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Review Article

Evaluation of the LightCycler® 1536 Instrument for high-throughput quantitative real-time PCR ☆

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ABSTRACT

Quantitative real-time PCR (qPCR) is a frequently used, sensitive and accurate method to study gene expression profiles. However, its throughput was so far limited for routine laboratories to 384 reactions per run based on the limitations of the available instruments. Recently, the LightCycler® 1536 Instrument was launched providing a high-throughput solution for qPCR with the analysis of 1536 reactions in approximately 45 min. We assessed the accuracy and sensitivity of this novel technology for the analysis of gene expression profiles in combination with the Innovadyne™ Nanodrop™ Express pipetting robot. We compared expression profiles obtained for 42 genes in 71 samples between the Universal ProbeLibrary and the LightCycler® 1536 Instrument and SYBR Green I and the ABI PRISM 7900HT system. We found that the results were highly reproducible between both systems. Beside the higher throughput, the advantage of the LightCycler® 1536 Instrument was the reduced consumption of reagents and sample material.

1. Introduction

Recently, we assessed gene expression profiles in 190 cardiac samples of patients with a broad range of congenital heart disease [1]. We identified regulatory networks based on profiling a set of 50 selected transcription factors and target genes. In line with our aim to extend this study, we evaluated the novel LightCycler® 1536 Instrument for its applicability as a high-throughput technology, which would reduce the costs per assay and moreover the sample material needed.

Quantitative real-time PCR (qPCR) is a commonly used, sensitive and accurate method for gene expression studies. The detection chemistry of all real-time PCR procedures are based on monitoring amplification products either through binding of double-stranded DNA (e.g. ethidium bromide [2] or SYBR Green I [3]) or through target-specific hybridization to single-stranded DNA (e.g. Molecular Beacons [4] or hydrolysis probe assays [5,6]). The latter are applicable for multiplexing approaches to increase the throughput capability of the instruments or to monitor housekeeping genes simultaneously [7]. So far the throughput was limited to 384 reactions per run by the limitations of the instruments applicable for routine laboratories (e.g. LightCycler® 480 Instrument, ABI PRISM 7900HT system). The recently launched LightCycler® 1536 Instrument offers the analysis of 1536 reactions per run with an approximate duration of 45 min. Here, we present an evaluation of the accuracy, sensitivity and robustness achieved with this novel technology for the application of quantitative real-time PCR. We compared gene expression profiles obtained by qPCR for 42 genes between the Universal ProbeLibrary and the LightCycler® 1536 Instrument and SYBR Green I and the ABI PRISM 7900HT system [1]. While reproducibility of the results was found to be highly comparable on both systems, the LightCycler® 1536 Instrument offers a 4-times higher sample throughput in a shorter time period with reduced volumes of reagents and sample material.

2. Materials and methods

2.1. Sample preparation, RNA isolation and cDNA synthesis

Sample preparation, RNA isolation and cDNA synthesis was performed as previously described [1]. In brief, total RNA of 93 cardiac tissues was extracted using TRIzol (Invitrogen) followed by DNase digest (Ambion). RNA concentration was measured using the NanoDrop spectrophotometer (NanoDrop Technologies) and RNA quality was further assessed by agarose gel electrophoresis. Five microgram of total RNA were reverse transcribed in cDNA using AMV reverse transcriptase (Promega) and random hexamer primers (Amersham Pharmacia Biotech) according to manufacturer's instruction. For subsequent expression analysis the cDNA was diluted 1:40 (1.25 ng/μl) in PCR grade water.

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2.2. Design of hydrolysis probe assays using the Universal ProbeLibrary

We selected a comprehensive set of 50 cardiac relevant genes based on previous experiments [1,8]. Hydrolysis probe assays consisting of primer sequences and respective hydrolysis probes were designed using the Universal ProbeLibrary and the ProbeFinder software (http://www.roche-applied-science.com/). All primers were intron spanning, with a melting temperature around 60 °C and an amplicon length of approximately 60–150 bp. Primer sequences were designed to recognize common regions of transcripts in cases of multiple transcript variants per gene. For normalization of the gene expression data assays for the human housekeeping genes *HPRT*, *B2M* and *GAPDH* were designed. Primer and probe sequences are available upon request from the authors.

2.3. Hydrolysis probe assays using the LightCycler® 480 Instrument

The reaction volume of 10 µl consisted of 2 µl cDNA dilution as template and 8 µl mastermix containing 1× RealTime ready DNA Probes Master (Roche), 300 nM primer and 400 nM probe (Universal ProbeLibrary, Roche). All assays were measured in triplicates using the following program: Enzyme activation: 95 °C for 10 min; amplification (45 cycles): 95 °C for 15sec (ramp: 4.8 °C/s), 60 °C for 30 s (ramp: 2.5 °C/s). The detection format was set to 'SYBR Green I/HRM Dye'.

2.4. Setup of the pipetting and sealing workflow

Pipetting into the 1536 well-plate (Roche) was performed with the Innovadyne™ Nanodrop™ Express robot (IDEX Health & Science LLC) using a 2 × 8 tip-head. The plates were sealed with PlateLoc Thermal Microplate Sealer with the '500' insert (Agilent Technologies) and the Clear Weld Seal Mark II foil (4titude Ltd.). The IDEX Health & Science Company developed a user-defined pipetting program with a plate setup of 96 samples in triplicates and five different assays. To ensure accurate results for replicates, we checked the workflow of the pipetting robot in several test runs using HEK293 cDNA as template and a subset of designed assays. To avoid contamination during the pipetting procedure we optimized the performance of the robot by increasing the washing steps and adding of Micro-90® Cleaning Solution (Cole-Parmer).

2.5. qPCR using the LightCycler® 1536 Instrument

The qPCR reaction contained 0.8 µl cDNA sample (1 ng) and 1.2 µl mastermix with 1× RealTime ready DNA Probes Master (Roche), 300 nM primer and 400 nM probe (Universal ProbeLibrary, Roche). The LightCycler® 1536 Instrument was used with the following program: Enzyme activation: 95 °C for 1 min; amplification (45 cycles): 95 °C for 1sec (ramp: 4.8 °C/s), 60 °C for 30 s (ramp: 2.5 °C/s); cooling: 40 °C for 30 s (ramp: 2.5 °C/s). The detection format was set to 'Mono Color Hydrolysis/UPL Probes' and the pipetting control to 'Master Control'.

3. Results and discussion

3.1. Pre-screening of hydrolysis probe assays using the LightCycler® 480 Instrument

To estimate the hydrolysis probe qPCR efficiency, a fourfold dilution series of standard HEK293 cDNA and 'no template control' was measured using the 384-well format of the LightCycler® 480 Instrument. The quality assessment was based on two general criteria (Fig. 1A). Considering the amplification curves, an assay was defined as 'good' if the calculated primer efficiency was between

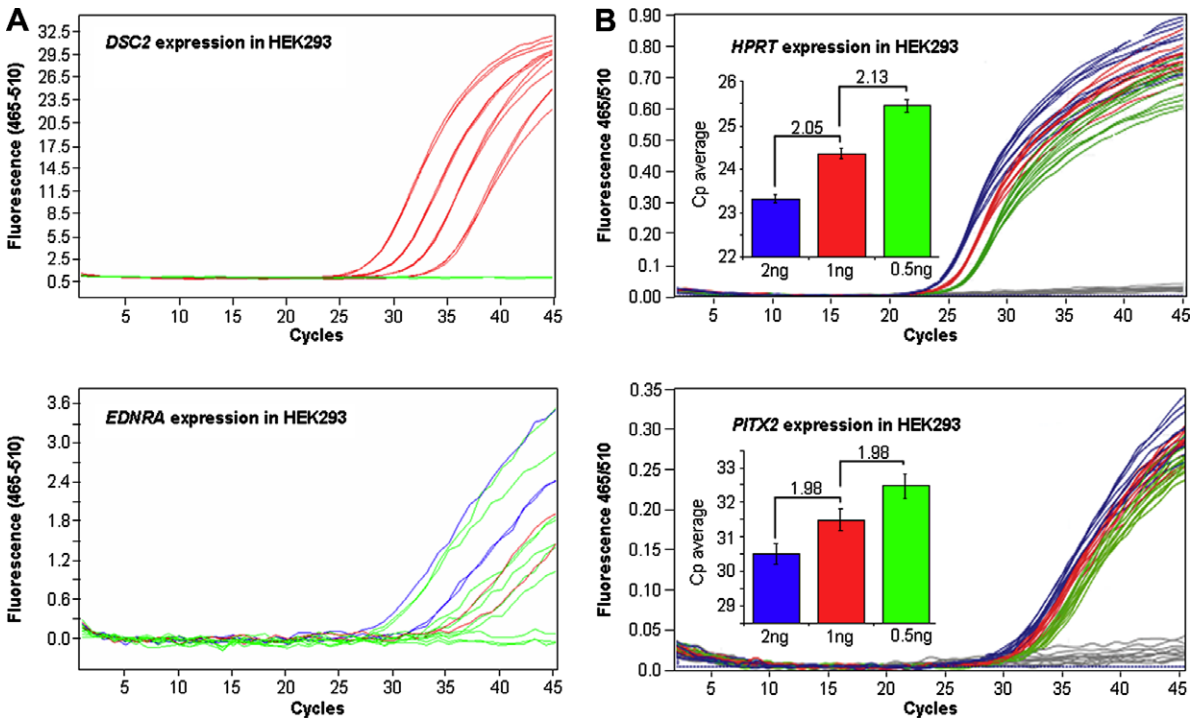

Fig. 1. Assessment of designed assays (A) and sensitivity of the LightCycler® 1536 Instrument (B). (A) Amplification curves of representative hydrolysis probe assays with serial dilutions of HEK293 cDNA (1:4, 1:16, 1: 64, 1:265 and no template control (NTC)) performed at the LightCycler® 480 Instrument. Shown are a 'good' assay (upper chart) and a 'failed' assay (lower chart) according to the minimal endpoint fluorescence. (B) Expression measurements obtained with the LightCycler® 1536 Instrument for *HPRT* and *PITX2* in HEK293 cells 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.

1.8–2.2 and the minimal endpoint fluorescence was greater than five. In total, only ~17% of designed assays failed and were excluded from the expression analysis.

3.2. Evaluation of optimized reaction chemistry and cDNA amount for the LightCycler® 1536 Instrument

To determine the minimal amount and volume of cDNA needed per reaction, we investigated the amplification of a high and a low expressed gene (*HPRT* and *PITX2*) in a serial 1:2 dilution of HEK293 cDNA (Fig. 1B). The obtained dilution factor corresponded well with the theoretical dilution factor of two. Even with 0.5 ng cDNA per reaction a linear amplification was achieved and the replicates were highly comparable. The similarity of replicates depended on the template concentration as well as the utilized volume. However, as a subset of selected cardiac genes was known to be expressed at low levels, we chose 1 ng cDNA in 0.8 µl volume for our study. This ensured that the corresponding C_p-value was below 35.

3.3. Gene expression analysis using the LightCycler® 1536 Instrument

We analyzed 42 pre-screened assays in 93 human heart samples and three controls, including HEK293 cDNA and 'no template control'. The analysis was carried out in triplicates. Fig. 2 shows a heat map chart of a representative 1536 well-plate, which is directly provided by the LightCycler® 1536 software and gives a fast overview of the preliminary results. It depicts the correct distribution of five different assays, the similarity of triplicates and the absence of contamination in the two water controls. For further analysis, we screened all amplification curves by hand in more detail to ensure that the detection threshold was correctly set. We calculated the average C_p-value for each set of triplicates and excluded outliers. Outliers were defined when the standard deviation

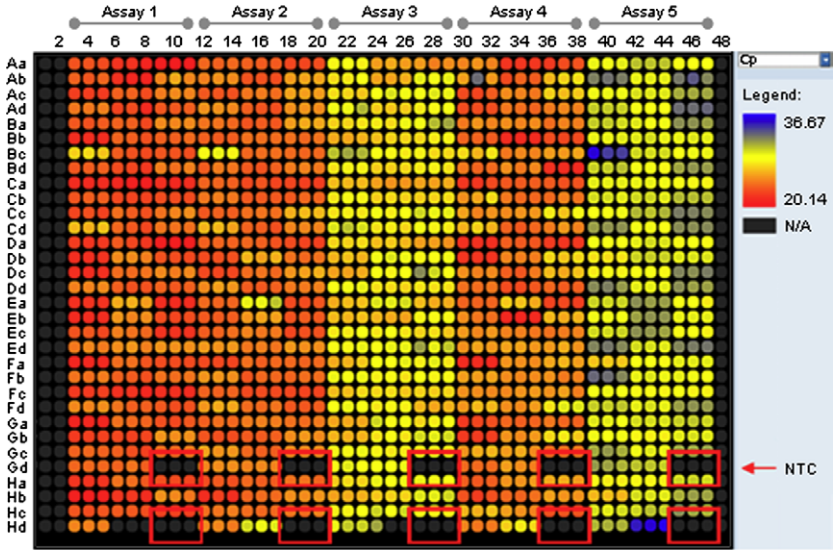

Fig. 2. Heat map chart as provided by the LightCycler® 1536 software. Shown are the C_p-values of five different assays with 96 cDNA samples including two no template controls (NTC) on one 1536 well-plate. The C_p-values are color-coded based on their intensity (see legend).

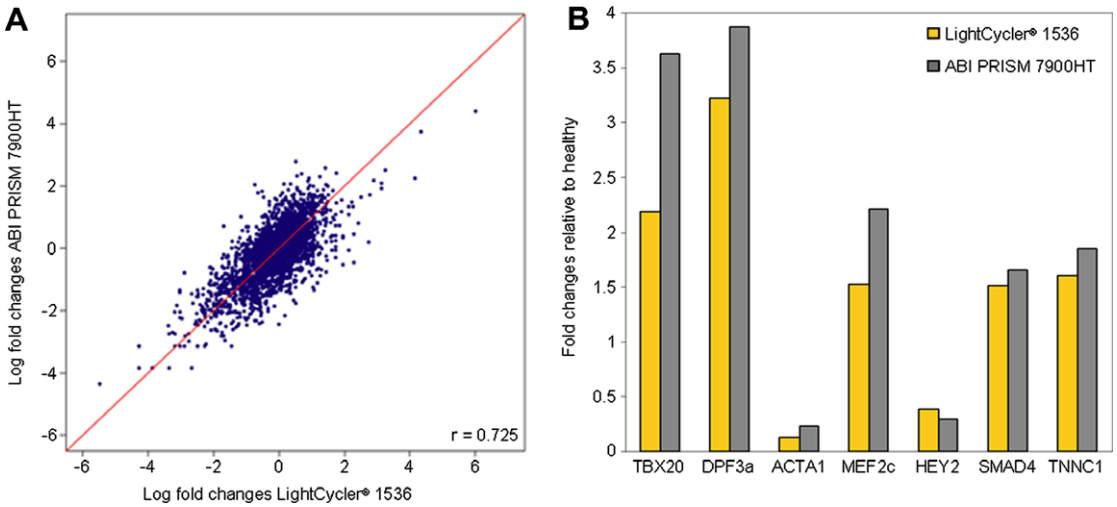

Fig. 3. Comparison of gene expression profiles obtained with the LightCycler® 1536 Instrument and the ABI PRISM 7900HT system (384-well format). Fold changes are normalized to *HPRT* and *B2M* and relative to healthy individuals. (A) Scatterplot with Pearson correlation coefficient ($r = 0.725$) of 42 genes in 71 samples. (B) Comparison of expression levels obtained for a subset of cardiac relevant genes.

of the triplicates was greater than 0.8. The expression levels were normalized using two out of the three screened housekeeping genes, as we found *GAPDH* to be deregulated in one distinct patient group which is in line with other studies [9]. Thus we normalized the data using the geometric mean of *HPRT* and *B2M* expression levels [10]. Finally, we calculated fold changes for the normalized expression values (ΔC_p-values) between the patients and healthy individuals.

3.4. Comparison of LightCycler® 1536 Instrument with ABI PRISM 7900HT system

We compared the gene expression profiles obtained with the LightCycler® 1536 Instrument with our previous data based on SYBR Green I and the ABI PRISM 7900HT system (348-well plates). Comparison of the normalized fold changes revealed a high correlation with a Pearson correlation coefficient of $r = 0.725$ as shown in Fig. 3A. In total, 42 genes measured in 71 human heart samples at both real-time PCR platforms were taken into account. Interesting candidate genes that have been previously found to be specifically deregulated in the studied samples [1] are displayed in Fig. 3B. Expression levels were found to be almost identical indicating that the obtained high-throughput data are reliable and robust.

4. Conclusion

We evaluated the accuracy, sensitivity and robustness of the LightCycler® 1536 Instrument in comparison with the 384-well plate ABI PRISM 7900HT system. We obtained highly reproducible results with both systems based on the analysis of 42 gene expression profiles over 71 samples. The LightCycler® 1536 Instrument offers a 4-times higher sample throughput in a shorter time period and moreover – based on its miniaturization – consumes reduced amounts of reagents and sample material. We found, that the pipetting workflow as well as LightCycler® 1536 software are user-friendly designed, which enables an easy-to-handle solution for a high-throughput qPCR application.

Acknowledgments

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