Doxorubicin Treatment of Cancer Cells Impairs Reverse Transcription and Affects the Interpretation of RT-qPCR Results

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Abstract. *Background: Doxorubicin (DOX) acts in a variety of ways including DNA damage and enzyme inhibition, which consequently causes changes in gene expression of cells treated with this agent. Practical validation of experimental results followed by appropriate normalization of the factors investigated is crucial for obtaining biologically relevant results in gene expression studies. Materials and Methods: Six candidates were evaluated regarding their validity as internal reference genes: RPS23, FLOT2, UBB, ABCF1, ACTB, HPRT1. Optimization for quantitative polymerase reaction (qPCR) included: sensitivity, specificity, amplification efficiency and linear dynamic range determination. The gene expression stability was evaluated by real-time quantitative polymerase reaction (RT-qPCR) in two human cervical cancer cell lines: HeLa and DOX-resistant KB-V1 Cells treated under various concentrations of DOX. Results: DOX treatment changed gene expression and led to re-optimization of the cDNA template amounts. ACTB, HPRT1, RPS23 and FLOT2 are proposed to be sufficient as internal reference genes. Conclusion: DOX may alter the reverse transcription and amplification reactions of RT-qPCR, thus creating a risk of misinterpretation of gene expression results.*

In vitro studies of cellular mechanisms related to response to chemotherapeutics offer a good means to find effective treatments for cancer. The ability of cancer cells to develop drug resistance against cytostatic drugs is considered one of the main causes of treatment failure. An understanding over the mechanisms underlying cancer cell behavior, including drug resistance, would hopefully have a large impact on future therapeutic efficacy, and elucidation of these mechanisms is undoubtedly greatly anticipated in the clinical environment (1).

Out of the various drugs used in anticancer therapy, doxorubicin (DOX) is the one typically receiving great amount of attention due to its wide use in the treatment of multiple types of cancers. DOX belongs to the anthracyclines, and its mechanisms of anti-mitotic and cytotoxic activity have been primarily linked to initiation of DNA damage *via* inhibition of topoisomerase II, intercalation between base pairs, direct membrane effects, and generation of reactive oxygen species (ROS) with subsequent lipid peroxidation and DNA damage (2, 3). Moreover, DOX inhibits polymerase activity and affects the regulation of expression of various genes (4).

The development of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) has facilitated detecting the changes in gene expression, allowing for more in-depth analysis of the effects induced by cytostatic drugs. This technique allows for detection of the fluorescence intensity of the synthesized DNA fragment of a particular target gene, produced from template cDNA during a series of sequential amplification reactions using specific primers (5, 6). RT-qPCR analysis is a multi-step process, but it is potentially the most sensitive, fastest and most precise technique that can be used for gene expression evaluation.

For obtaining biologically relevant results, appropriate normalization is critical. In RT-qPCR, this involves comparison of the expression of the target genes with the stable, constitutively expressed internal reference genes. Expression of a reference gene is a standard measure for evaluating the number of target cDNA copies in each sample, as it is subject to the same errors during cDNA preparation as the target gene (7, 8). The use of reference genes also allows for controlling sample-to-sample variation throughout the experiment (9). However, gene expression levels are labile and difficult to predict, and no single gene is able to fulfill the criteria of a universal reference gene for every experimental condition (10). Housekeeping genes are involved in basic cell maintenance and therefore, their expression is expected to be constant between the cells of

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different tissues and under different experimental conditions (11). The most commonly used reference genes used as internal controls in expression studies include actin B (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*), tubulins, cyclophylin (*CYC*), albumin, 18S or 28S rRNA, but there are also many other genes selected for individually designed experiments (12). Expression of some popular and traditionally used housekeeping genes, however, has been found to vary considerably depending on the tissue, organism or experiment type. Using an inapropriate internal reference based on presumed gene expression stability may result in overlooking or even misinterpretation of the study findings, a fact that is highlighted in many recent research papers (13-16).

A standard deviation of less than 2-fold from the mean expression level of the gene is usually a requirement for suitability as a reference gene (16). There exist many factors contributing to expression stability: the type of cells under investigation, gene locus, structure and sequence, its function, predicted expression level, presence or absence of pseudogenes and mobile genetic elements (17). Microbiological contaminants can also create a serious issue and affect both cell viability and expression stability. It is especially important to take these factors into account in experiments with cancer cells, that are often found to have altered expression levels of many genes, including housekeeping genes (12, 17-23). In general, cancer cells lines are considered naturally unstable and prone to genetic rearrangements, which make maintaining satisfactory gene expression consistency very difficult. Tumor cells are often characterized by more variable phenotypic changes than non-malignant cells, which is related to activation or quenching of different group of genes due to genome instability. Treatment of the cells with cytostatic drugs (like DOX) affecting DNA can also unpredictably change the gene expression pattern. It is, therefore, critical to perform the practical validation to confirm the expression stability of each candidate reference gene before they are selected for the normalization of RT-qPCR data generated in the studies (24, 25).

A normalization strategy based on multiple validated reference genes is much more accurate than the use of a single non-validated reference gene. In general, it is recommended to use between two and five validated stably-expressed reference genes for normalization of the expression data (26, 27). There exist a few methods for the selection of internal reference genes (28). There are software-based approaches, like the Global Pattern Recognition Analysis Tool used to analyze the results obtained from StellARray System. Based on the uploaded amplification results, the program determines the most stable genes from a tested panel, which allows for simultaneous use of many reference genes in the study. Although accurate and fast, this approach requires the dedicated software and, in some cases, has limited applications. The most common strategy of selection consists of comparison of a wide variety of potential reference genes on the basis of existing literature and databases and narrowing the range to the genes that meet the criteria for optimal primer design. Gene function in the cell should also be taken into account, as there is a risk that the gene expression would be affected in the given experimental conditions. Regardless of the results of *in silico* analysis, each candidate for internal reference gene must also be experimentally validated for use in each individual study.

In the present study, six candidate internal reference genes were validated. Firstly, the best performing primer concentrations for a given assay were determined. Then, the amplification effectiveness within a range of template concentrations was measured. Then the expression stability of the selected genes was evaluated in two cervical cancer cell lines: HeLa and DOX-resistant KB-V1 cells, following treatment of the cells with DOX.

Materials and Methods

Cell lines and culture. All cell lines were checked for the presence of *Mycoplasma* spp. with qPCR technique using an in-house validated detection method (manuscript in preparation).

Human B-lymphocyte EBV transformed GM14467 cells were obtained from Coriell Cell Respositories, USA. GM14467 cells were cultured at 37˚C in 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma, Saint Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (Lonza Walkersville Inc., Walkersville, MD, USA) and 1x Antibiotic Antimycotic (Sigma), with final concentrations of 0.1 Units penicillin, 0.1 mg streptomycin and 0.25 μg Amphotericin B per ml. The cells were cultured by exchange or dilution of the culture medium every 2 to 3 days to maintain the cell density between 2×10^5 and 4×10^5 cells per ml. Prior to DNA isolation cells were pelleted by centrifugation at 1,500 rpm for 5 min, then washed with calcium and magnesium ion free PBS (IITD, Wrocław, Poland) and centrifuged again. Cell pellets were used immediately for nucleic acid isolation.

HeLa cells were purchased from American Type Culture Collection, Manassas, VA, USA and MDR resistant KB-V1 cells - from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. KB-V1 is a multidrug-resistant sub-clone derived from KB-3-1, which itself is a derivative of HeLa cells. The KB-V1 cell line, developed through a selection with vinblastine, expresses the multidrug-resistant phenotype, comprising of resistance to colchicine and DOX (29). In order to maximize gene expression results consistency, cell cultures used in each independent repetition of the experiment originated from the same passage and were derived from the common cell masterbanks. After thawing, each cell culture undergone repetitive culturing and treatment schedule. HeLa and KB-V1 cells were cultured at 37˚C in 5% CO_2 in Minimum Essential Eagle's Medium (Sigma, Saint Louis, USA), supplemented with 10% heat-inactivated fetal bovine serum (Lonza Walkersville Inc.) and 1x Antibiotic Antimycotic (Sigma), with final concentration of 0,100 units penicillin, 0.1 mg streptomycin and

0.25 μg Amphotericin B per ml. Cells were passaged using 75 cm^2 cell culture flasks at a density 2×10^6 cells per flask and maintained until reaching 90% confluence. Cell dissociation was routinely achieved by removal of the culture medium followed by washing with calcium and magnesium ion free PBS (IITD, Wrocław, Poland) and subsequent incubation for 2 min at 37˚C in the presence of 0.5% Trypsin-EDTA 10x, without phenol red (Gibco, Carlsbad, Germany). The trypsinized cells were resuspended in culture medium and seeded into a new flask.

Cell treatment and preparation. After seeding at a density of 2×10^6 cells per flask HeLa and KB-V1 cells were incubated for 24 h at 37˚C in 5% CO_2. Culture medium was removed, replaced with fresh medium in the presence or absence of DOX (Sigma) and incubated for another 24 h. The tested DOX concentrations were as follows: (i) 4.5 μM DOX and (ii) 9.0 μM DOX (HeLa) or (i) 4.5 μM DOX and (ii) 69.0 μM DOX (KB-V1). The higher DOX concentrations (9.0 μM and 69.0 μM) used throughout the study were the same as in our previous experiments (30) and were about 80% of the IC50 values established for the respective cells. The lower DOX concentration (4.5 μM), was a half the concentration used for the sensitive HeLa cells. Following cell treatment, the medium was discarded, and cells were washed with calcium and magnesium ion free PBS, trypsinized and suspended in fresh culture medium. Cells were centrifuged at 1,500 rpm for 5 min, then washed 3 times with calcium and magnesium ion free PBS (IITD, Wrocław, Poland) and centrifuged again. Cell pellets were used immediately for nucleic acid isolation.

DNA isolation. DNA was isolated from $5x10^6$ cells using the GeneMATRIX Cell Culture DNA Purification Kit (EURx, Gdańsk, Poland), according to the manufacturer's instructions. The DNA concentration was measured using the NanoDrop 2000™ UV-Vis Spectrophotometer (Thermo Scientific) and quantified by absorbance measurements and 260/280 and 260/230 ratios analysis. DNA samples were stored at –20˚C.

RNA isolation and purification. Total RNA was isolated from $5x10^6$ cells using the GeneMATRIX Universal RNA/RNAi Purification Kit (EURx, Gdańsk, Poland), according to the manufacturer's instructions. The RNA concentration and quality was assessed using the NanoDrop 2000™ UV-Vis Spectrophotometer (Thermo Scientific) by absorbance measurements and 260/280 and 260/230 ratios analysis. After isolation, two RNA purification steps were performed to ensure the source and quality of nucleic acid in further qPCR amplification. To prevent RNA degradation by RNases A, B and C, the Ribolock Ribonuclease Inhibitor (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was added at a final concentration of 1 Unit per 1 μl RNA. Purification from the residual DNA contamination was performed using the RapidOut DNA Removal Kit (Thermo Fisher Scientific Inc., Vilnius, Lithuania), according to the manufacturer's instructions. Total RNA samples were stored at –70˚C.

RNA purity evaluation. Total RNA extracted from the samples was examined by qPCR prior to and following RNase and DNA purification to exclude the possibility of genomic-DNA contamination. The non-treated RNA samples showed the presence of specific amplicons from genomic DNA, whereas after two-step purification no signal was detected.

Reverse transcription (RT). RT reactions were carried out using the PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa Bio Inc., Otsu, Japan). The cDNA was synthesized starting from 500 ng of purified total RNA. The reactions in a final volume of 20 μl contained 1x PrimeScript Buffer, PrimeScript RT Enzyme Mix I, and the combination of 25 pmol oligo(dT) and 50 pmol random hexamer primers. Samples were incubated at 37˚C for 30 min, and then the RT enzyme was inactivated by heating to 85˚C for 5 sec. cDNA samples were stored at -20˚C.

Quantitative PCR (qPCR). qPCR was carried-out with the Takara SYBR®Premix Ex Taq (Tli RNaseH Plus) (TaKaRa Bio Inc., Otsu, Japan) in a final volume of 20 μl, with final concentrations of 1x PrimeScript Buffer, the appropriate forward and reverse primer, and 10 ng of cDNA or DNA template. Varying amounts (that were established during optimization) of the forward and reverse primers were used. Fluorescent detection was performed using the Mx3005P System (Stratagene, La Jolla, CA, USA) with the following thermal cycling conditions: initial polymerase activation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 2 min and annealing/extension at 60˚C for 30 sec. After amplification, dissociation (melting) curve analysis was performed (95˚C for 1 min, 55˚C for 30 sec and 95˚C for 30 sec) to analyze the product melting temperature. Each sample was amplified in duplicate or triplicate wells. Negative (no template) and positive (genomic DNA template) controls were included in each assay. Results were analyzed using MxPro3005P Software. The threshold cycle (Ct) at which the amount of amplified target reached a fixed threshold was determined.

Gene selection and primer design. On the basis of literature data the initial list of the most commonly used internal reference genes was prepared (31-36). DNA sequence from mRNA of each gene was retrieved from GenBank (www.ncbi.nlm.nih.gov) and analyzed *in silico* using Beacon Designer software v. 7.7 from Premier BioSoft International. Attempts to design the amplicons of desired length located within the long coding DNA sequences (CDS) resulted in narrowing the gene candidates list. Primer design met the following thermodynamic and sequence criteria set on the basis to the commonly used qPCR guideline (37): i) Primer length within the range of 18 to 22 bp; ii) Amplicon length of maximum 150 bp; iii) 50% GC pair content; iv) Melting temperature of Fw and Rv primers close to 60˚C; v) Similar melting temperatures of forward and reverse primers (±1˚C); vi) Low risk of forming of secondary structures (hairpins, self-dimers, cross-dimers).

BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) was performed to ensure that the chosen primer sequences were sufficiently different from the rest of the other species transcriptome. Comparative analysis with human and mouse genomic sequences was performed. The above-described strategy allowed for selection of sets of primers with the best physicochemical characteristics. The genes eventually used for RT-qPCR assay design were: ribosomal protein S23 (*RPS23*), flotillin 2 (*FLOT2*), ubiquitin B (*UBB*), ATP binding cassette subfamily F (*ABCF1*), actin B (*ACTB*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), as outlined in Table I. Taking into account the amplified gene functions, were not (except for *ABCF1*) known to be directly involved in drug resistance mechanisms, which could be affected by given experimental conditions. All primers were obtained from Oligo (Oligo at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland).

Table I. *Nucleotide base sequences of primers designed for RT-qPCR assays.*

Gene name	Gene symbol	Gene ID	Accession number	Primer sequence (5' → 3')	Position (bp)	Amplicon length (bp)	Design method
Ribosomal protein S23	*RPS23*	6228	NM_001025	Fw: TAGGATCAAGAGCAGAACC Rv: GCAAGGAACCATAGTAACAG	1005-1079	75	Beacon Designer software v. 7.7 (Premier BioSoft International)
Flotilin 2	*FLOT2*	2319	NM_004475	Fw: GAAGGAGATGCTGGATGT Rv: CTCACTGAAGGCTGACTT	717-807	91	
Ubiquitin B	*UBB*	7314	NM_018955	Fw: AAGCCTAAACTGCCTCTC Rv: GTTGCGTCACTTATCACC	163-257	95	
ATP binding cassette subfamily F	*ABCF1*	23	NM_001090	Fw: AAGACCACACTCCTCAAG Rv: CTCACACAGCAACACATC	1005-1079	75	
Actin B	*ACTB*	60	NM_001101	Fw: TCGTGCGTGACATTAAGGAG Rv: GAAGGAAGGCTGGAAGAGTG	707-882	176	(35)
Hypoxanthine phosphoribo-syltransferase 1	*HPRT1*	3251	NM000194.2	Fw: GTTGGATACAGGCCAGACTTTGTTG Rv: GATTCAACTTGCGCTCATCTTAGGC	681-842	163	(36)

Results

qPCR optimization. Optimization steps were performed using genomic DNA obtained from GM14467 cells as a reference template, and included determination of sensitivity, specificity, amplification efficiency and linear dynamic range.

Primer concentration optimization. Two primer concentrations: 200 nM and 300 nM and their combinations were investigated, with template concentration and all other reaction conditions kept unchanged. Primer concentration combinations that were optimal in terms of qPCR reaction sensitivity, efficiency and specificity were selected on the basis of: Ct value, fluorescence intensity and amplicon Tm and melting curve shape. The best primer concentration combinations were the ones that resulted in the best (most acute) amplification curve, and lowest Ct value. In case of comparable results, a primer set with a strong amplification curve was preferable on the one with the low Ct. Obtained data are presented in Figure 1. The performance of the primer concentration combination for *RPS23*, *FLOT2* and *UBB* genes were almost the same in terms of Ct values and amplification curve geometry. For *ABCF1* there was one set that gave clearly the best results in terms of obtained fluorescence intensity (Figure 1). For all tested primer concentrations, specific amplicons of expected size were obtained and no non-specific secondary structures were reported (based on the melting curve analysis). The optimal concentrations of *ACTB* and *HPRT1* primers had been previously established in our laboratory and were 300 nM/300 nM for both amplicons (manuscript in preparation).

Efficiency of qPCR with various template concentrations. The qPCR with the optimal primer combination was further tested for the efficiency of the amplification reaction within the broader range of the template concentrations. The linearity of the reaction was assessed for all tested genes. Genomic DNA concentrations ranging from 0.01 ng/μl to 100 ng/μl in 10-fold serial dilutions were prepared and tested in triplicate. A calibration curve approach was used to establish the obtained results. The slope of the standard curve was calculated to describe the efficiency of qPCR.

The resulting standard curves obtained for *FLOT2, ACTB, UBB* and *ABCF1* (Figure 2), with high correlation coefficiency (r^2>0.99), indicated strong linear relationships over the 4-log range. The standard curves obtained for two genes, *RPS23* and *HPRT1*, with the highest Ct values, showed the linear correlation over the 3-log and 2-log range, respectively. *HPRT1* was the only gene that gave the r^2<0.99, that is related to the lowest value of the dynamic range with comparable efficiency to the other tested genes. qPCR amplification efficiency for each assay was calculated from the slope of the standard curves, and ranged from 104.1% to 109.5%. A template amount of 10 ng per reaction resulted in the best assay performance and measurable Ct values, and was chosen for further experiments.

After establishment of the above-described test parameters using GM14467 reference DNA, pilot runs with HeLa and KB-V1 DNA and cDNA templates were performed, and the specificity of each of the amplification products was confirmed (data not shown).

Normalization-selection of endogenous reference genes. Normalization was performed using cDNA obtained from

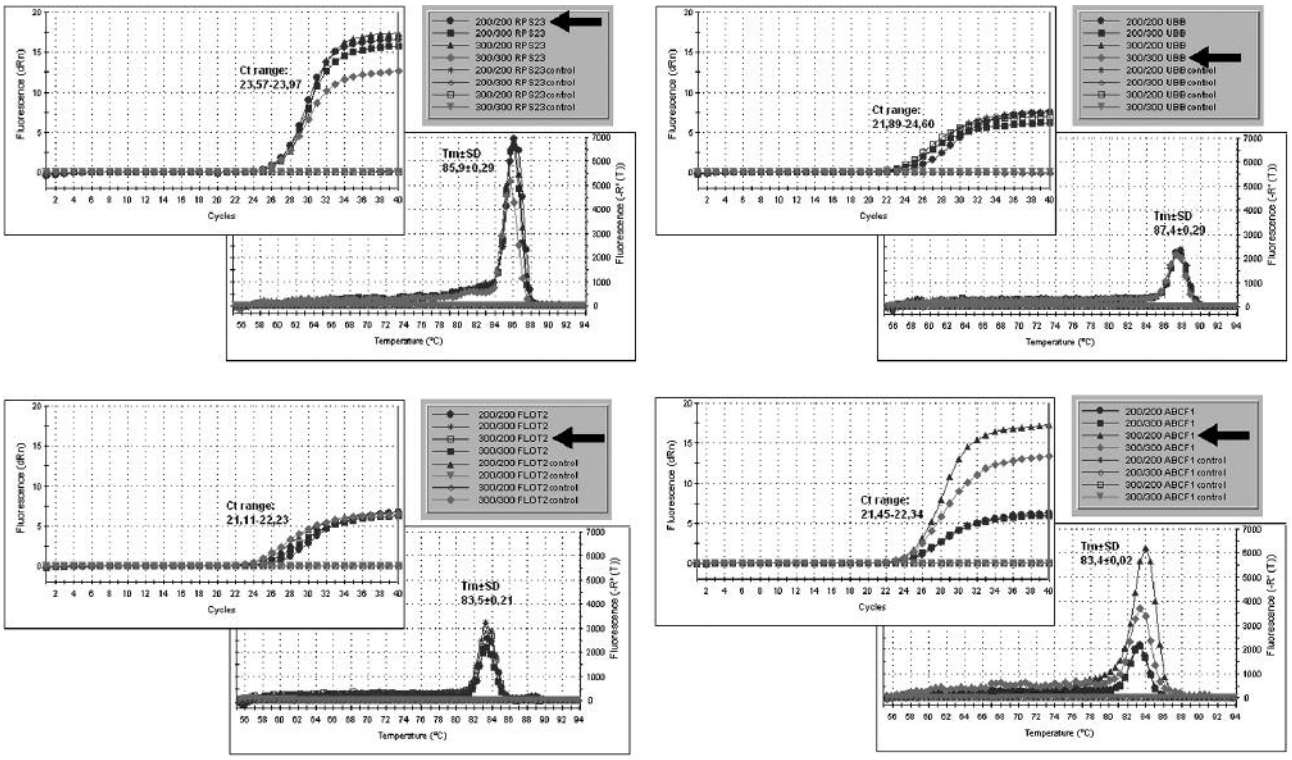

Figure 1. *Optimization of the primer concentrations used for qPCR. Amplification curves for the primer concentration combinations and the respective melting curves are presented. qPCR reactions were performed using genomic DNA obtained from GM14467 cells. No amplification was reported for the negative (no template) controls. No non-specific amplification products were obtained. The mean Ct values and melting temperatures (Tm) were calculated from the amplification curves. The optimal concentration combinations for each primer are indicated by arrows. The primer concentration combinations are given in nM of the forward/reverse primer per amplification reaction.*

HeLa and KB-V1 cells, in order to ensure that the cDNA synthesis was consistent, in the meaning that the Ct values obtained for the selected genes were similar in different experimental settings (see Cell treatment and preparation), demonstrating whether the target gene expression was affected by treatment with DOX.

Data obtained from 3 independent series of experiments showed that the expression levels of genes were reproducible and consistent. However, in all repetitions of the experiment exposure of cells to DOX resulted in increase of the Ct values for about 2-3 cycles. The negative effect of DOX on the Ct values obtained for all the tested genes was positively correlated with the DOX concentration used in the study. In order to optimize the results the amount of the template cDNA was re-calculated. To lower the Ct for 1 cycle the amount of the cDNA template was doubled, and was 20 ng per qPCR reaction. In the case of HeLa cells treated with 9.0 μM DOX this modification resulted in acceptable Ct values. For the corresponding template obtained from KB-V1 cells exposed to 69.0 μM DOX, doubling of the template input still did not give satisfactory normalization results (SD>1 cycle). After increasing the cDNA amount to 25 ng per reaction acceptable results were obtained. After optimization of the template amounts normalization was then performed again, with all the other experimental settings kept unchanged.

The results obtained after re-optimization are presented in Tables II and III. The Ct values observed after treatment of cells with DOX were compared against results of untreated cells. The genes that met the criteria as reference genes in the tested experimental conditions (set as SD of the obtained Ct values varying for max 2 cycles) are: *HPRT1, ACTB, FLOT2, RPS23* in both cell lines, and additionally *ABCF1* in KB-V1 cells. The *UBB* gene expression stability was found to be insufficient to use this gene as a normalizer.

Inhibition of RT reactions by DOX. The correlation between DOX concentrations used in the study and the obtained RT-qPCR results has raised the concern of whether the observed relationship was a result of simultaneous lower expression of all tested genes, or the inhibitory effect of DOX residue in the samples. It was, therefore, necessary to verify the possibility of DOX affecting the RT reactions, that may

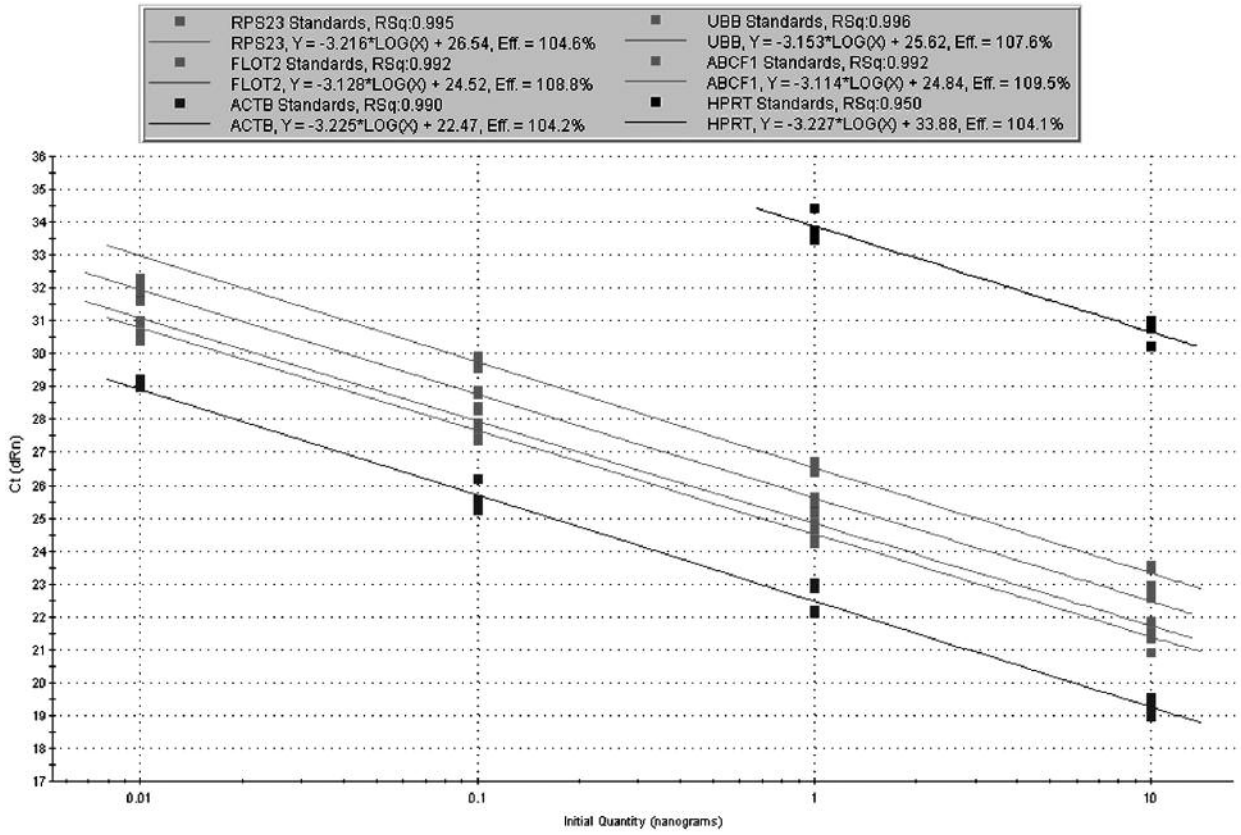

Figure 2. *Standard curves for calculation of qPCR efficiency. qPCR reactions were performed using genomic DNA obtained from GM14467 cells. The slope of the plot is -1/log (efficiency). The intercept is the log of the amount of template DNA at threshold divided by the log of the efficiency. The standard curves were obtained from the values from three replicates of each sample and were calculated using the MxPro Software.*

result in a lower amount and quality of the produced cDNA, leading to misinterpretation of the RT-qPCR results.

To assess whether the presence of DOX has an impact on reverse transcriptase activity, the RT reaction mixtures were prepared using RNA obtained from the control (non-treated) HeLa cells and loaded with different DOX concentrations: 0.001 μM, 0.1 μM, 10 μM, 100 μM, 500 μM and 1000 μM. After cDNA synthesis, the RT-qPCR amplification of *ACTB* and *HPRT1* gene fragments was performed and the obtained Ct results were analyzed. The choice of *ACTB* and *HPRT1* for analysis was justified, according to their different Ct values, to evaluate the impact of DOX on genes with both high (*ACTB*) and medium (*HPRT1*) expression levels. The quality of the obtained cDNA was assessed using the NanoDrop 2000™ UV-Vis Spectrophotometer (Thermo Scientific) by measurements of absorbance 260/280 and 260/230 ratios.

The obtained results are presented in Figure 3. DOX concentrations of 10 μM and lower did not change the Ct values of *ACTB* and *HPRT1* amplicons. However, concentrations of 100 μM, 500 μM and 1000 μM significantly increased the obtained Ct values of both genes, compared to the unloaded samples. At the same time, a significant decrease in cDNA quality (A_{260}/A_{230}>1.5) was reported. The loss of cDNA quality was proportional to the DOX concentration in RT reaction mixtures, indicating that it may have an inhibitory activity against the reverse transcriptase.

Although the effects on RT reactions were proportional to DOX concentration, they were diversified between the tested genes (Figure 3). While DOX used at the concentration of 100 μM increased the *ACTB* and *HPRT1* Ct values equally by 1 cycle, the concentration of 500 μM resulted in the increase by 11 and 7 cycles; and 1000 μM – by more than 27 and by 18 cycles, respectively.

Inhibition of RT-qPCR by DOX. After the evaluation of the impact of DOX on the RT reaction it was reasonable to confirm its effect directly on RT-qPCR amplification. The qPCR reactions were loaded with DOX concentrations equal to those used for RT reactions: 0.001 μM, 0.1 μM, 10

Table II. *Normalization results in HeLa cells treated with DOX.*

	RPS23	*FLOT2*	*UBB*	*ABCF1*	*ACTB*	*HPRT1*
HeLa	23.78±0.00	20.90±0.05	23.74±0.00	20.09±0.00	12.90±0.05	20.78±0.30
HeLa+4.5 μM DOX	24.87±0.12	22.05±0.14	25.08±0.17	22.23±0.24	13.43±0.20	21.91±0.24
HeLa+9.0 μM DOX	24.95±0.04	22.39±0.39	27.40±0.10	21.32±1.16	13.32±0.26	22.12±0.13
MEAN	24.53	21.78	25.41	21.21	13.22	21.60
Fold change	1.17	1.49	3.66	2.14	0.52	1.35

Table III. *Normalization results in KB-V1 cells treated with DOX.*

	RPS23	*FLOT2*	*UBB*	*ABCF1*	*ACTB*	*HPRT1*
KB-V1	25.22±0.00	20.44±0.06	22.77±0.00	19.19±0.00	13.71±0.12	20.45±0.04
KB-V1+4.5 μM DOX	26.16±0.09	20.54±0.17	24.00±0.13	19.90±0.17	13.93±0.14	21.34±0.14
KB-V1+69.0 μM DOX	27.04±0.06	22.10±0.46	26.16±0.06	20.88±0.16	15.42±0.14	22.44±0.15
MEAN	26.14	21.03	24.31	19.99	14.35	21.41
Fold change	1.82	1.66	3.39	1.69	1.72	1.99

μM, 100 μM, 500 μM and 1000 μM. After amplification of *ACTB* and *HPRT1* genes the obtained Ct results were analyzed. The obtained results are presented in Figure 3.

It was observed that the presence of DOX in the qPCR reaction mixtures at concentrations below 10 μM did not significantly increase the Ct values of the tested genes. At the concentration of 10 μM, DOX increased the Ct of *ACTB* by 7 cycles, and the Ct of *HPRT1* – by 3 cycles. When it was used at a concentration of 100 μM or higher the *ACTB* and *HPRT1* signals were undetectable, and therefore no Ct values were reported (Figure 3).

Discussion and Conclusion

DOX acts in a variety of ways and its cytotoxic properties are valuable in anticancer therapy. It is known that DOX inhibits the DNA polymerase activity, forms complexes with DNA by intercalation between base pairs and generates free radicals that oxidize bases resulting in DNA break-down. It can also inhibit topoisomerase II activity by stabilizing the DNA-topoisomerase II complex, which prevents certain regions of DNA from being transcribed by polymerase. The very early studies of conducted in the 80's showed that DOX and its derivatives have a strong inhibitory effect on RT enzymes of human immunodeficiency virus and Rauscher leukemia virus. These early findings provided a basis for use of this drug in combined anti-retroviral therapy (38-41). The present study showed that the above-mentioned properties of DOX can be problematic in *in vitro* studies of gene expression.

In RT-qPCR analysis, not only the levels of gene expression, but also the effectiveness of the enzymatic reactions: RT and qPCR amplification affect the strength of the detected signal. The difficulties noticed during normalization in the study, that eventually led to the necessity of re-optimization of the template input, brought to our attention the possibility of these reactions being inhibited by DOX residues remaining in the samples. The concern was whether DOX activity can affect the reverse transcription and qPCR amplification. In fact, it has been proven in the study that DOX under certain concentrations can significantly change the effectiveness of both reactions. Alteration of their efficiency had led to decrease in cDNA amount, quality and lower specificity of the amplification products, and created a serious risk of misinterpretation of the expression results. Moreover, the obtained results indicated that the inhibitory activity of DOX affected particular regions of cDNA differently. This makes the prediction of the possible impact of DOX on the gene expression pattern even more problematic.

Surprisingly, high concentrations of DOX in the reaction mixtures were required to eventually change the Ct of the tested genes, *ACTB* and *HPRT1*. This finding suggests that HeLa and KB-V1 cells are able to accumulate and sustain very high amounts of DOX, which may further be transferred during RNA isolation and contaminate the sample. The visual examination made throughout the study indicated that DOX can be accumulated during exposure of cells, as DOX-treated cells were stained intensely red, whereas untreated cells remained colourless (data not

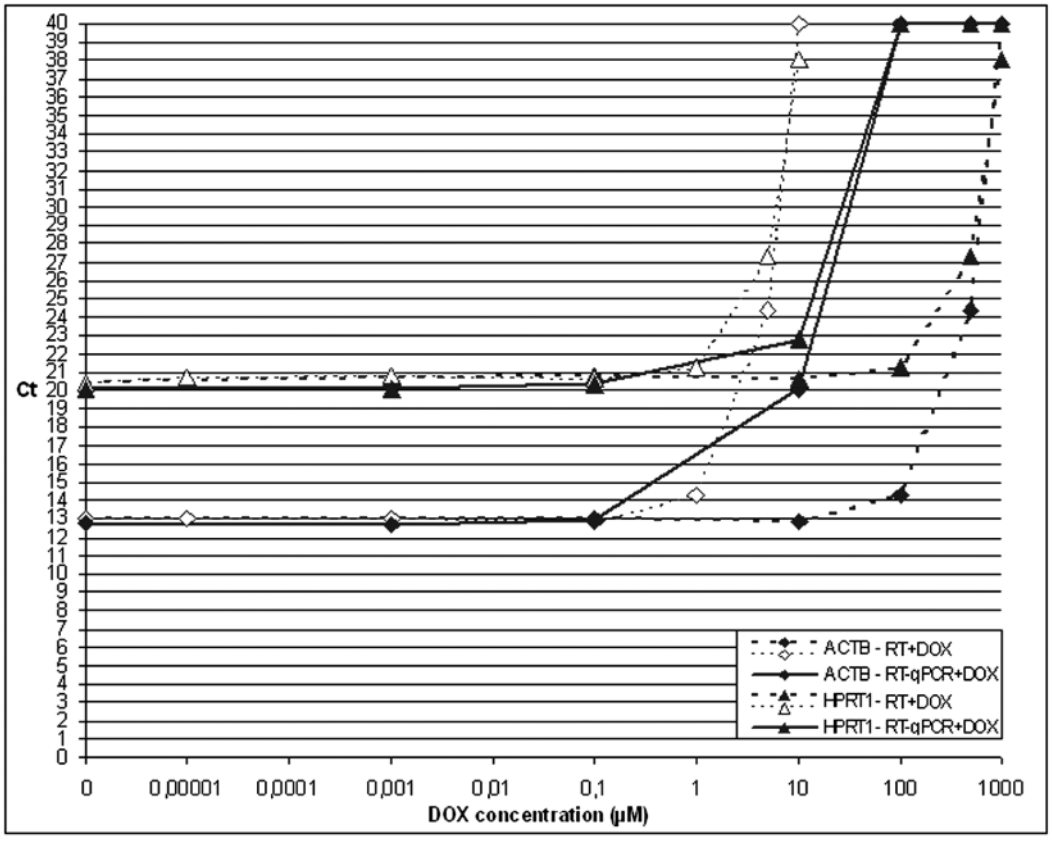

Figure 3. *Effect of DOX on RT and RT-qPCR. A comparison of the results obtained for ACTB and HPRT1 amplification after loading of the RT or RT-qPCR reaction mixtures with various concentrations (0.00001-1,000 μM) of DOX. RNA and cDNA templates were obtained from control (non-treated) HeLa cells. Thick, dashed plots represent the Ct values obtained after loading of the RT reactions with DOX, referred to DOX concentrations in the reaction mixtures. Thin, dashed plots represent the same Ct values referred to the final DOX concentrations in the corresponding RT-qPCR mixtures. Solid plots represent the Ct values obtained after direct loading of the RT-qPCR reactions with DOX. The mean values from two replicates of each sample were calculated using the MxPro Software algorithm. The mean values are non-arithmetic, therefore Standard Deviations are not shown.*

shown). The human melanoma cells used in a similar study in our laboratory did not demonstrate similar abilities during treatment with DOX (data not shown). Therefore, the mechanism of DOX resistance in HeLa and KB-V1 cells seems to be quite specific and not related to the active transport of the drug outside the cells, yet it still needs to be investigated.

Lastly, the best performing genes: *ACTB, HPRT1, RPS23* and *FLOT2* are proposed to be sufficient for reliable normalization of RT-qPCR data in both tested cell lines treated with DOX. The *ABCF1* can serve as a normalizer only for KB-V1 cells, not for HeLa cells. The *UBB* gene was found to have low expression stability, and therefore will not be used as a normalizer in the study.

The present study confirms the importance of extended validation for the proper normalizing of RT-qPCR data, especially in experiments with cytostatic drugs. These types of studies definitely require evaluation of whether the tested compounds can affect the RT and qPCR amplification and possibly alter the normalization results. Regardless of the potential inhibitory mechanism of these compounds, it is important to take it into consideration when analyzing for the final expression results.

The best normalization strategy still seems to be the one based on thorough normalization using a panel of reference genes, in order to minimize the risk of misinterpretation. It is also important to note that the normalization results obtained for certain cell types or experimental conditions cannot be extrapolated without re-validation.

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