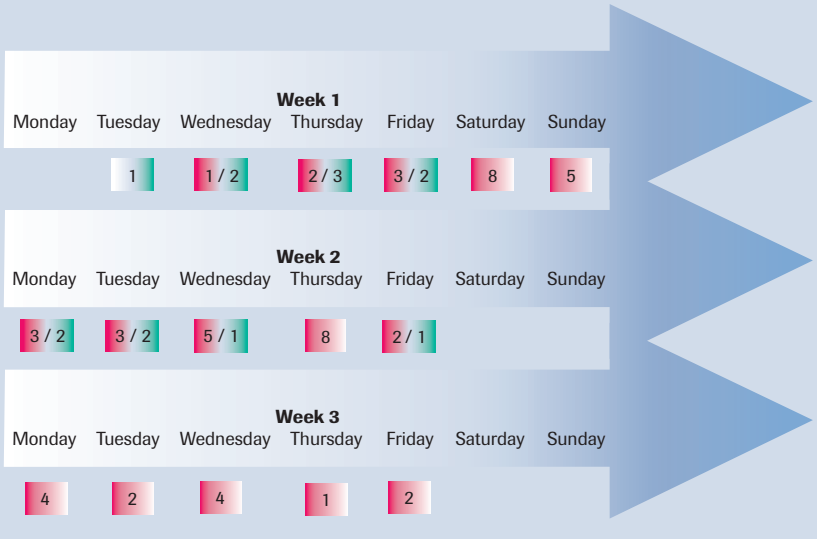

Figure 2: Schedule of the performance study. Numbers in red and green represent amount of multiwell plates performed with the LightCycler® 480 System for gene expression and genotyping analysis, respectively.

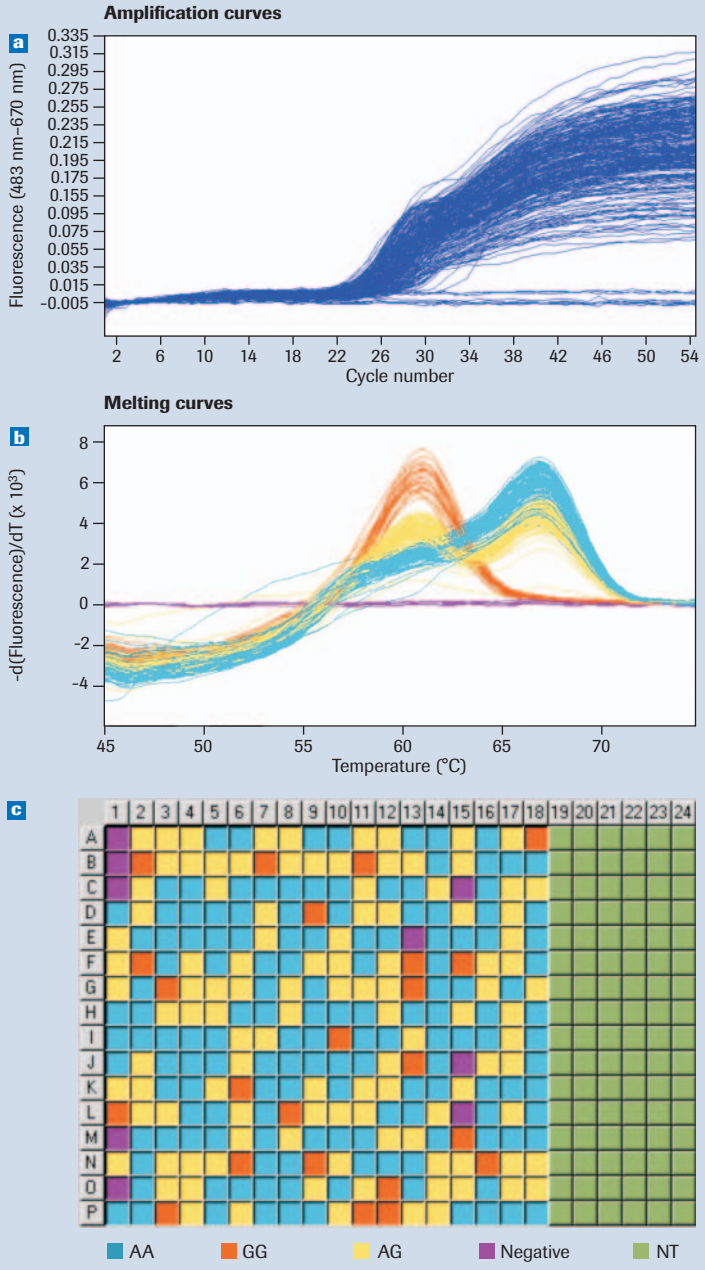

Figure 3: (a) Amplification curves, (b) melting curves, and (c) genotyping table for the DRD3 (rs6280) genotyping assay. All positive samples were amplified within 10 PCR cycles with no relevant ceiling effect. Genotyping failed for both NTCs (J15 and L15) and seven additional positions (2.4%; A1, B1, C1, M1, O1, E13, and C15). Observed genotypes did not differ from expectations under Hardy-Weinberg equilibrium.

ciency. As the minor PCR performance observed on the first trial for six further assays was attributed to pipetting problems in the automated workflow, the initial success rate increased to 79.5% (30 of 39 assays). The quality of these 30 assays was excellent without the need for time-consuming establishment. This is reflected by an optimal PCR efficiency and a low coefficient of variation within a single group of replicates (< 5%).

For the remaining eight target genes of the study (20.5%) it was shown that the PCR performance was reduced (high Cp values, high Cp value variations, non-specific amplification) due to the assay design (analyzed target sequences) and conditions (minor RNA quality). These results were confirmed by corresponding microarray data.

Genotyping analysis

Initial attempts to perform all SNP assays under the same standard conditions showed that robust amplification was possible for 4 of the 14 investigated targets. For 10 targets, a touchdown PCR protocol, starting at 65°C with 1°C reduction per cycle down to 55°C, gave better results. Three of these assays were optimized further when asymmetric PCR conditions (unequal amounts of primers: 16 pmol and 10 pmol, respectively) were used. For only one target was it not possible to satisfactorily establish PCR with any chosen strategy.

When performed in a smaller volume (10 µl instead of 20 µl), 10 of the 13 established assays worked without significant losses in signal strength, thus demonstrating that it is possible to reduce reagent cost per SNP data point.

For ten targets, the standard running time of 90 minutes (including PCR and melting curve analysis) was cut down to less than 75 minutes by shortening the denaturation and annealing steps.

With respect to allele calling, 8 of the 13 successfully established SNP assays (targets ANKK1, CYP2A6, DRD3, TPH1, CHRNA4, CHRNB2, DRD4, and CHRNA7) gave excellent and highly informative results, the quality and accuracy of which perfectly met our expectations. Unexpected drop-outs of individual samples on an assay plate (*e.g.*, wells A1, B1 and C1 in Figure 3) occurred at a rate below 2% and were related to pipetting errors when very small amounts of DNA solution were used.

Two assays (SLC6A4 and HTR2A) gave excellent amplification and melting curves but showed no allele discrimination at all, indicating possible data-base annotation errors or absence of heterozygosity. For three SNPs (RAPGEF3, COMT and MAOA) the assays resulted in insufficient allele discrimination or uneven melting peaks. This might be explained by putative secondary structures present in the target sequence for one allele or the presence of additional unknown SNPs. Based on data available from oligohouses (*e.g.*, TIB MOLBIOL), it is expected that a redesign of the HybProbe probes used

could help to deal with such more complex situations and to establish robust assays for these SNPs as well. It should also be mentioned that this study was carried out with a beta version of the LightCycler® 480 Genotyping Software that did not yet include all functionalities of the final version (*e.g.*, regarding the sensitivity of curve grouping).

In summary, the success rate for genotyping in this study (*i.e.*, number of cases where at least one allele of the respective SNP could be clearly identified) was more than 90%, with no evidence for a technical performance failure of the LightCycler® 480 Instrument, even when used as a pilot version under conditions of very high-throughput conditions.

Conclusions

The study demonstrated that the LightCycler® 480 System is a highly versatile and reliable platform for rapid gene expression and genotyping analysis offering enhanced throughputs and automation options for streamlined workflows. Based on the flexible software management and the speed of the PCR protocols, comprehensive work loads (50 multiwell plates for gene expression analysis, 14 multiwell plates for genotyping) were processed in a limited time frame (15 working days; Figure 2). The ease-of-use of the system allowed the fast adjustment to the new real-time PCR system by seven people handling the samples in this study. Based on the improved ready-to-use enzyme composition, the sensitive and robust LightCycler® 480 masters minimized cumbersome and time-consuming assay establishment activities and accelerated the entire PCR workflow setup. ■

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Order INFO

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	96-well or 384-well instrument	04 640 268 001 04 545 885 001
LightCycler® 480 SYBR Green I Master	5 x 1 ml (2x conc.)	04 707 516 001
LightCycler® 480 Genotyping Master	4 x 384 µl (5x conc.)	04 707 524 001
LightCycler® 480 Multiwell Plate	96-well plate 384-well plate	04 729 692 001 04 729 749 001
LightCycler® 480 Genotyping Software	1 software package	04 727 860 001
MagNA Pure Compact Instrument	1 instrument	03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit (32 isolations)	03 730 964 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit (50 reactions)	04 379 012 001