Published online 11 July 2011

Nucleic Acids Research, 2011, Vol. 39, No. 18 ***e124***
doi:10.1093/nar/gkr505

Quantification noise in single cell experiments

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Received February 8, 2011; Revised May 30, 2011; Accepted June 3, 2011

ABSTRACT

In quantitative single-cell studies, the critical part is the low amount of nucleic acids present and the resulting experimental variations. In addition biological data obtained from heterogeneous tissue are not reflecting the expression behaviour of every single-cell. These variations can be derived from natural biological variance or can be introduced externally. Both have negative effects on the quantification result. The aim of this study is to make quantitative single-cell studies more transparent and reliable in order to fulfil the MIQE guidelines at the single-cell level. The technical variability introduced by RT, pre-amplification, evaporation, biological material and qPCR itself was evaluated by using RNA or DNA standards. Secondly, the biological expression variances of GAPDH, TNFα, IL-1β, TLR4 were measured by mRNA profiling experiment in single lymphocytes. The used quantification setup was sensitive enough to detect single standard copies and transcripts out of one solitary cell. Most variability was introduced by RT, followed by evaporation, and pre-amplification. The qPCR analysis and the biological matrix introduced only minor variability. Both conducted studies impressively demonstrate the heterogeneity of expression patterns in individual cells and showed clearly today's limitation in quantitative single-cell expression analysis.

BACKGROUND

In many aspects cells are unique in their characteristics, even in homologous cultures or tissues. They differ in cell type, size, protein level and especially in the amount of expressed mRNA or microRNA transcripts. Biological data obtained from complex tissue samples composed of a heterogeneous cell population, are averaged from multiple-thousands of individual cells. The application of global expression result in a biological sample can not be assumed to reflect the behaviour of each individual cells (1,2). Global transcriptome measurements provide the average gene expression in the sample, hence the most abundant signatures will be captured (3). It has been suggested that the heterogeneity could arise from stochastic noise in the gene expression of each individual cell. The amplitude and the dynamic of the gene expression are controlled by various internal or external factors, e.g. gene regulation, transcription abundance, genetic or epigenetic factors (4). In many aspects individual cells exhibit a large degree of variability. Responses to identical stimuli may be very different between different cell types and even within homogeneous cell populations (5–8). This effect becomes essential for dynamic gene expression studies, especially in biomarker identification or expression profiling studies.

The mRNA and microRNA expression level is 1–2% of total RNA, hence the total RNA amount expected in one solitary cell is <1 pg (9). Low concentration in single cells are reliably detected by methods such as quantitative reverse transcription (RT) followed by polymerase chain reaction (RT–qPCR), quantitative next generation sequencing, digital PCR (dPCR), microarray analysis after linear pre-amplification, or high resolution imaging technologies, like RNA fluorescence *in situ* hybridization (FISH) (3,10–15). The critical part in single-cell real-time RT–qPCR analysis is the very low amount of nucleic acids present, and therefore high variations are expected during the quantification workflow. These variations can be either due to natural biological variance of the expressed mRNA or can be introduced externally by technical setup, such as sampling, storage, nucleic-acid stabilization, extraction, RT, pre-amplification, quantitative PCR, or by the quantification process, like using an inappropriate normalization procedure (16,17). Both the biological and the technical variances have negative effects on the quantification procedure and therefore should be eliminated or at least kept to a minimum. The aim should be to reach highest reproducibility and therefore lowest technical variance in the whole RNA quantification workflow, in order to measure RNA quantities, gene expression differences and the biological regulation afterwards (16,18).

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While the variances and deviation of conventional qPCR studies are already reported (19,20), little is known about the sensitivity and reproducibility of the single-cell based analysis system and the pre-amplification step (13). The detection of a specific transpcript on single-cell mRNA level is possible by flow cytometry sorting of single lymphocytes, the subsequent pre-amplification of the transcriptome in low volume applications (1 µl) on glass slides, followed by a real-time RT–qPCR amplification. In this study a slide cycler system designed for single-cell based gene analysis was investigated in combination with a classical real-time PCR cycler to determine the source of technical variances induced by reverse transcription, pre-amplification, single cells and qPCR itself. The aim of this study was to establish an optimal workflow and a reliable protocol for quantitative single-cell experiments on DNA and RNA level. Determination of the technical variability at both levels was done, by using artificial nucleic acid DNA and RNA standards in combination with a biological matrix herein a single lymphocytes.

In a second study a gene expression profiling experiment was performed to get information on the biological transcriptional noise in lymphocytes. Therefore single lipopolysaccharide (LPS) induced lymphocytes were analyzed for a selection of genes: a stable expressed housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and tumor necrosis factor alpha (TNFα), interleukin 1β (IL-1β), or toll-like receptor 4 (TLR4) known to be expressed and regulated in white blood cells (21,22). The goal was to investigate the natural variability and expression ranges between individual cells. Aditionally the suitability of a slide based system for this quantitative approach was determined to finally establish a valid protocol for fully quantitative mRNA measurements on single cells.

MATERIALS AND METHODS

Cell culture and flow cytometry

A concentrate of lymphocytes (LYM) isolated by Ficoll separation from female human donor's blood was purchased commercially (Labor Pachmann, Bayreuth). The lymphocytes were resuspended in Ham's tissue culture medium (Biochrom, Berlin, Germany) and kept cool at 4°C until flow cytometry cell sorting. For gene expression analysis lymphocytes were treated with LPS to final concentration of 1 µg/ml (Sigma Aldrich, St Louis, USA) for 2 h at 37°C. Cell sorting was done using flow cytometry service by DRFZ (Berlin, Germany). Cells were stained with Hoechst dye H33342 (final concentration 3.33 µg/ml, Sigma-Aldrich, Hamburg, Germany) for optical detection of cell deposition success on the AmpliGrid slides afterwards. The Hoechst dye H33342 was added to the cell suspension and incubated 10 min at room temperature before sorting. For separation of living cells propidium iodide (final concentration 50 µg/ml, Sigma Aldrich) was added to the cell suspension just before cell sorting. Single cells were directly deposited on AmpliGrid AG480F slides (Beckman Coulter Biomedical GmbH (BCB), Munich, Germany) using a MoFlo™Legacy flow cytometer (Beckman Coulter, Fullerton, USA) with a 70-µm nozzle. Following sort criteria were used: single cell modus, lymphocyte gate in FSC/SSC plot, Hoechst positive and propidium iodide negative gate. Slides deposited with cells were stored at 4°C until PCR experiments were performed. The integrity of the cells was verified optically by the presence of a round, compact nucleus in the DAPI filter by using a Olympus BX 61 fluorescence microscope (Olympus Deutschland GmbH, Germany) (Figure 1).

Single-cell analysis system

The single-cell AmpliGrid analysis system (BCB) consists of a reaction glass slide (AmpliGrid® AG480F slides, BCB) and a slide cycler (AmpliSpeed, BCB). AmpliGrid comprises 48 reaction sites each capable of safely holding 1 µl of reaction solution due to surrounding hydrophilic and hydrophobic rings. Since the AmpliSpeed thermocycler is not equipped with an online fluorescence sensor, qPCR needed to be carried out on a conventional qPCR cycler, herein the realplex real-time PCR cycler (Eppendorf, Hamburg, Germany).

Standard materials

DNA and RNA standards. One DNA and one RNA standard were investigated. A DNA plasmid standard (pMS1) with perfectly matching forward and reverse primers (50 µM) were ordered at AJ Roboscreen (Leipzig, Germany). A RNA standard was *in vitro* transcribed from pMS1 and purified by the manufacturer. Both standards were diluted and stabilized at a final concentrations of 10, 10^2, 10^3, 10^4, 10^5 and 10^6 single- or double-stranded molecules/µl, respectively. As stabilizing agents, a transfer-RNA (tRNA) and a 10 mM TRIS buffer (pH 7.4) was added to the RNA standard (23). For further analysis, 1 µl of the respective standards dilutions (as described above) were then applied to each of the AmpliGrid slide spots (BCB). Each RNA or DNA standard was pipetted separately and solely on AmpliGrid slides ('DNA standard') and on four AmpliGrid slides containing a single lymphocytes in each spot ('DNA standard + LYM' and 'RNA standard + LYM'). Immediately after pipetting, standard dilutions were dried on the AmpliGrid slide by short evaporation at 37°C on the slide cycler and all slides were stored at −20°C until analysis.

Standard curve for gene expression profiling. For the absolute quantification in mRNA expression study, PCR product based standard curves were created from each of the measured genes (GAPDH, TNFα, IL-1β and TLR4) obeying MIQE guidelines (16): PCR-products of six PCR runs were pooled and purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The DNA concentration was measured multiple times by NanoDrop 1000 system (Thermo Fisher Scientific, Waltham, USA). The number of copies per pool were calculated and the standard curves were established by

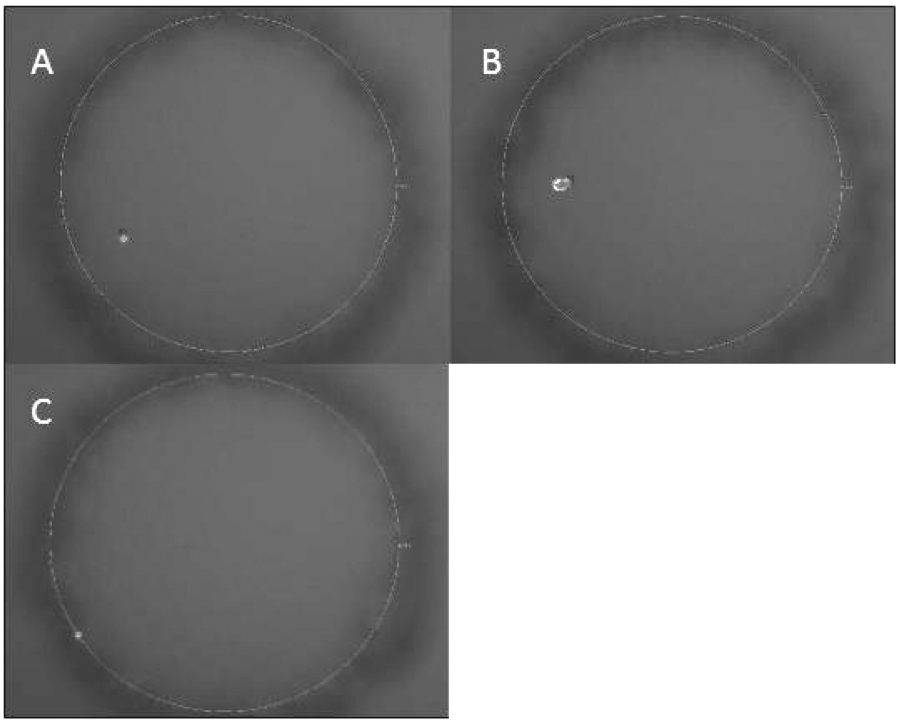

Figure 1. Deposition categories of flow cytometry cell sorting of lymphocytes on glass slides. (**A**) One single cell. (**B**) One diffuse cell. (**C**) One cell at the edge of the spot.

dilution to final concentrations of 10, 10^2, 10^3, 10^4, 10^5 and 10^6 molecules/2 µl.

Experimental setup

DNA standard analysis. To determine the quantification range, sensitivity and variability just caused by the quantitative qPCR assay, the DNA standard curve (named 'DNA standard solely') was amplified on the real-time PCR cycler (realplex, Eppendorf).

To show effects on the quantification variance, induced by pre-amplification or by the biological sample (LYM) or in combination further sub-studies and standard curves were applied. Using DNA standards, we compared the pre-amplification without ('DNA standard + PreAmp') and with biological material herein LYM ('DNA standard + PreAmp + LYM').

RNA standard analysis. A comparable setup was chosen for RNA standards to show the influence of the RT reaction in combination with single LYM. RT and subsequent qPCR were done with RNA standards to look for technical variations induced by sampling and amplification process, starting at RNA level. Furthermore the impact of evaporation during sampling on RNA stability and quantification variance was investigated using RNA standards. Comparison of 'RNA standard' with 'RNA standard + LYM' slides should show biological inhibition and quantification variance at low RNA molecule level.

Gene expression profiling. LPS treated (LPS^+) and untreated LYM (LPS^-) were analyzed for further detection of natural biological mRNA variances in single-cell gene expression profiling. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was expected to be used as unregulated high expressed housekeeping gene and tumor necrosis factor alpha (TNFα), interleukin 1β (IL-1β) and toll-like receptor 4 (TLR4) were selected as classical LPS sensitive genes.

DNA standard pre-amplification. Pre-amplification was done only with the 'DNA standard + PreAmp' and 'DNA standard + PreAmp + LYM'. PCR master mix was prepared using Maxima SYBR Green Kit (Fermentas, USA) to 1 µl total PCR reaction. An amount of 0.04 µl Primer mix (10 µM), 0.5 µl Maxima SYBR mix and 0.46 µl nuclease-free water were added on each spot and covered with 5 µl sealing solution (BCB). Ten cycles pre-amplification was done following the protocol: pre-denaturation (95°C, 10 min), 10 cycles of denaturation (95°C, 15 s) and annealing (60°C, 40 s). Assuming an optimal doubling in each pre-amplification PCR cycle, a 10 cycle course would equal a theoretical 1024-fold pre-amplification (2^{10}). To restore the original concentration after pre-amplification 4 µl nuclease-free water were added to each reaction spot (dilution 1:5). An amount of 4 µl of this volume were taken and transferred to a fresh 0.2-ml reaction tube. The pre-amplified sample was finally diluted by a final dilution factor of 1:100.

Reverse transcription of RNA standards. Complementary DNA (cDNA) synthesis out of 'RNA standard' and 'RNA standard + LYM' were performed using the RevertAid Premium Reverse Transcriptase-Kit

(Fermentas International Inc., Burlington, Canada). Total reaction volume was 1 µl, including RevertAid H^{Minus} MuLV Reverse Transcriptase (0.1 µl), dNTP Mix (10 mM, 0.1 µl), 5× Reaction Buffer (0.5 µl) and a 1:1 (v/v) mix of random hexamer primers (0.1 µl) and oligo-dT_{18} primers (0.1 µl). An amount of 1 µl reaction mix was pipetted on six spots of each RNA standard dilution. One spot was used as negative RT control, without adding reverse transcriptase. The 1-µl reaction mix was immediately covered with 5 µl sealing solution to avoid evaporation. Primer extension was done at 30°C for 10 min, followed by cDNA synthesis at 37°C for 30 min and assay termination at 85°C for 5 min. Before qPCR 3 µl nuclease-free water were added to each spot (dilution 1:4). An amount of 4 µl of this volume were taken and transferred to a fresh 0.2-ml reaction tube. The pre-amplified sample was diluted to a final dilution factor of 1:100.

Gene expression profiling. To determine the expressions patterns of lymphocytes with (LPS^+) and without LPS treatment (LPS^-), a pre-amplification of 10 cycles was performed for four AmpliGrid slides covered with 24 LYM LPS treated (LPS^+) and 24 non-treated LYM (LPS^-) per slide. Pre-amplification was performed on the AmpliSpeed (BCB) using the single cell one-step RT–PCR Kit (BCB) to 1 µl total PCR reaction. An amount of 0.05 µl primer mix of each gene (in total four primer pairs) were multiplexed (10 µM), 0.5 µl single cell RT reaction buffer, 0.02 µl RNase Inhibitor (10 U/µl), 0.15 µl single cell RT enhancer and 0.28 µl nuclease-free water were added on each spot and covered with 5 µl sealing solution. Pre-amplification was done following the protocol: Start RT reaction (42°C, 10 min), primer extension (50°C, 10 min), RT reaction termination (95°C, 10 min); 10 cycles denaturation (95°C, 10 min), annealing (60°C, 40 s) and elongation (72°C, 60 s). For every PCR cycle a doubling of DNA amount was expected. Assuming an optimal doubling in each pre-amplification PCR cycle, a 10-cycle course would equal to a theoretical 1024-fold amplification (2^{10}). To restore the original concentration after pre-amplification 4 µl nuclease-free water were added to each reaction spot (dilution 1:5). An amount of 4 µl of this volume were taken and transferred to a fresh 0.2-ml reaction tube. The pre-amplified sample was diluted to a final dilution factor of 1:50. The measurement was repeated with four slides.

Primer design

Primers were designed using published nucleic-acid sequences of the human genome GenBank (NCBI, Bethesda, USA) (Table 1). Primer design and optimization was done using primer design program Primer 3 v.0.4.0 (http://frodo.wi.mit.edu/primer3/) and checked with Netprimer (Premier Biosoft, Silicon Valey, USA) with regard to primer dimer formation, self-priming formation and similar primer annealing temperature. Newly designed primers were ordered and synthesized by TIB Molbiol (Berlin, Germany). Primer testing was performed on human white blood cells sample pool and a negative control (RNAse free water) for each primer set. Specificity of the primer was controlled by melting curve analysis and agarose gel electrophoresis. Primer sequence and location was checked with PrimerBLAST (NCBI, Bethesda, USA). Primer design was done MIQE compliant (16).

Quantification by qPCR. All qPCR experiments starting from either standard DNA or cDNA (from reverse transcribed standard RNA) were done with the realplex system (Eppendorf). Samples were measured in transparent, fully skirted 96-well plates (Eppendorf) which were heat-sealed using highly transparent films and the 4s2 automated heat sealer (4titude, Berlin, Germany) to prevent from any evaporation during qPCR (18).

DNA samples were measured in five replicates and additionally a non-template control in qPCR. RNA samples were measured as well in five replicates in the cDNA synthesis and in the qPCR for each dilution step. As controls a negative RT sample (without RT enzyme) and a non-template control (RNAse free water) were included, according to the MIQE guidelines (16).

Maxima SYBR Green qPCR Kit (Fermentas) was used for preparation of 11 µl total reaction volume. This was prepared using 0.6 µl primer mix (10 µM), 7.5 µl Maxima SYBR mix and 2.9 µl nuclease-free water. An amount of 4 µl template was added to each reaction mix. Quantitative PCR was performed by the following protocol: Predenaturation at 95°C, 10 min; 30 cycles (DNA) or 40 cycles (RNA) of repeated denaturation at 95° for 15 s and annealing at 60°C for 40 s. At the end of the run an additional melt curve step was included from 65 to 95°C, rising 0.5°C/s.

Table 1. List of primer pairs designed for gene expression analysis in single cells

Gene	Primer name	Primer sequence 5′–3′	Annealing temperature (experimental)	Product length (bp)	NCBI Accession number
GAPDH	GAPDH for	GAA GGT GAA GGT CGG AGT CAA	60	233	NM_002046
	GAPDH rev	GCT CCT GGA AGA TGG TGA TG	60		
TNFα	TNFα for	AGG GAC CTC TCT CTA ATC AGC	60	104	NM_000594
	TNFα rev	CTC AGC TTG AGG GTT TGC TAC	60		
IL-1β	IL1β for	GGA CAG GAT ATG GAG CAA CAA G	60	121	NM_000576
	IL1β rev	AAC ACG CAG GAC AGG TAC AG	60		
TLR4	TLR4 for	TTC CCG GTG TGG CCA TTG	60	202	NM_138554.3
	TLR4 rev	GCC TGA GCA GGG TCT TCT CC	60		

Gene expression profiling. Quantitative analysis of the LPS treated cells was done following the protocol described in the 'RNA standard' qPCR measurements. An amount of 2 µl sample of pre-amplified diluted PCR product and of purified standard were used for qPCR analysis. Standards, samples, positive control (LYM pool) and non-template control were measured in duplicates. All four lymphocytes slides were measured in one qPCR run to avoid inter-run differences.

Cq values (quantification cycle) and copy numbers were evaluated by the realplex software (version 2.0, Eppendorf). Baseline was automatically corrected by the analysis software. The threshold was defined using the 'best correlation' algorithm (r^2). PCR efficiency (E) of each amplified transcript was calculated via dilution row using the formula (16,24):

$$E = 10^{(-1/a)} - 1$$

Cq and copy numbers were analyzed as soon as a single and specific product peak could be detected in the melting curve analysis. Samples which could not be detected or showed unspecific products were eliminated from further analysis.

Statistical analysis

To compare individual samples and slides within the DNA and RNA standard array, a standard curve was drawn for each 48-well AmpliGrid slide and the actual number of copies per sample was figured out using the linear regression formula:

$$Y = a^*x+b$$

where Y = Cq-value; a = slope of curve; x = copy number in log-scale; b = y-intercept of curve.

Mean value and standard deviation for each dilution step was determined and intra-run variance ($n = 4$) and inter-run variance ($n = 4$) was calculated as the mean variation (standard deviation SD divided by mean of copies) of each slide. For calculation of mean copy numbers in the gene expression profiling the geometric mean was taken (2,3).

For statistical evaluation of significance in LPS induced gene regulation in expression profiling experiments the 'Mann–Whitney Rank Sum Test' was applied. Test was chosen due to none normal (Gaussian) distribution of the expression data and the inhomogeneous data groups in all analyzed genes and treatments. For normality testing of cellular copy numbers and log-normal distribution the robust 'Kolmogorow Smirnow Test' (KS-test) was used. The KS-test has the advantage of making no assumption about the distribution of data. All calculations were performed in Microsoft Excel (Microsoft, Redmond, USA), illustrated using SigmaPlot 11.0 (Systat Software Inc., Richmond, USA) and statistical evaluations were performed in Sigma Stat version 3 (Systat Software Inc.).

RESULTS

Deposition of lymphocytes

The position of cells on AmpliGrid slides was checked under the microscope for the presence of a fluorescent nucleus. Exact recognition and deposition of single cells via flow cytometry cell sorting is difficult but deposition success was well below average in terms of quantity and quality compared to other studies using the AmpliGrid system. Around 40% of AmpliGrids spots were empty, ambiguous or less often occupied with two cells. The remaining 60% of the spots could be classified in one of three categories (Figure 1): (i) one single cell with a round nucleus; (ii) one single cell with a diffuse nucleus; and (iii) one cell at the edge of the spot.

While the round and diffuse cells (most likely cells burst because of the impact) should allow for optimal access for further quantitative analysis, cells at the edge of a spot may have the possibility of not being properly covered by the mastermix and therefore lead to impaired results. To retain a significant number of samples all these three types were used for RT–qPCR analysis. Empty spots without cells were not counted or included in any analysis. A correlation between the different cell spotting types and variances in Cq values could not be detected.

DNA standard array

As expected the DNA measured in qPCR only (solely realplex amplification system; $n = 4$) showed highest sensitivity, best linearity and the lowest variation of entire quantification range from 10 to 10^6 start molecules with a PCR efficiency of 96.7% ($r^2 = 0.997$) (Table 2). Over the quantification range we could observe a very low variability of 7.03%, which was dominated by the variability at lowest concentration. Including the pre-amplification (PreAmp) directly on the slide the variance increased to 26.19% (PCR efficiency 84.4%; $r^2 = 0.986$). Variability did not change significantly down to 27.81% by adding single LYM, but PCR efficiency dropped to 74.2% ($r^2 = 0.981$). Adding biological material we determined similar quantification ranges and PCR dynamics. Compared to the solely 'DNA standard' we discovered in both approaches relatively higher quantification noise which exhibit no potentially effect of biological material on quantification variability, but a loss in PCR amplification efficiency. Variation induced by pre-qPCR steps (drying, pre-amplification, handling and dilution steps) could be roughly estimated by subtracting the discovered variances and result in a theoretical technical variation of ~20% introduced by pre-amplification handling steps. For PreAmp an optimal quantification range from 10^2 to 10^6 start molecules could be found. Therefore a decrease in efficiency, reproducibility and sensitivity induced by pre-amplification was clearly visible.

To measure the effect of a single LYM and hence the impact of biological matrix on the measured copies, the input copy numbers were plotted to the calculated output copy number. Three scenarios are shown: qPCR 'solely' in the realplex cycler, the 'PreAmp' and 'PreAmp + LYM' scenarios, performed on the slide cycler (BCB) and on

Table 2. Technical variations for DNA or RNA standard amplification curves induced by pre-amplification, reverse transcription and cellular material ($n = 4$, standard deviation = SD)

DNA standard	DNA standard (solely)		DNA standard + PreAmp		DNA standard + PreAmp + LYM	
	Mean copies (SD)	Variation (%)	Mean copies(SD)	Variation (%)	Mean copies (SD)	Variation (%)
10	11.13 (1.81)	16.25	205.62 (145.70)	2.27	16.40 (17.59)	32.92
10^2	102.46 (8.17)	7.97	200.57 (82.67)	29.65	53.45 (37.77)	24.69
10^3	1058.81 (66.50)	6.28	2426.47 (1084.18)	33.46	87.65 (152.15)	24.45
10^4	10 681.79 (370.02)	3.46	77067.76 (19280.66)	40.08	8453.64 (3438.72)	21.02
10^5	109 308.79 (3957.16)	3.62	983485.03 (814075.06)	18.67	103827.70 (34518.88)	24.22
10^6	1 041 723.33 (48 070.20)	4.61	2453015.76 (1383551.87)	32.99	483012.82 (170583.55)	39.56
r^2/Mean variation	$r^2 = 0.997$	7.03	$r^2 = 0.986$	26.19	$r^2 = 0.981$	27.81
RNA standard			RNA standard		RNA standard + LYM	
			Mean copies (SD)	Variation (%)	Mean copies (SD)	Variation (%)
10			107.23 (134.03)	124.99	237.83 (108.21)	45.50
10^2			106.82 (14.15)	13.25	157.64 (110.29)	69.96
10			898.38 (394.42	43.90	232.43 (162.77)	70.03
10^4			5794.50 (3835.54	66.19	1094.97 (598.80)	54.69
10			105 840.86 (40630.58	38.39	153475.83 (101068.43)	65.85
10^6			2 977 310.24 (3314177.18	111.31	19348193.24 (14290252.09)	73.86
r^2/Mean variation			$r^2 = 0.970$	66.34	$r^2 = 0.962$	63.32

the realplex system (Eppendorf). Regressions and 95% confidence intervals (CI) are shown (Figure 2A–C). As expected the highest consistency was found in the regression analysis of solely qPCR (standard error of estimate = 0.2598, $r^2 = 0.978$, $P = 0.0001$). The variation of slides with LYM biological material were slightly higher (standard error of estimate = 0.478, $r^2 = 0.9345$, $P = 0.0002$) than without (standard error of estimate = 0.439, $r^2 = 0.921$, $P = 0.0003$).

RNA standard array

The efficiency of the reverse transcription reaction was determined by comparing reverse transcribed RNA with DNA standard dilutions on multiple AmpliGrid glass arrays ($n = 4$). As average a RT efficiency of 67.7% was obtained ranging from 46.7% to 129% over the entire quantification range of 10–10^6 copies.

RT–qPCR results of pure 'RNA standard' slides amplification showed acceptable PCR efficiencies ~77.4% but in general lower efficiencies than measurements with DNA standard. Similar results could be seen for 'RNA standard + LYM', one slide over-estimated efficiency because of inconsistent standard curve correlations and high slopes of the standard curve caused by outliers in copy number. A variation of 66.34% was calculated for the 'RNA standard' only, 63.32% were calculated for 'RNA standard + LYM'. Similar to the results in the DNA standard curve, no higher variation was induced by LYM cellular material (Table 2). The theoretical variation introduced by RT at low copy number range could be calculated ~55–58% (total variability minus solely qPCR variability).

To visualize the variance implemented by the lymphocytes on RNA level, the input and output copy number were plotted and 95% CI were calculated. The higher variation could be easily seen by eye for slides including LYM (standard error of estimate = 0.976, $r^2 = 0.7442$, $P = 0.0025$) compared with others without LYM which mirrors the expected heterogeneity of the cells (standard error of estimate = 0.4867, $r^2 = 0.920$, $P = 0.0001$) (Figure 3).

Evaporation on RNA standard array

The reason for such high technical variations in RNA measurement at single-cell level was introduced in the first experimental setup by single-cell processing. We assumed that the cell evaporation and the drying, RT and handling caused this variability. To fix RNA, the standard was initially evaporated at 37°C on the AmpliGrid slide to simulate conditions of a fixed cell and that later RT reaction could be pipetted in 1 µl reaction volume on each spot (see 'Materials and Methods' section). To reduce the implemented variation through this additional evaporation step, a second experiment run without evaporation on the slides was performed. The 'RNA standard' experimental setup was repeated with a RNA dilution series from 10 to 10^5 copies on two additional slides. The RT protocol was optimized and RNA standard was included to the RT reaction mix (0.1 µl each dilution) instead of nuclease-free water. The result showed a decrease of RT–qPCR variation from 57.34 down to 8.89% and therefore the effect of cell evaporation and RNA stabilization by buffer components on the quantitative RNA measurement. Further the test linearity and sensitivity could be improved from 100 down to 10 RNA molecules resulting in a higher concentration range of the RNA quantification system (Table 3).

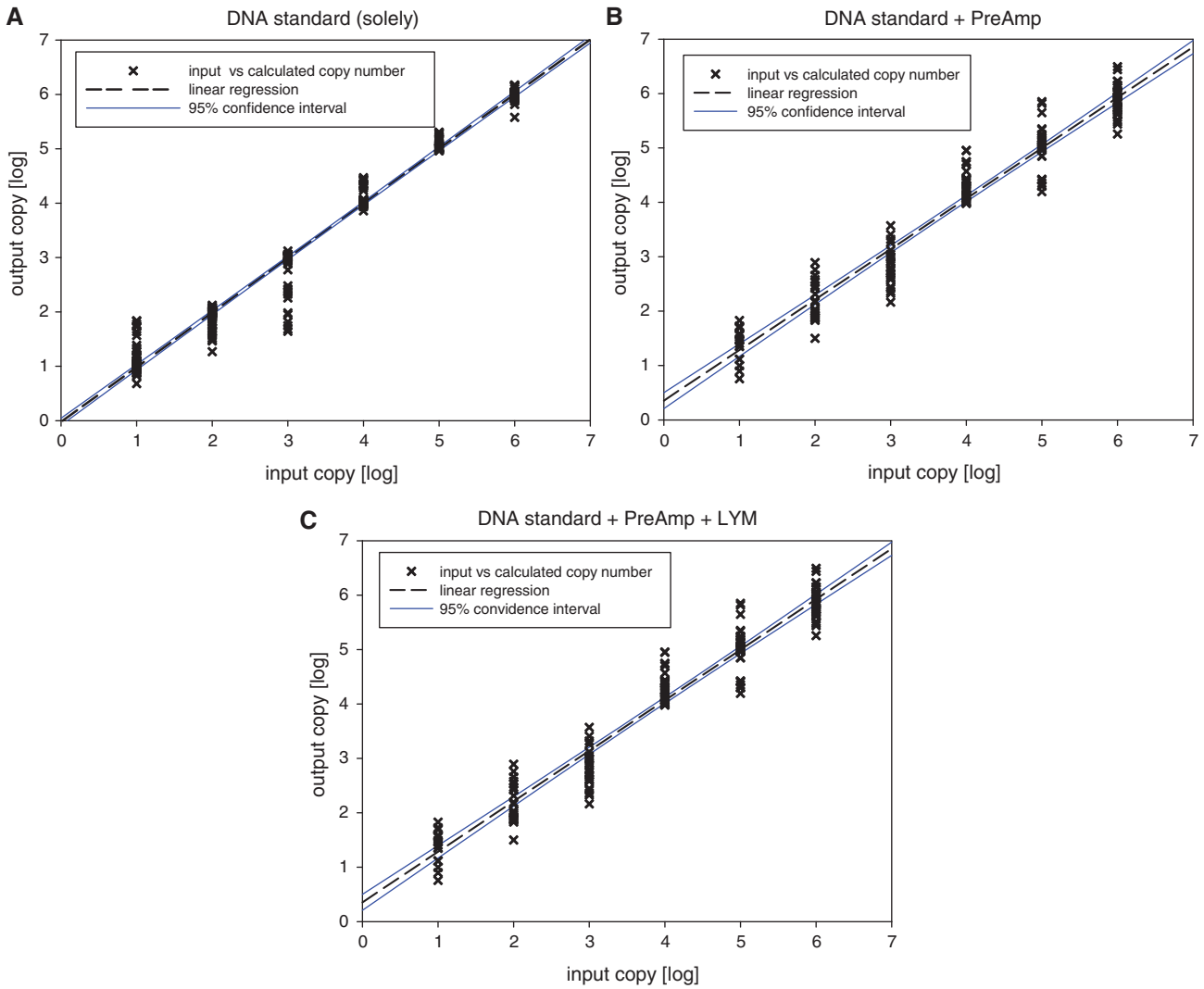

Figure 2. Input copy numbers versus calculated output copy numbers including linear regression line and 95% confidence interval (CI) for DNA standard (**A**) solely qPCR (without pre-amplification and without LYM); (**B**) DNA standard amplification with pre-amplification but without LYM; (**C**) DNA standard amplification with pre-amplification and with a single LYM.

Gene expressions profiling

All four quantitative performed assays (GAPDH, TNFα, IL-1β and TLR4) showed a quantification range of 10–10^6 molecules and acceptable PCR efficiencies in the range between 94.2 and 118.0% (Table 4).

As expected, most of the analyzed cells (73.5%) showed a GAPDH expression due to the ubiquitously high expression level and its well known function as house keeping gene (Figure 4). For the remaining analyzed transcripts (TNFα, IL-1β and TLR4) slight primer dimer formation were generated for extreme low copy concentrations. Therefore only cells with a specific product peak in the melting curve analysis were taken for further analysis, according to the strict requirement of the MIQE guidelines (16). All biological samples with no specific PCR product peak were removed from the study. We are aware that cells which express mRNA levels below the limit of detection (LOD) of the assays (herein 10 molecules) can not be separated from negative cells (16).

This reduced the number in which gene expression could be reliably quantified down to 144 cells for GAPDH, 56 cells for TNFα, 32 cells for IL-1β and 86 cells for TLR4, compared to the existence of 196 spotted cells on four slides. Primer dimer formation might be related to the multiplex pre-amplification step in which all four primer pairs were included. This assumption was confirmed by melting curve analysis of the standards and positive controls which showed solely specific gene peaks. For the TNFα transcript 66 copies was the lowest average copy number detected, based on the geometric average, reflecting the high sensitivity of the pre-amplification technology (Table 5). In a single event 13 copies could be detected in average for LPS negative LYM.

The LPS treatment showed significant effects on mRNA expression profiles (Figure 4) on IL-1β ($P = 0.003$) and TLR4 ($P = 0.014$), and no effect on GAPDH ($P = 0.925$) and TNFα ($P = 0.357$). For TNFα the theoretical lowest gene expression in one cell could be measured

with around three theoretical copies (below LOD of our assay) and the highest copy numbers with over 1 million copies for IL-1β. This reflects the high heterogeneity in single-cell gene expression, showing that only a few highly expressed genes per cell are necessary to get a significant gene expression. Variation was lowest for GAPDH expression (CV = 54%) in the LPS negative group and >550% in the LPS positive group. This high variation in GAPDH was mainly caused by one extreme 'outlier' expression sample measured in the LPS treated LYM group.

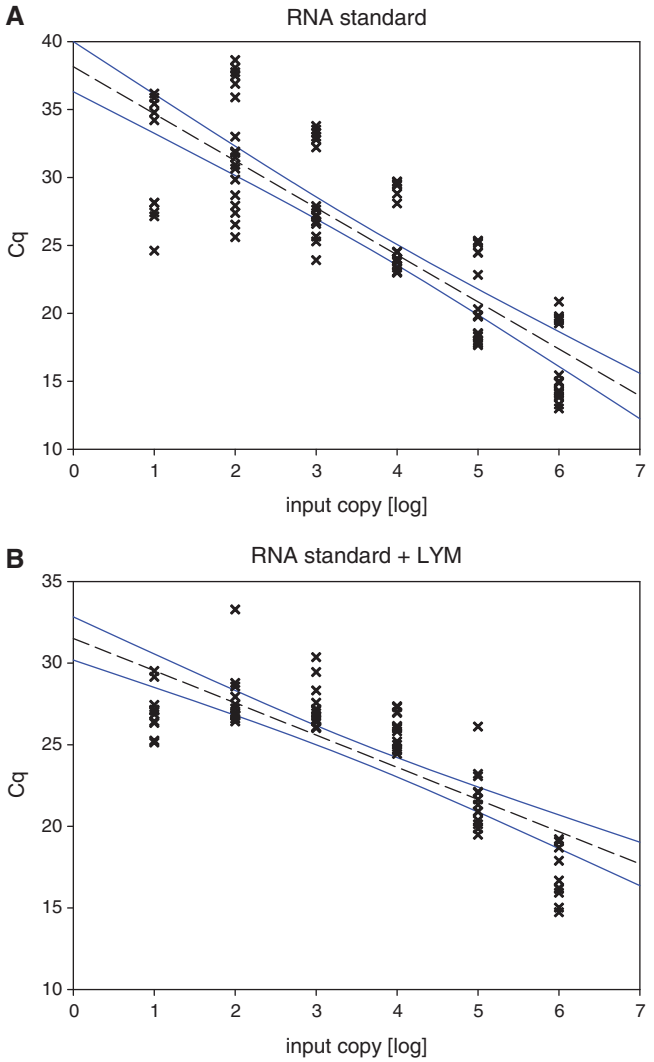

Figure 3. Input copy numbers versus threshold cycle value (Cq) including linear regression line and 95% confidence interval (CI) for (**A**) RNA standard amplification without LYM and (**B**) RNA standard amplification with LYM.

DISCUSSION

Comprehension of transcriptomics regulations on the single-cell level has become a new and challenging issue in the field of molecular biology and diagnostics (1,4,9,15). Especially in cancer research the analysis of circulating tumor cells (CTC) are based on the dynamics of mRNA marker gene expression of solitary cells (25). Real-time qPCR seems an ideal tool for single cell analysis, because it addresses researcher's need for single-cell expression profiling, ultra high sensitivity and a wide linear quantification range. It is well reported that RT–qPCR is the most sensitive technique with the widest quantification range for rare copy quantification in limited cell material (24). Gene expression analysis on single cellular level show high heterogeneity, so researchers try to understand the origin of such phenomena and which conclusions can be drawn for the cell physiology (2,6–8). The knowledge of biological derived transcriptional noise in single-cells makes it essential to decrease all possible technical variations and quantification noise of single-cell expression profiling experiments (18,19).

In this study we investigated the quantification and transcriptional noise using a combined analytical platform, reverse transcription or pre-amplification on single-cell array on glass slide and subsequent quantitative real-time PCR technology. Different experimental steps were investigated to induce technical variance like the flow cytometry cell sorting, the evaporation of standard on glass array, the pre-amplification step, the dilution of samples and the PCR amplification step itself. For any RNA quantitative study and especially for single-cell analysis it was already clearly shown that the sampling step induces most of the technical variance in the whole workflow, followed by the cDNA synthesis step (23,26). We investigated the source of expression noise in the pre-PCR steps, and we could confirm the expected high technical variability in the pre-PCR experimental steps.

The deposition failure on glass slides was relatively low and could be neglected because of the optical control by

Table 3. Differences in variation of RNA standard analysis, with and without sample evaporation step on the AmpliGrid slide (standard deviation = SD)

RNA standard	With evaporation		Without evaporation	
	Mean copies (SD)	Variation (%)	Mean copies (SD)	Variation (%)
10	107.23 (134.03)	124.99	16.55 (2.16)	13.03
10^2	106.82 (14.15)	13.25	74.26 (6.12)	8.24
10^3	898.38 (394.42)	43.90	702.46 (31.72)	4.52
10^4	5794.50 (3835.54)	66.19	9532.42 (876.84)	9.20
10^5	105840.86 (40 630.58	38.39	148757.37 (14082.09)	9.47
Mean	22549.56 (9001.74)	57.34	31816.61 (2999.78)	8.89

microscopy at the beginning of the experiment. Therefore the optical quality control is essential and only possible by using glass arrays and a microscope to proof single-cell presence and integrity. The central problem in single-cell deposition on any platform and using any consumables are: 'Is a single-cell there or not?' 'How many cells are deposited?' Of further importance is the location on the reaction spot on array or reaction vessel: 'Is the cell accessible for the chemicals, stabilizers, and enzymes?' And finally of interest is the cell integrity: 'Is the cell intact or disrupted?' 'Is the cellar nucleic acid integer or fragmented?' Further the accessibility of the chemicals, mainly the RNA stabilizers and the RNAse inhibitors, is essential and impacts the subsequent assay sensitivity and variability.

The quantitative PCR analysis performed on the realplex system showed the expected lowest technical variance (7 and 8%) over the entire quantification range down to 10 DNA molecules. The technical variation of one-step RT–qPCR experiments with RNA standard was shown to be mainly influenced by the RT step which was earlier reported as highly variable (26,27) and being influenced by the analyzed gene sequence and the type of reverse transcriptase enzyme.

Pre-amplification-qPCR experiments showed higher technical variations than without pre-amplification, mainly due to an additional dilution step. This step followed by nucleic acid dilution caused a technical variation of ~20%. Herein a major source of variability was induced by evaporation of the standard on the glass slides. By excluding the drying step and by optimizing the standard deposition directly in buffer the technical noise could be reduced to a minimum. The initial drying of RNA standard caused a very high technical variation and a lost of sensitivity, especially at very low concentrations. RNA might be degraded by the heating step wherefore such high variations within the replicates could be explained. Hence the RNA templates should be included directly into the reaction mix to allow RNA stabilization, prevent degradation and variation in later quantitative measurements. Following this advanced method for RNA quantification the technical variance could be limited to 4.5–13%, depending on the concentration range of the RNA standard. This shows that the single-cell quantification can be sensitive and accurate upon evaporation is prevented. The single-cell analysis system of BCB in combination with real-time qPCR detection seems to be sensitive enough to detect various copies at low concentrations out of one solitaire cell.

The results of the expression profiling trial impressively demonstrate the heterogeneity of expression patterns in individual cells and a log-normal distribution as already described by Bengtsson and co-workers (9). Some cells had up to 1000-times more mRNA copies per gene than others in their respective group. Even highly abundant housekeeping genes like GAPDH varied considerably

Table 4. PCR efficiencies, correlation coefficients (r^2) and slope of standard curves done for gene expression profiling analysis of GAPDH, IL-1β, TNFα and TLR4

	GAPDH	TLR4	IL1b	TNFα
PCR efficiency (%)	108.20	97.20	118.00	94.20
r^2	0.981	0.990	0.989	0.998
Slope	−3.14	−3.39	−2.96	−3.47

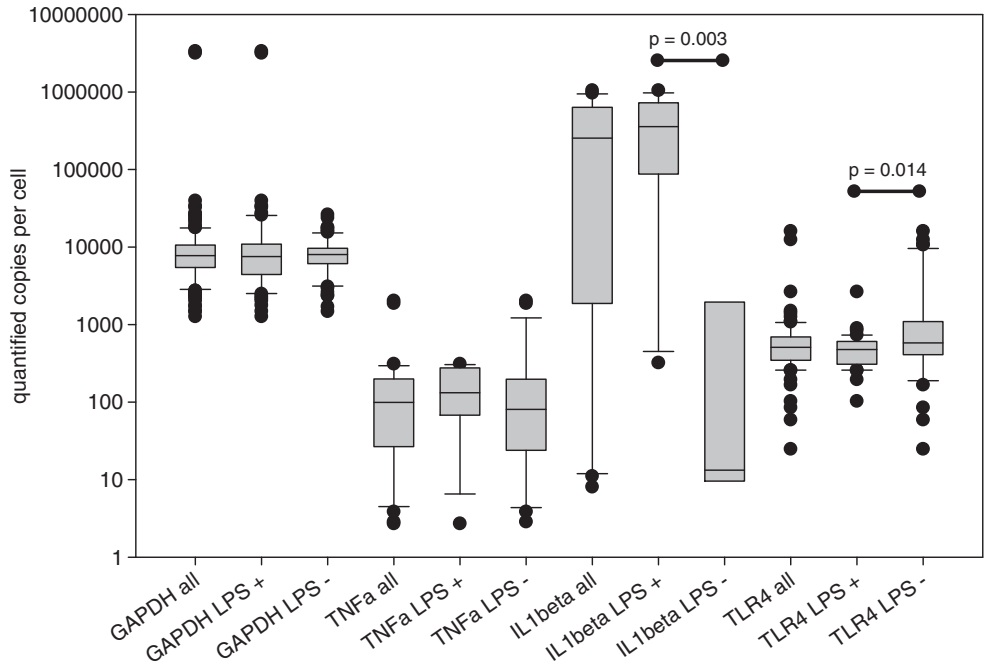

Figure 4. Box plot of quantified copies per cell for following marker genes: GAPDH, TNFα, IL-1β and TLR4. The median and the 10, 25, 75 and 90th percentiles are plotted.

Table 5. Overview of calculated copy numbers for GAPDH, TNFα, IL-1β and TLR4 including number of analyzed cells, geometric and arithmetic mean of copy numbers, median, standard deviation (SD), standard error (SE), minimal and maximal expression within group, coefficient of variation (CV), 95 and 99% confidence intervals (CI) and *P*-value between LPS treated and untreated group

	GAPDH			TNFα			IL-1β			TLR4		
	All	LPS^+	LPS^-	All	LPS^+	LPS^-	All	LPS^+	LPS^-	All	LPS^+	LPS^-
n	144	70	74	56	22	34	32	20	12	86	45	41
Geo. mean	8040	8510.57	7500.49	73.14	91.39	65.76	28315.2	141997.62	85.33	500.68	448.25	728.48
Arr. mean	54273.16	102527.09	8627.55	218.55	147.91	252.33	331819.69	423773.06	787.58	891.91	520.26	1970.87
Median	7734.75	7529.64	8026.84	99.17	131.93	80.58	254033.8	357373.84	13.26	509.07	476.35	579.79
SD	382711.08	546744.53	4650.49	449.18	103.88	542.26	361457.41	357624.41	1064.95	2174.61	387.15	3704
SE	31892.59	65348.47	540.61	77.03	31.32	113.07	75369.08	84292.882	476.26	240.15	60.46	578.467
Min. expr.	1269.12	1269.12	1479.93	2.71	2.71	2.85	8.08	321.56	8.08	24.82	102.93	24.82
Max. expr.	3356211.2	3356211.2	26225.88	2027.38	311.29	2027.38	1049671.6	1049671.6	2027.38	16052.66	2655.05	16052.66
CV (%)	705.16	533.27	53.9	205.53	70.23	214.9	108.93	84.39	135.22	243.82	74.41	187.94
95% CI	63043.01	130369.1	1077.45	156.73	69.78	234.5	156309.04	177846.09	1322.29	477.82	122.2	1169.15
99% CI	83265.94	173118.62	1429.93	210.57	99.27	318.74	212463.4	244319.82	2192.19	633.53	163.53	1564.55
P-value			0.925			0.357			0.003			0.014

between cells of the same treatment group as well as between LPS treated and untreated cells, whereby most of this variability was introduced by one outlier.

According to the MIQE guidelines in qPCR analysis a RNA quality check and normalization steps should always be included to avoid false positive results (16,17,28). RNA quality and quantity control is impossible in this experimental single-cell setup, due to lacking sensitivity of any RNA integrity analytical platform. The RNA 6000 pico chip for the 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, USA) is designed for RNA integrity detection from 200 to 5000 pg/μl RNA and was successfully tested with samples taken by laser micro dissection (29). For single-cells measurements the given RNA concentrations were out of the detection range and outperform the Bioanalyzer detection limit wherefore the RNA integrity measurement were also excluded (own data not shown). Hence optical quality control of single cell integrity on glass array is state of the art.

The classical normalization procedure applied in expression profiling is done on the level of validated stable expressed reference gens (16,17). For data normalization GAPDH was thought to be an optimal reference candidate, but the high variations in the copy numbers in different cells showed that in this study GAPDH could not be taken for further expression normalization. We tried to calculate the normalized expression on the level of each individual cell (using GAPDH), and we found out that it makes no sense. Single-cell expression data normalized with GAPDH showed boosted relative quantification values and high variability in treatment groups, which is in contrary to the normalization strategy with the goal to minimize technical variance (data not shown). Hence we fully agree, that data normalization on the basis of reference genes in not applicable (9,19). The relative gene expression strategy seems to be not valid for single-cell analysis, because cells individually vary significantly in expression magnitude, pattern and cellular timing of mRNA expression bursts.

'But how can the expression profiles of cell populations are compared?' It was proposed by Bengtsson and co-workers (9,19) that copy numbers are log-normal distributed over a cell population. We could confirm log-normal distribution using the KS-Test for almost all analyzed genes and applications, depending on sample size. Only groups with less replicates seem to follow no distribution, not even a $\log^{10}$-normal or ln-normal distribution. Therefore a box plot on an exponential scale was used for visualization. But as well the comparison of different groups of single-cells with individual treatments on the basis of the median expression level is still a point for future discussions!

CONCLUSION

This study could provide evidence that the gene detection and quantification out of one cell is technically possible down to a few DNA or RNA molecules per cell. But we have to take in mind that quantification noise is higher for RNA compared to DNA due to reverse transcription or pre-amplification noise. Natural variance of mRNA expression in single cells is much higher that the variance introduced by handling and the absolute quantification system. Nearly no effects on variation are introduced by the biological material on single-cell level, herein LYM, but this may change in other tissue types. Successfully a reliable quantification protocol for single-cell specific real-time RT–qPCR could be established. Anyhow many questions remain, regarding sample integrity control, cell dependent variability and data analysis in single-cell expression profiling experiments.

ACKNOWLEDGEMENTS

M.R. and B.K. were carrying out the real-time RT–qPCR, gene expression and data analysis and wrote the manuscript. H.M. and C.H. organized the cell culture experiments and did the flow cytometry cell sorting. M.P. had

the initial idea and has been involved in the data analysis part, supervising the lab work and in drafting the manuscript. W.M. and C.H. revised the manuscript for important content and final approval.

FUNDING

Beckmann Coulter Biomedical GmbH and BioEPS GmbH research funds. Funding for open access charge: Beckmann Coulter Biomedical GmbH and BioEPS GmbH.

Conflict of interest statement. None declared.

REFERENCES

1. Cai,L., Friedman,N. and Xie,X.S. (2006) Stochastic protein expression in individual cells at the single molecule level. *Nature*, **440**, 358–362.
2. Levsky,J.M. and Singer,R.H. (2003) Gene expression and the myth of the average cell. *Trends Cell. Biol.*, **13**, 4–6.
3. Itzkovitz,S. and van Oudenaarden,A. (2011) Validating transcripts with probes and imaging technology. *Nat. Methods*, **8(4 Suppl.)**, S12–S19.
4. Gao,W., Zhang,W. and Meldrum,D.R. (2011) RT-qPCR based quantitative analysis of gene expression in single bacterial cells. *J. Microbiol. Methods*, **85**, 221–227.
5. Stahlberg,A. and Bengtsson,M. (2010) Single-cell gene expression profiling using reverse transcription quantitative real-time PCR. *Methods*, **50**, 282–288.
6. Raj,A. and van Oudenaarden,A. (2008) Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell*, **135**, 216–226.
7. Raj,A. and van Oudenaarden,A. (2009) Single-molecule approaches to stochastic gene expression. *Annu. Rev. Biophys.*, **38**, 255–270.
8. Raj,A., Rifkin,S.A., Andersen,E. and van Oudenaarden,A. (2010) Variability in gene expression underlies incomplete penetrance. *Nature*, **463**, 913–918.
9. Bengtsson,M., Stahlberg,A., Rorsman,P. and Kubista,M. (2005) Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA level. *Genome Res.*, **15**, 1388–1392.
10. Warren,L., Bryder,D., Weissman,I.L. and Quake,S.R. (2006) Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. *Proc. Natl Acad. Sci. USA*, **103**, 17807–17812.
11. Kurimoto,K., Yabuta,Y., Ohinata,Y., Ono,Y., Uno,K.D., Yamada,R.G., Ueda,H.R. and Saitou,M. (2006) An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. *Nucleic Acid Res.*, **34**, e42.
12. Lao,K.Q., Tang,F., Barbacioru,C., Wang,Y., Nordman,E., Lee,C., Xu,N., Wang,X., Tuch,B., Bodeau,J. *et al.* (2009) mRNA-sequencing whole transcriptome analysis of a single cell on the SOLiD system. *J. Biomol. Tech.*, **20**, 266–271.
13. Stahlberg,A., Andersson,D., Aurelius,J., Faiz,M., Pekna,M., Kubista,M. and Pekny,M. (2010) Defining cell populations with single-cell gene expression profiling: correlations and identification of astrocyte subpopulations. *Nucleic Acids Res.*, **39**, e24.
14. Tang,F., Barbacioru,C., Wang,Y., Nordman,E., Lee,C., Xu,N., Wang,X., Bodeau,J., Tuch,B.B., Siddiqui,A. *et al.* (2009) mRNA-Seq whole-transcriptome analysis of a single cell. *Nat. Methods*, **6**, 377–382.
15. Tang,F., Lao,K. and Surani,M.A. (2011) Development and applications of single-cell transcriptome analysis. *Nat. Methods*, **8(4 Suppl.)**, S6–S11.
16. Bustin,S.A., Benes,V., Garson,J., Hellemans,J., Huggett,J., Kubista,M., Mueller,R., Nolan,T., Pfaffl,M.W., Shipley,G.L. *et al.* (2009) The MIQE guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.*, **55**, 611–622.
17. Vandesompele,J., Kubista,M. and Pfaffl,M.W. (2009) Chapter 4: Reference gene validation software for improved normalization. In Logan,J., Edwards,K. and Saunders,N. (eds), *Real-Time PCR Technology Publisher*. Caister Academic Press, London, pp. 47–64.
18. Reiter,M. and Pfaffl,M. (2008) Effects of plate position, plate type and sealing systems on real-time PCR results. *Biotechnol. Biotechnol. Equip.*, **22**, 824–828.
19. Bengtsson,M., Hemberg,M., Rorsman,P. and Stahlberg,A. (2008) Quantification of mRNA in single cells and modelling of RT-qPCR induced noise. *BMC Mol. Biol.*, **9**, 63.
20. Taniguchi,K., Kajiyama,T. and Kambara,H. (2009) Quantitative analysis of gene expression in a single cell by qPCR. *Nat. Methods*, **6**, 503–506.
21. Miquet,S., Dopfer,E.P., Pollmer,C., Freudenberg,M.A., Glanos,C., Reth,M., Huber,M. and Schamel,W.W. (2008) Enhanced B-cell activation mediated by TLR4 and BCR crosstalk. *Eur. J. Immunol.*, **38**, 2475–2487.
22. Li,J., Peet,G.W., Balzarno,D., Li,X., Massa,P., Barton,R.W. and Marcu,K.B. (2001) Novel NEMO/Ikappa B kinase and NF-kappa B target genes at the pre-B to immature B cell transition. *J. Biol. Chem.*, **276**, 18579–18590.
23. Stahlberg,A., Kubista,M. and Pfaffl,M.W. (2004) Comparison of reverse transcriptases in absolute and relative gene expression analysis. *Clin. Chem.*, **50**, 1678–1680.
24. Pfaffl,M.W. (2004) Quantification strategies in real-time RT-PCR. In Bustin,S.A. (ed.), *The real-time PCR Encyclopaedia A-Z of quantitative PCR*, 1st edn. International University Line, La Jolla, CA, pp. 87–120.
25. Yeh,C.S., Wang,J.Y., Wu,C.H., Chong,I.W., Chung,F.Y., Wang,Y.H., Yu,Y.P. and Lin,S.R. (2006) Molecular detection of circulating cancer cells in the peripheral blood of patients with colorectal cancer by using membrane array with a multiple mRNA marker panel. *Int. J. Oncol.*, **28**, 411–420.
26. Tichopad,A., Kitchen,R., Riedmaier,I., Becker,C., Stahlberg,A. and Kubista,M. (2009) Design and optimization of reverse-transcription quantitative PCR experiments. *Clin. Chem.*, **55**, 1816–1823.
27. Stahlberg,A., Hakansson,J., Xian,X., Semb,H. and Kubista,M. (2004) Properties of the reverse transcription reaction in mRNA quantification. *Clin. Chem.*, **50**, 509–515.
28. Becker,C., Hammerle-Fickinger,A., Riedmaier,I. and Pfaffl,M.W. (2010) mRNA and microRNA quality control for RT-qPCR analysis. *Methods*, **50**, 237–243.
29. Kerman,I.A., Buch,B.J., Evans,S.J., Aki,l.H. and Watson,S.J. (2005) Combining laser capture microdissection with quantitative real-time PCR: Effects of tissue manipulation on RNA quality and gene expression. *J. Neurosci. Methods*, **1153**, 71–85.