High-Throughput Cardiac Gene Expression Analysis Using the Universal ProbeLibrary and the Novel LightCycler® 1536 Real-Time PCR System


Martje Tönjes

*Martje Tönjes[1], Jenny Schlesinger[1], Markus Schüler[1, 2], Ilona Dunkel[1], Jörn Bethune[1, 3], and Silke R. Sperling[1]**
[1]Cardiovascular Genetics, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany
[2]Department of Computational Molecular Biology, Max Planck Institute for Molecular Genetics, Berlin, Germany
[3]Department of Mathematics and Computer Science, Free University Berlin, Germany
**Corresponding author: sperling@molgen.mpg.de*

Introduction

Although congenital heart malformations are the most common birth defects in humans, the underlying pathomechanisms remain widely unknown. Through linkage analyzes and candidate-gene approaches, several gene mutations causing congenital heart defects (*e.g.*, *CITED2*, *GATA4*, *NKX2-5*) have been identified [1-3]. However, most heart malformations display variable expressivity and penetrance, which indicates a multifactorial and multigenic basis. This suggests that the regulatory context of transcription factors plays an important role in the manifestation of the defects. In order to better understand the molecular pathways in cardiogenesis and disease we studied the global genetic network that is deregulated in malformed hearts.

We had previously assessed gene expression profile disturbances of 42 genes by quantitative real-time PCR (384-well format) in 190 cardiac samples from individuals with different heart malformations [4]. We were able to identify regulatory dependencies and disease-specific molecular portraits with interesting candidate genes. Several of the found interactions were confirmed by chromatin immunoprecipitation and RNA interference (unpublished data). In order to extend the obtained networks, we selected another 250 cardiac genes and assessed their expression levels in 93 cardiac tissue samples from individuals with congenital heart disease and healthy individuals.

For high-throughput mRNA profiling, we used the novel LightCycler® 1536 Real-Time PCR System. To evaluate the performance, accuracy, and speed of this new system, all genes measured in the previous analysis (using a competitor system) were also included for comparison.

While the reproducibility of the results was found to be highly comparable on both systems, the novel LightCycler® 1536 Instrument allowed for higher sample throughput in a short time and reduced volumes of reagents and samples.

Materials and Methods

Samples

All cardiac samples were obtained from the German Heart Institute during cardiac surgery with approval by the institutional review committee and informed consent of the individuals or their parents. Biopsies were taken from the right ventricle of individuals with different cardiac malformations as well as from normal human hearts. All samples were snap-frozen in liquid nitrogen directly after excision and stored at -80°C.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from 93 cardiac tissue samples using TRIzol according to manufacturer's instructions; 5 µg of total RNA were reverse transcribed using AMV reverse transcriptase and random hexamer primers, and diluted 1:40 (1.25 ng/µl).

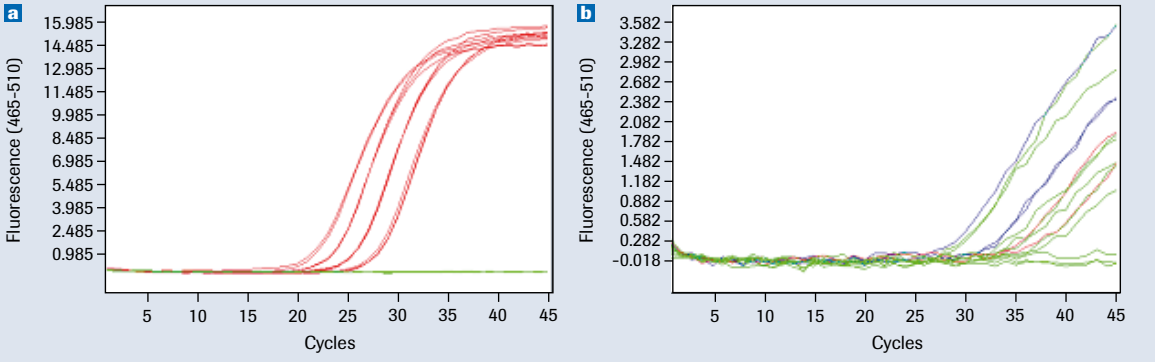

Figure 1: Assessment of designed assays. Amplification curves of test assays with serial dilutions of standard HEK293 cDNA (1:4, 1:16, 1:64, 1:265, and no template control) were performed using the LightCycler® 480 Instrument. Presented are **(a)** a good assay and **(b)** a failed assay with too low end-point fluorescence (see y-axis).

Assay design and quantitative real-time PCR

A comprehensive set of 300 cardiac genes was selected and primers were designed using the Universal ProbeLibrary and the ProbeFinder software, which is freely accessible at www.universalprobelibrary.com. Primers were designed to recognize common regions of genes that are comprised of multiple transcript variants; they were synthesized by TIB MOLBIOL. To estimate qPCR efficiency, fourfold dilution series of standard HEK293 cDNA were measured in test assays using the 384-well format of the LightCycler® 480 Real-Time PCR Instrument (PCR setup [total reaction volume 10 µl]: 2 µl template and 8 µl master mix containing 1x RealTime ready DNA Probes Master, 300 nM primer, and 400 nM probe). The quality of the amplification curves was assessed based on primer efficiency (1.8–2.2) and a minimal end-point fluorescence (EPF>5); examples are shown in Figure 1.

For the PCR setup of the LightCycler® 1536 Multiwell Plates, the Innovadyne Nanodrop Express pipetting robot and the Agilent PlateLoc Thermal Microplate Sealer were used. First, the suitable amount and volume of cDNA was determined with serial 1:2 dilutions of HEK293 cDNA (Figure 2). We compared amplification curves of a gene with high and one with low expression levels (*HPRT* and *PITX2*) and decided to use 1 ng cDNA per reaction.

Then, 250 prescreened assays were chosen and expression analysis in 93 samples and three controls – including HEK293 cDNA and no template control – was carried out in triplicate using the LightCycler® 1536 System with the following program: initial denaturation: 95°C for 1 minute; amplification (45 cycles): 95°C for 1 second (ramp: 4.8°C/second), 60°C for 30 seconds (ramp: 2.5°C/second); cooling: 40°C for 30 seconds (ramp: 2.5°C/second); software settings: Mono Color Hydrolysis/UPL Probes; pipetting control mode: Master Control. The qPCR contained 0.8 µl sample and 1.2 µl master mix containing 1x RealTime ready DNA Probes Master, 300 nM primer and 400 nM probe. The housekeeping gene *HPRT* was used for normalization. A total of 50 LightCycler® 1536 Multiwell Plates with five different assays per plate were analyzed.

Results and Discussion

The aim of our study was to assess the performance and speed of the novel LightCycler® 1536 System as a high-throughput system for gene expression analysis. In order to expand our cardiac regulatory networks, 300 qPCR assays were designed and prescreened on the LightCycler® 480 Instrument. Of these, 17% failed in the first test run and were excluded from the expression analysis. To determine the minimal amount of cDNA needed per reaction, we investigated the amplification of a gene with high and one

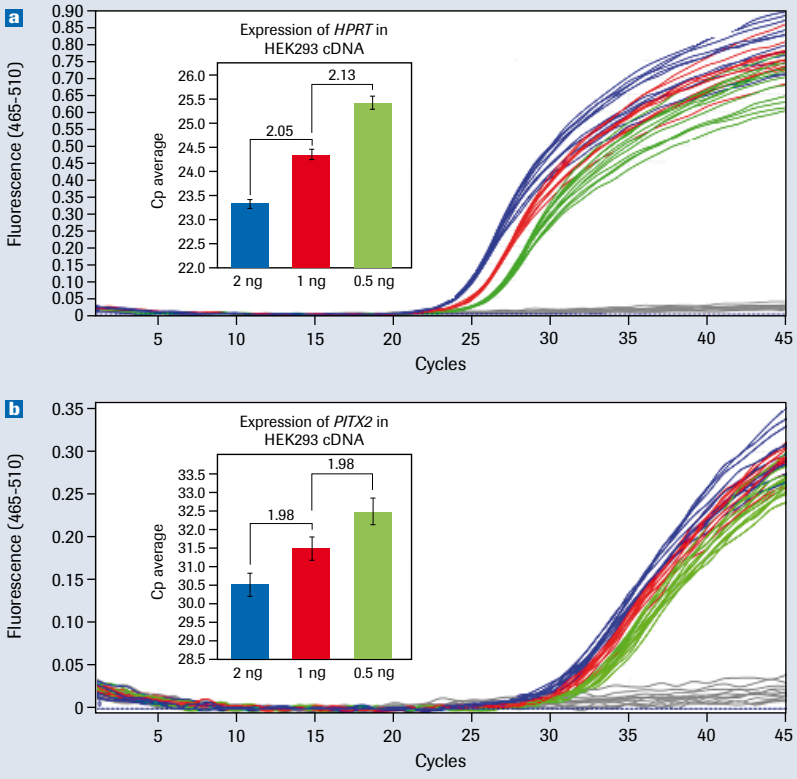

Figure 2: Comparing amplification curves of genes with different expression levels. Expression levels of *HPRT* and *PITX2* in HEK293 cells were measured with the LightCycler® 1536 Instrument using a dilution series of cDNA (2 ng in blue, 1 ng in red, 0.5 ng in green and NTC in grey). The determined dilution factor corresponded well with the theoretical dilution factor of 2. Measurements were performed in 12 replicates.

with low expression levels in a serial dilution of HEK293 cDNA (Figure 2). The theoretical dilution factor of 2 matched well with the measurements. Even when using 0.5 ng cDNA per reaction, a linear amplification was achieved and replicates were almost identical. However, as many cardiac genes are known to show low expression levels and as the crossing-point (Cp) value should not exceed 35, we used 1 ng cDNA in 0.8 µl in our expression analysis. The similarity of replicates depended on the template concentration; therefore, the sample volume was higher than expected for our application.

Subsequently, we performed a gene expression analysis of 250 genes in 93 right ventricular heart samples from individuals with congenital heart disease and healthy individuals. A total of 50 LightCycler® 1536 Multiwell Plates with five different assays on each plate were measured within 7 days (Figure 3). Only 7.5% of the performed assays failed in the majority of heart samples. This was in most cases due to a low level of transcript expression, as the HEK293 cDNA control showed functionality of the assays.

In all steps of the workflow – including pipetting, sealing, and quantification – the LightCycler® 1536 System proved

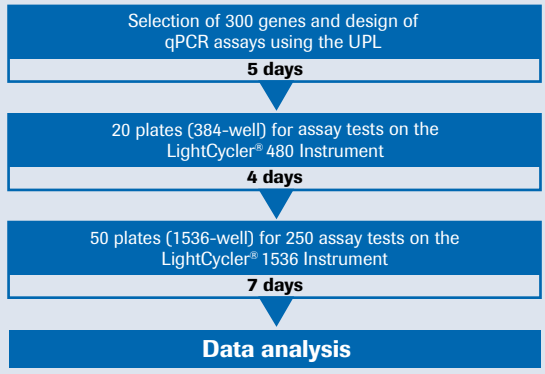

Figure 3: Time frame of expression analysis. The presented workflow includes preparation and testing of the test assays (UPL: Universal ProbeLibrary).

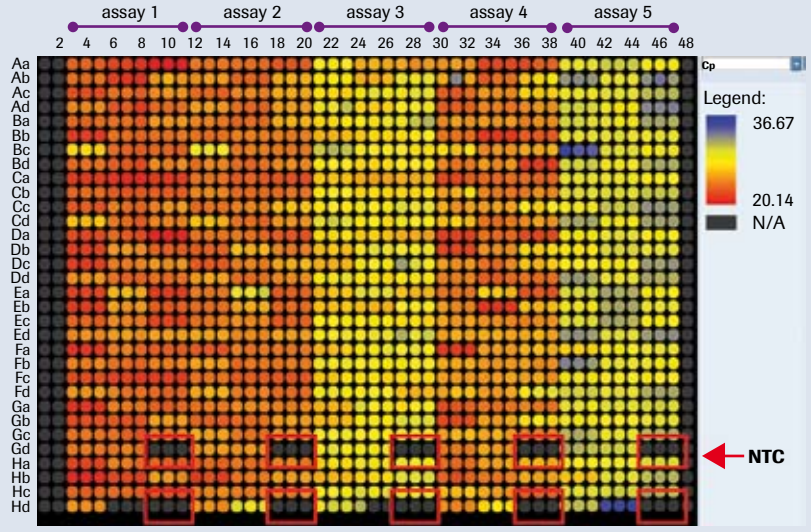

Figure 4: Heatmap chart of the LightCycler® 1536 Software. Shown are the Cp values of five different assays with 96 cDNA samples including two no-template control samples (NTC) on one plate. The Cp values of the samples are displayed as a continuous spectrum from blue (highest Cp) to red (lowest Cp; see legend).

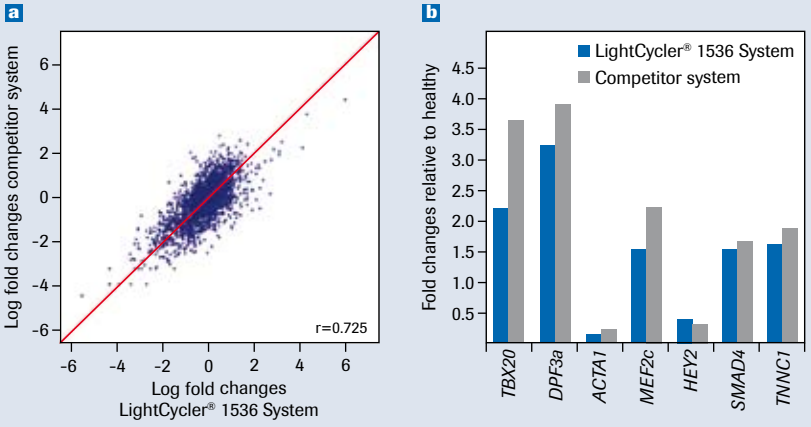

Figure 5: Comparison of gene expression fold changes between the LightCycler® 1536 System and a competitor real-time PCR system (384-well format). Fold changes are normalized to *HPRT* and relative to healthy individuals. **(a)** Dot plot with Pearson correlation coefficient (r=0.725) of 40 genes and 71 samples. **(b)** Comparison of a subset of transcripts relevant to the heart in a group of individuals with tetralogy of Fallot individuals.

to be convenient, fast, and robust. The system allowed the performance of specific, sensitive, and comparable assays. In addition, the LightCycler® 1536 Software is very user-friendly, gives a fast overview of the preliminary results, and provides easy data-export options. A very convenient feature is the heatmap tool shown in Figure 4. It directly indicated correct distribution of the five conducted assays, similarity of triplicates, and absence of contamination in the two water controls.

The resulting expression profiles were highly similar to the ones obtained earlier using a competitor system (384-well format). Comparison of the normalized fold changes from the LightCycler® 1536 System with the data from the competitor system revealed a very good correlation (Pearson correlation coefficient r=0.725) as shown in Figure 5a. In total, 40 genes measured in 71 human heart samples were taken into account. Interesting candidate genes included a subgroup that had been shown to be specifically deregulated in a population with the complex disease tetralogy of Fallot [4] (Figure 5b). The expression levels were found to be almost identical between the two systems, indicating that the obtained LightCycler® 1536 high-throughput data are reliable and give us the opportunity to expand our cardiac regulatory networks.

Conclusion

In this study, the LightCycler® 1536 Real-Time PCR System was found to be a very suitable instrument for high-throughput gene expression analysis and provided a convenient alternative to microarrays. In comparison to the previously used competitor system, the novel LightCycler® 1536 Instrument makes it possible to produce expression data in a shorter time frame due to its higher throughput (1536 data points/PCR run) combined with faster PCR protocols, and additionally enables researchers to significantly scale down reagent and sample inputs. ■

References

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Order INFO

Product	Pack Size	Cat. No.
LightCycler® 1536 Instrument	1 instrument	05 334 276 001
LightCycler® 1536 Multiwell Plate	10 x 10 plates	05 358 639 001
RealTime ready DNA Probes Master (5 x conc.)	5 x 1 ml (12,500 x 2 µl reactions)	05 502 381 001
Universal ProbeLibrary Assays	For detailed information, please visit www.universalprobelibrary.com	