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Postmortem mRNA profiling II: Practical considerations☆

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ABSTRACT

Using human postmortem tissues for gene expression studies is particularly challenging. Besides the problem of impaired RNA one has to face a very high degree of biological variance within a sample set. Variations of individual parameters like age, body mass, health, but also the cause and circumstances of death and the postmortem interval lead to a rather inhomogeneous collection of samples.

To meet these problems it is necessary to consider certain precautions before starting a gene expression project. These precautions include the sample collection and the determination of the RNA integrity, the number of replicates needed and the methods used for reverse transcription and quantitative polymerase chain reaction, but also the strategy for data normalisation and data interpretation.

In this article practical issues are discussed to address some of the problems occurring in the work with postmortem human samples obtained during medico-legal autopsy.

1. Introduction

Studying gene expression patterns in human postmortem tissue is becoming an ambitious field in molecular forensic research. For the isolation of RNA from autopsy tissue it is necessary to strictly avoid any further degradation during handling and processing of the samples. Thus, certain considerations concerning the organisation of the workplace have to be made to ensure an RNase-free environment. The quality of RNA should be as high as possible, but nevertheless, a certain degree of degradation cannot be avoided when working with postmortem samples. Thus, the knowledge of RNA integrity and its impact on quantitative gene expression data is indispensable [1].

Besides partial degradation, one is confronted with the problem of a rather inhomogeneous sample set. When working with animal models it is possible to control the conditions before death and to minimise the biological variance within a sample collection. In humans, the samples comprise a number of highly varying parameters; including age, body mass, health, fitness and life style. Additionally, different causes and circumstances of death and varying postmortem intervals further add to the biological variances. These problems have implications for the sample collection, the number of biological and technical replicates needed and the data normalisation strategy.

Finally, the interpretation of quantitative gene expression data obtained from postmortem tissue should be performed very carefully. It is necessary to keep in mind the possibility of impaired and adulterated results, which might occur due to low RNA integrities or a high biological variance within the sample set.

In this article we aimed to discuss several practical considerations to address some of the above mentioned parameters.

2. Avoiding RNase mediated degradation

Working with RNA requires some special precautions to avoid further RNA degradation during handling and processing of the samples. The main focus should be on a strictly RNase-free environment. RNases are omnipresent and are produced by all organisms. In contrast to DNases, they do not need any co-factors, like Mg^{2+}, and are extremely stable, which explains their extraordinarily high reactivity [45]. Contact between samples/extracts and RNases via contaminated surfaces, tubes, glassware or pipette-tips should be avoided consequently. Since RNases show extreme stability, they cannot be destroyed by conventional surface cleaning and disinfection using for example detergents and alcohol. Additionally, autoclaving plastic and glassware is not sufficient to inactivate RNases. Further precautions are necessary to avoid RNase contamination in the first place and to destroy or inactivate RNases that are already present in consumables, buffers or within the sample itself.

A workplace dedicated exclusively to RNA handling including an extra set of pipettes, racks, tubes and pipette-tips is useful for the creation of a nearly RNase-free environment. It is not recommended to use a workplace for extraction of RNA close to

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the working area for extraction of DNA from crime scene stains. On the one hand, tissue samples contain high amounts of genomic DNA and thus, DNA contamination might occur. Additionally, at the end of RNA extraction, a DNase treatment of samples is necessary to eliminate residual genomic DNA. Thus, there should be no contact between any items that might be used for DNase handling and DNA extracted from stains.

Additionally, thorough cleaning of working surfaces in the RNA handling area and RNase inactivation is necessary. The most common method for RNase inactivation in water and buffers is treatment with diethylpyrocarbonate (DEPC), which is added to the solution and incubated overnight before it is autoclaved. DEPC binds primary and secondary amines (e.g. histidine) and builds covalent bindings, which inactivates RNases [2,3]. In aqueous solutions, DEPC is hydrolysed to CO_2 and ethanol, a reaction which is greatly accelerated by Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) and other primary amines, which themselves can be destroyed in this process. Thus, DEPC is not suitable for the treatment of buffers containing amines [45]. Glassware and consumables can be baked at 200 °C for 2 h or rinsed with hydrogen peroxide. An easy-to-use alternative are commercially available RNase inactivation solutions that can be used to clean surfaces as well as plastic and glassware (e.g. ''RNaseAway'' from Molecular Bio Products, ''RNaseZap'' from Applied Biosystems, ''RNase-Off'' from PureBioTech or ''RNase-ExitusPlus'' from AppliChem). Additionally, the use of RNase-free aerosol-resistant pipette-tips can avoid the transfer of RNases through the pipettes.

3. Sample collection

When working with postmortem human tissues one is confronted with a rather heterogeneous sample set. The influence of parameters like age, gender [40,43], body mass and of course cause of death as well as specificities of the agonal phase [4,5] on the expression and the half-lives of certain gene transcripts is still widely unknown [6,7,43]. Thus, a careful selection of samples is crucial and within a sample set the above mentioned parameters should show a variance as low as possible.

To ensure comparability, samples should always be taken consistently from analogous areas from all individuals included in a study. Additionally, samples should be taken from undamaged areas without macroscopic signs of putrefaction.

Furthermore, when targeting biomarkers for the analysis of certain causes of death or specificities of the agonal phase, like hypoxia, it is crucial to have strict inclusion criteria. In general, results from a very well defined, but rather small group of individuals can be expected to provide more reliable data compared to those obtained from a large but rather heterogeneous group.

It is helpful to obtain as much information about the sample source as possible. By doing so, the influence of certain parameters, other than the one the study aimed to analyse, can be identified. Thus, besides the cause and circumstances of death and the postmortem interval the main results of the autopsy as well as body mass, height, gender and age at death need to be recorded. Further important forensic data may be the location in which the body was found, its temperature and clothes/covers and the time of storage at ambient and low temperature.

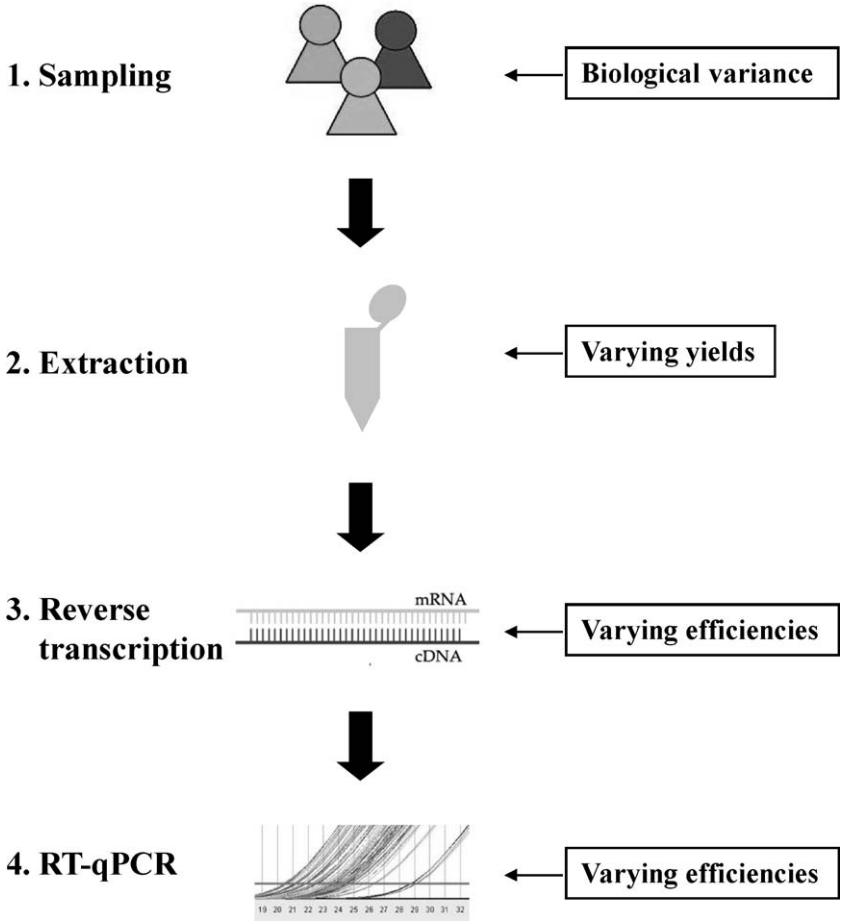

Fig. 1. Overview of sources of variation that occur throughout the workflow.

4. Replicates

In quantitative gene expression studies two types of variation affecting the results can be distinguished (Fig. 1): on the one hand one has to deal with a certain degree of biological variation that is present within the sample set. Partially, these variances can be controlled by the use of biological replicates. At least three to four biological replicates are commonly used for each group analysed. In contrast to animal models, there is no control on antemortem parameters when working with human postmortem tissue samples and thus, different individuals cannot be regarded as true biological replicates. This problem can partially be solved by the inclusion of a higher number of individuals, for example 20 or more for each group analysed.

The second type of variation is caused by slight differences in the handling of the samples and in varying efficiencies of extraction, reverse transcription and PCR. This type of variation can be controlled by technical replicates starting at least from the reverse transcription step while it is even better to start with the extraction replicates. A typical experiment would be to collect a sample and perform the reverse transcription (RT) in three replicates. Again, the subsequent quantitative polymerase chain reaction (qPCR) would be implemented in three replicates from each RT-replicate leading to a total of nine cycle-of-quantification (C_q, [8]) values per sample. The minimal number of replicates required for adequate data interpretation is not constant but can be determined by calculation of the coefficient of variance [8].

5. Methods

Besides the careful selection of samples, their accurate handling and processing is important to obtain high-quality and biologically meaningful data. Thus, each method has to be validated carefully and the protocol should be identical for all samples analysed [8,9]. For instance, the quality and integrity of RNA is affected by the extraction method. From our own work we know that RNA extracted by different methods from the same source produces different RNA integrity numbers. For different tissue types (e.g. fibre rich tissues versus fatty tissues) different extraction techniques have proven to give best results indicating that the extraction technique should be validated for each tissue type (data not published yet). Following the RNA extraction an additional DNase treatment has been proven to eliminate remaining genomic DNA [10]. During PCR genomic DNA may yield co-products (Fig. 2) that might affect the quantitative analysis by reducing the efficiency of the main reaction targeting cDNA. Thus, the extracted RNA should fulfil at least the following four criteria [11]: it should be of the highest possible quality, it should be free of DNA to avoid co-amplification of genomic sequences, it should not contain any inhibitors which might affect the reverse transcription, and it must not contain any nucleases to avoid further degradation of the samples.

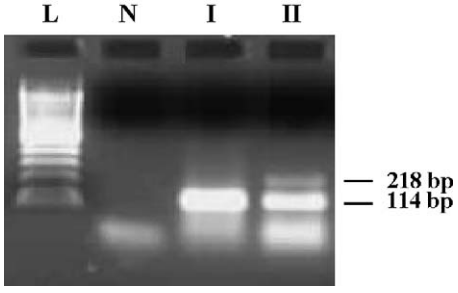

Fig. 2. Example for the use of DNase treatment to remove residual genomic DNA from the RNA extract. The cDNA sample that received DNase treatment before reverse transcription (I) shows one sharp band representing the expected PCR product with a length of 114 bp. The sample without DNase treatment (II) shows a second band at 218 bp, because here, not only cDNA (covering an exon–exon-boundary) but also genomic DNA was amplified, in which the intron sequence of 104 bp is still present [10] (L, size standard; N, negative control).

5.1. Assessing the RNA integrity

Furthermore, the photometric assessment of quantity and purity at 260 and 280 nm, respectively, as well as the measurement of RNA integrity of each sample seem to be indispensable. There are different ways to assess RNA integrity. Formerly, the most common method was the use of denaturing agarose gel electrophoresis followed by an assessment of the intensity of bands representing 18S and 28S rRNA. But recent works could show that this method lacks sensitivity and specificity, mainly regarding smaller differences in RNA integrity [12–14]. A more sensitive and user-independent method uses chip-based capillary electrophoresis. From the electrophoretical data an RNA integrity number (RIN, Bioanalyzer 2100, Agilent) or an RNA quality index (RQI, Experion, Bio-Rad) is calculated [15–17] including not only the peaks representing rRNA fragments, but also the background and the intensity of possible degradation products. RIN and RQI-values may range from 1 (= completely degraded) to 10 (= completely intact; Fig. 3). Many laboratories use a RIN/RQI of at

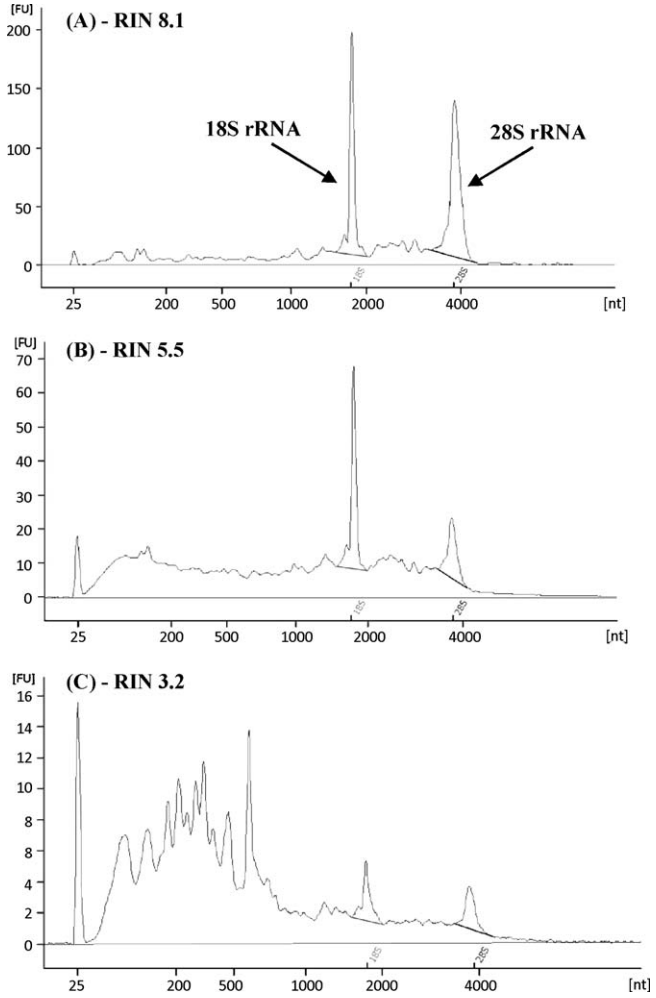

Fig. 3. RNA integrity numbers (RINs) generated using a Bioanalyzer instrument (Agilent Technologies, Böblingen, Germany). In samples with a relatively high RIN (A) both peaks representing ribosomal RNA fractions are clearly visible. In samples with medium integrities (B) the overall fluorescence intensity (fluorescence units, FU) is lower and the 28S rRNA fraction is diminished. In samples with low integrities, the ribosomal RNA peaks are very small while the background of degradation products with lengths of up to 1000 nucleotides (nt) increases.

least 5 as a cut-off value for RNA integrity [13]. While this value is based on individual experience, Weis et al. [12] calculated that a RIN of at least 3.95 is sufficient to obtain reliable gene expression data from human postmortem brain tissue. A recent study could show that different techniques for determination of RNA integrity show very good correlation and that RNA integrity clearly correlates with the quality of quantitative gene expression data [1].

An alternative to this rather cost-intensive method is the 3′-5′-assay for the determination of RNA integrity. This method is based on the fact that during degradation most transcripts show an unequal decay of their 3′- and 5′-ends. The quantitative analysis of two different regions of a transcript—one based in the 3′- and one in the 5′-region allows the calculation of a 3′:5′ ratio which in turn gives information on a samples degradation state [1]. Additionally, the determined RNA integrity values were shown to indeed influence the RT-qPCR results [13]. With our own work we could show that different degradation states lead to RIN-dependent shifts of C_q-values of approximately 1.5–2. These observations were based on degradation by heat exposure of a commercially available high-quality total RNA sample and a very limited number of genes [18].

5.2. Reverse transcription

In reverse transcription it is important always to use constant RNA quantities because RNA overload might impair the reaction efficiency of this step [11]. The priming strategy (gene specific primers versus oligo-(dT) or random hexamer) should also be considered carefully. Oligo-(dT) primers selectively bind to the poly-A-tail at the 3′-end of mature mRNAs. Thus, intact mRNA transcripts are reversely transcribed while in degraded RNA samples, reverse transcription is likely to fail due to a lack of the poly-A-tail. Random hexamer primers bind randomly at RNA and reverse transcription starts at different points of the transcript simultaneously. Thus, this might yield a well-balanced covering of the whole transcriptome. When working with potentially impaired material the use of random hexamer primers or a mixture with oligo-(dT) has been approved [8].

5.3. Quantitative real-time PCR

In quantitative real-time PCR a careful selection of primers and probes as well as the validation of the assay are highly important factors to obtain reliable data. To ensure that the primers are specific for cDNA only, it is helpful to design an assay to span over an exon–exon–boundary to insure amplification of cDNA only and to validate this assay using a minus-RT control. Amplification of genomic co-products should be avoided strictly because it would diminish the efficiency of the main reaction, which complicates or even falsifies transcript quantification. Additionally, it is essential to analyse the real amplification efficiency and to include it in the data analysis process. Efficiency can be detected by establishing a standard curve based on serial dilution data of a sample with known concentration (Fig. 4). For instance, a three-fold serial dilution with a slope of −3.32 would conform to 100% efficiency. This correlates with an amplification rate of two per cycle. Though, in reality one has to expect efficiencies ranging between 90% and 110% when working with well-validated assays [11,44]. The real amplification rate is proposed to be used for the calculation of the calibrated, relative, normalised quantities [19]. In contrast, the alternative "delta–delta–C_t-algorithm" [20] is based on the assumption of a PCR efficiency of 100%, which usually is not achieved.

6. Data normalisation

Working with human postmortem samples means to be confronted with a number of varying parameters. Thus, several antemortem factors, as well as factors concerning the gene expression process, may result in varying RNA qualities and quantities, respectively. Moreover, one has to consider variability in the amount of RNA used for cDNA synthesis. Besides of differences in the amount of starting material there also may be variability within the extraction protocol, the reverse transcription efficiency and the PCR itself [43].

Finally, the quantitative real-time PCR is a highly sensitive technique. Even marginal changes of the template amount may be detected reproducibly [9]. Therefore, the consideration of possible factors affecting the constitution of the RNA pool is essential for a correct data interpretation. In order to avoid incorrect interpretation of gene expression profiles and to control or balance such sources of error, it is important to find an adequate normalisation strategy for the particular set of samples [13,21,22,41]. Based on controlled animal gene expression studies we know that different normalisation strategies can be applied. The pros and cons of these strategies are discussed by Huggett et al. [21] and Bustin [9] and a brief overview is given in Table 1.

6.1. Normalisation against the total amount of tissue

Normalising against the total amount of tissue deployed into the extraction process is a first step to reduce the existing experimental error [21]. However, one cannot assume that different samples of the same tissue types have exactly identical cellular constitutions. Even when working with cell culture this normalisation strategy may lead to an erroneous data interpretation, because cells occur in a varying morphology or may cluster with each other. Moreover, normalisation against the total amount of tissue does not comprise variation of the efficiency of RNA extraction, reverse transcription or PCR, respectively.

6.2. Normalisation against the total amount of DNA or RNA

Normalisation of quantitative data against the total amount of genomic DNA of the same tissue type was described by Talaat et al. [23]. One drawback of this method is that it is not suitable to normalise for any variances in the reverse transcription [24] or the PCR itself [21]. Furthermore, many RNA extraction protocols do not enable an additional DNA purification and the amounts of different RNA and DNA extracts may vary considerably.

Another way of normalisation refers to the extracted amount of total RNA. This may be quantified by the assessment of the optical density or by fluorescence labelling. However, this normalisation strategy also does not enable the control of possible variation in the efficiency of reverse transcription or the PCR itself [24]. Furthermore, extract of total RNA contains predominantly rRNA molecules, which does not implicitly represent the mRNA fraction, which is much smaller [25].

6.3. Normalisation using external controls (spike-in RNA)

A further strategy for data normalisation is the inclusion of an artificial RNA molecule which is added at the extraction step [26]. This technique was first described by Lockhart et al. [27] for its use in early genome-wide gene expression profiling studies. These in vitro-synthesised RNA molecules are also known as external controls [26], spike-in RNA or "alien molecules" [21]. These molecules are artificially synthesised from an expression vector and can be selected from plant or bacterial gene transcripts. Even completely synthetical RNA molecules can be designed. The advantage of this method is that it enables to compare different experiments, different reaction platforms and even data obtained from different laboratories. Even though this technique is primarily used for normalisation of microarray data sets (e.g.

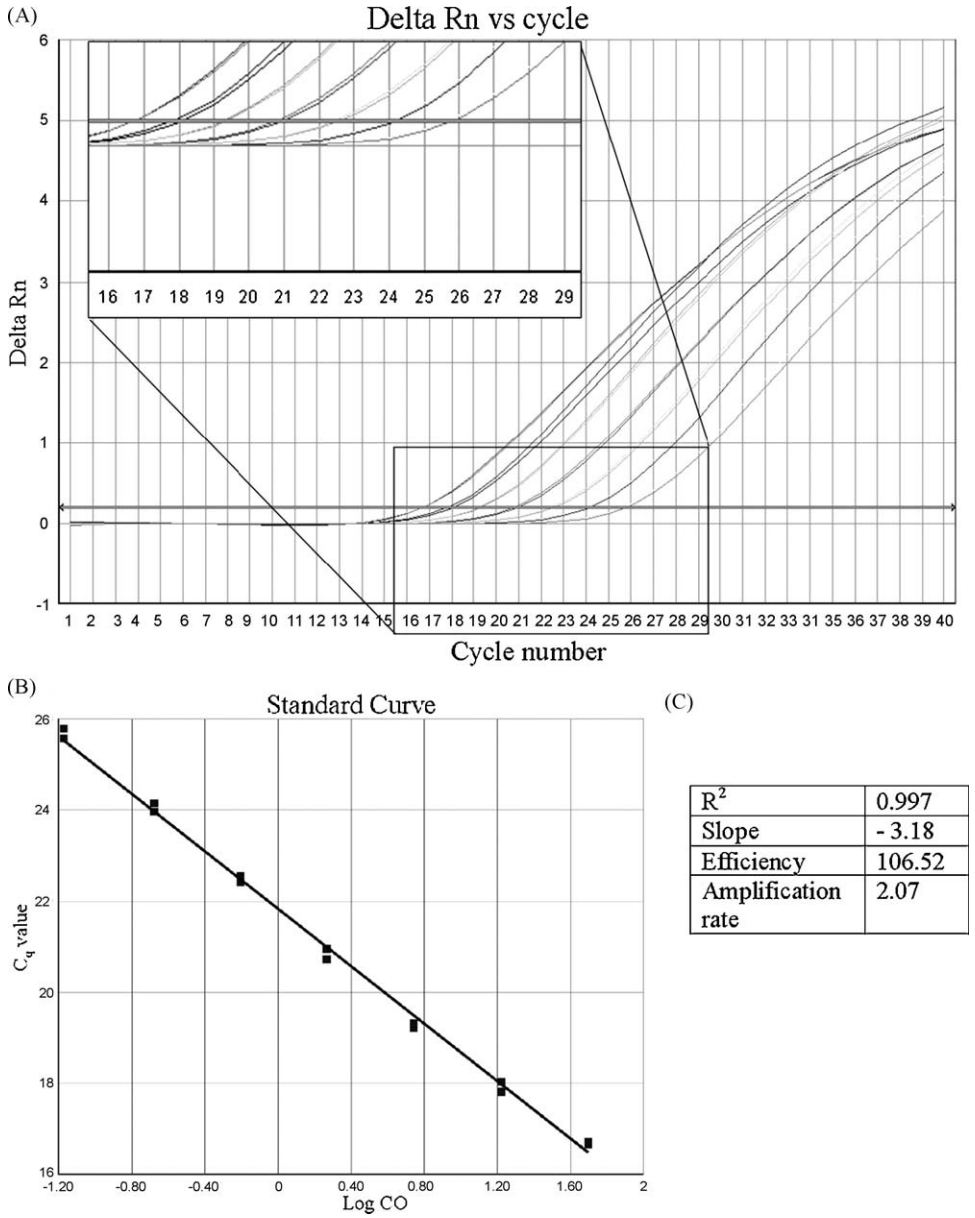

Fig. 4. Example of a three-fold serial dilution of cDNA generated from a commercially available human heart total RNA. The TaqMan Assay detects the transcript amount of β-actin (Assay ID: Hs00357333_g1, Applied Biosystems). (A) Amplification plot of the three-fold serial dilution (starting concentration 50 ng/μL). Each dilution step was performed in duplicates. Each curve is crossing the threshold in an interval of about 1.5 cycles. (B) Standard curve resulting from the serial dilution. (C) R^2 describes the accuracy of pipetting. The slope of −3.18 represents a PCR efficiency of 106.52% calculated using the formula $E = 10^{(-1/\text{slope})} - 1$ and a mean amplification rate of 2.07.

Table 1
Overview of different strategies for normalising RT-qPCR data.

Normalisation strategy	Advantages	Disadvantages	Note
Total amount of tissue	Easiest way of normalisation	Different samples might not comprise similar cellular constitution	Does not normalise variations in the workflow or the efficiencies of e.g. RT and PCR
Total DNA/RNA content	Easy to perform, straight forward	DNA does not control for varying RT and PCR efficiencies	Total RNA contains mostly rRNA species and might not be representative for mRNA
External control	Allows inter-laboratory and inter-experimental comparison	Laborious and time-consuming in vitro-synthesis and validation	Currently used for normalisation of microarray data
Single endogenous control gene	Easy to perform, multiplexing with gene of interest is possible	Difficult to find a stable and ubiquitously expressed gene	Most single genes do not show stable expression
Set of validated endogenous control genes	Expressed in the same cell as the genes of interest, stable as a set	Careful validation is necessary	Recommended in current literature

[28]), it also represents a potentially excellent method for normalising RT-qPCR data. The drawback of this method is that it is extremely time-consuming and laborious to design and synthesise these external controls. Additionally, they might need extended validation because they are not extracted from within the cells, unlike the RNA of interest or endogenous controls [21]. Recently, it has been proposed to generate such standards which will be commercially available [39]. This might facilitate their use in the future.

6.4. Normalisation against endogenous reference genes

During the last years, normalisation against endogenous reference genes became one of the most commonly used normalisation strategies. Meanwhile, it is used in numerous studies, which investigate gene expression levels [43]. Working with so-called endogenous reference genes, which incipiently were noted as "housekeeping genes" [9], came out to be very advantageous because the transcript used for normalisation is running through exactly the same working steps as the gene of interest does. Thus, it passes the same working conditions as well as the same varying efficiencies of reverse transcription and qPCR.

For a long time, "housekeeping genes" have been considered to show ubiquitous and stable expression [29], which means that they are constantly expressed in each tissue type without any influence from environmental or pathological conditions, because they regulate basic cellular functions [30]. Commonly used reference genes are for example glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin (ACTB), 18S rRNA and beta-2-microglobulin (B2M). In the past their expression was considered as stable and independent of varying experimental parameters. However, the work with highly sensitive RT-qPCR did not show stability for any of those genes. On the contrary, they were characterised by changing expression patterns in response to experimental conditions and different types of tissue (e.g. [25,31,32]). Therefore, the term "housekeeping genes" seems to be no longer appropriate [8]. In fact, current literature proposes the use of a set of at least three very well-validated endogenous control genes, which show expression stability as a set of transcripts, while individual stability of each transcript is not necessary [25].

6.5. Validation of endogenous reference genes

The knowledge of postmortem transcript stability of potential endogenous control genes is the precondition for reliable data normalisation [25]. Hence, in an own study [33] we checked ten functionally different gene transcripts for their suitability to serve as endogenous control genes in gene expression studies of postmortem human tissue samples. Their transcript variability was analysed to assess which transcripts are most stable depending on the type of tissue. Samples were taken from cardiac muscle, skeletal muscle (M. iliopsoas) and brain tissue, because these tissue types emerged to be applicable for gene expression studies using postmortem human tissue [10]. Fundamental information like gender, age at death, cause of death and postmortem interval were recorded. In contrast to further findings, the actual study revealed relatively high transcript stability for genes like hydroxymethylbilane synthase (HMBS), Ubiquitin C (UBC), succinate dehydrogenase complex subunit A (SDHA) and cyclophilin a (CYCA) depending on several parameters. Furthermore it was shown that at least four very well-validated control genes are necessary for a correct data normalisation. These four control genes were used to normalise the expression levels of rather instable genes like 18S rRNA, B2M, hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), and UBC. In doing so, we found that the specific cause of death and the type of tissue might have an influence on transcript amounts of certain genes commonly used as endogenous control genes. With this work, a general basis is created for numerous future gene expression studies using human postmortem tissue, that aim to assess changing gene expression patterns by using very well-validated stable reference genes and at least four endogenous control genes [33].

Validation of adequate endogenous control genes can be performed using various software applications for the determination of transcript stability [34], for example the freely available Microsoft Excel applet geNorm [25]. With this software, mean C_q-values were used for calculation of ΔC_q-values. The exponential function $E^{-\Delta C_q}$ determined the relative, non-normalised quantity of each transcript with regard to the specific amplification efficiency E (see above). To identify the control genes with the most stable amount of transcripts in the given set of samples and to quantify the variation of the ten endogenous control genes relative, non-normalised quantities were imported into the geNorm VBA applet. geNorm calculates the gene expression stability measure M for a reference gene as the average pairwise variation V for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability (geNorm manual: http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf).

The use of at least three reference genes for normalisation is recommended and a normalisation factor (NF) is given for the three most stable genes by calculating the geometric mean of these genes (NF_n, $n = 3$). To determine whether further genes have an impact on NF, a stepwise inclusion of genes with lower stability is performed until the $(n + 1)$th gene has no significant impact on the new normalisation factor (NF_{n+1}). To assess the possible need for more than three genes the pairwise variation ($V_{n/n+1}$) between two sequential normalisation factors (NF_n and NF_{n+1}) is calculated for all samples investigated [25,33].

Another regularly used software for the determination of transcript stability in candidate reference genes is called NormFinder [35]. This strategy is based on a mathematical model that enables the estimation of the overall variation of potential reference genes and also gives information on the variation between sample subgroups.

A third software that should be mentioned in this context is the Microsoft Excel based program BestKeeper, which has some similarities with geNorm, but uses another algorithm for the determination of transcript stability within a set of candidate genes. The underlying theory is that reference genes should show similar expression patterns and thus, a Pearson correlation is calculated and all highly correlated genes are considered stably expressed. Similar to geNorm, the normalisation factor is calculated as the geometric mean of the most stable genes [36].

7. Data interpretation

For data analysis, several software tools were generated to facilitate quantitative analysis of raw data and to reveal fold-changes in gene expression levels, e.g. the relative expression software tool (REST, [41,42]), GenEx (MultiD) or qBasePlus [19].

In general, one should be extremely cautious when interpreting quantitative gene expression data [37] generated from human postmortem tissue. As mentioned above, a number of parameters might influence transcript levels in these tissues, including individual differences, but also pathological findings, cause and circumstances of death, the postmortem interval and storage conditions of the bodies. In order to reveal biologically meaningful data that really answer the questions asked in a particular study, one has to make sure that the parameters mentioned above do not

adulterate or even mask changes in gene expression levels. Thus, for each sample included in a study as much information as possible should be available. Even if no distinct parameter was found to influence the data it is possible (and likely) that due to partial degradation of certain transcripts there might be a bias in the mRNA pool leading to over- or underestimation of individual transcript levels.

When reporting quantitative gene expression data, it is important to ensure that published data are reliable and reproducible. Thus, to maintain the integrity of scientific literature and to increase experimental transparency, guidelines for the minimum information for publication of quantitative real-time PCR experiments (MIQE guidelines) were published recently. These guidelines address issues of experimental design, sample collection, all methods included in the workflow and their validation as well as the data analysis procedure. A checklist for authors, reviewers and editors was established containing all necessary information that should be included in manuscripts reporting quantitative gene expression data [8,38].

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