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REVIEW

Real-time RT-PCR normalisation; strategies and considerations

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Real-time RT-PCR has become a common technique, no longer limited to specialist core facilities. It is in many cases the only method for measuring mRNA levels of vivo low copy number targets of interest for which alternative assays either do not exist or lack the required sensitivity. Benefits of this procedure over conventional methods for measuring RNA include its sensitivity, large dynamic range, the potential for high throughout as well as accurate quantification. To achieve this, however, appropriate normalisation strategies are required to control for experimental error introduced during the multistage process required to extract and process the RNA. There are many strategies that can be chosen; these include normalisation to sample size, total RNA and the popular practice of measuring an internal reference or housekeeping gene. However, these methods are frequently applied without appropriate validation. In this review we discuss the relative merits of different normalisation strategies and suggest a method of validation that will enable the measurement of biologically meaningful results.
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Introduction

Real-time reverse transcription PCR (real-time RT-PCR) is an established technique for quantifying mRNA in biological samples. Benefits of this procedure over conventional methods for measuring RNA include its sensitivity, large dynamic range, and the potential for high throughout as well as accurate quantification. Its enhanced specificity is particularly useful for immunological research, which frequently involves analysis of proteins derived from different splice variants of the original transcript.[1] Furthermore, many of the key proteins (eg cytokines and transcription factors) are found in such low abundance that real-time RT-PCR quantification of their mRNAs represents the only technique sensitive enough to measure reliably their expression *in vivo*.[2,3]

Although real-time RT-PCR is widely used to quantitate biologically relevant changes in mRNA levels, there remain a number of problems associated with its use. These include the inherent variability of RNA, variability of extraction protocols that may copurify inhibitors, and different reverse transcription and PCR efficiencies.[4] Consequently, it is important that an accurate method of normalisation is chosen to control for this error. Unfortunately, normalisation remains one of real-time RT-PCRs most difficult problems.[5]

Several strategies have been proposed for normalising real-time RT-PCR data. These range from ensuring that a similar sample size is chosen to using an internal housekeeping or reference gene (Table 1). These approaches are not mutually exclusive and can be incorporated into a protocol at many stages (Figure 1). Here we discuss the respective advantages and disadvantages of each technique.

Normalisation; sample size

Ensuring a similar sample size is obtained, by sampling similar tissue volume or weight, is the first stage of reducing experimental error. This may appear to be straightforward, but experimental sample groups of similar size are often not representative. It can be difficult to ensure that different samples contain the same cellular material. A good example is blood, which is relatively easy to sample and ensure similar volumes are compared. However, this can be misleading, as is illustrated when sampling a similar volume of blood from HIV + ve patients (Figure 2). Patients with HIV that have less advanced immunosupression (CD4 counts $\geqslant$200 cells/ml) will yield a higher amount RNA than patients with CD4 counts $\leqslant$200 cells/ml. This is simply because there are fewer cells per millilitre of blood in the

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Table 1 Comparison of the actual amount of RNA used in different reverse transcription reactions with the respective amount of HuPO cDNA measured by real time RT-PCR

Normalisation strategy	*Pros*	*Cons*	*Note*
Similar sample size/tissue volume	Relatively easy	Sample size/tissue volume may be difficult to estimate and/or may not be biological representative	Simple first step to reduce experimental error
Total RNA	Ensures similar reverse transcriptase input. May provide information on the integrity (depending on technique used)	Does not control for error introduced at the reverse transcription or PCR stages. Assumes no variation in rRNA/mRNA ratio	Requires a good method of assessing quality and quantity
Genomic DNA	Give an idea of the cellular sample size.	May vary in copy number per cell. Difficult to extract with RNA	Rarely used. Can be measured optically or by real time PCR
Reference genes ribosomal RNAs (rRNA)	Internal control that is subject to the same conditions as the RNA of interest. Also measured by real time RT-PCR	Must be validated using the same experimental samples. Resolution of assay is defined by the error of the reference gene	Oligo dt priming of RNA for reverse transcription will not work well with rRNA as no polyA tail is present. Usually in high abundance
Reference genes messenger RNAs (mRNA)	Internal control that is subject to the same conditions as the mRNA of interest. Also measured by real time RT-PCR	Must be validated using the same experimental samples. Resolution of assay is defined by the error of the reference gene	Most, but not all, of mRNAs contain polyA tails and can be primed with oligo dt for reverse transcription
Alien molecules	Internal control that is subject to most of the conditions as the mRNA of interest. Is without the biological variability of a reference gene	Must be identified and cloned or synthesised. Unlike the RNA of interest, is not extracted from the within the cells	Requires more characterisation and to be made available commercially

There is good correlation between the RNA concentration used and the real time PCR estimation of the different amounts of HuPO cDNA (using omniscript reverse transcriptase).

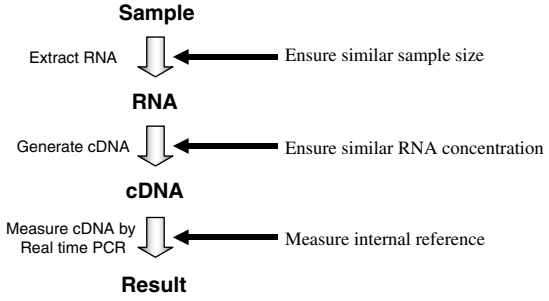


Figure 1 Processes required to generate a real time RT-PCR result. Black arrows indicate points, which should be considered for a good normalisation strategy.

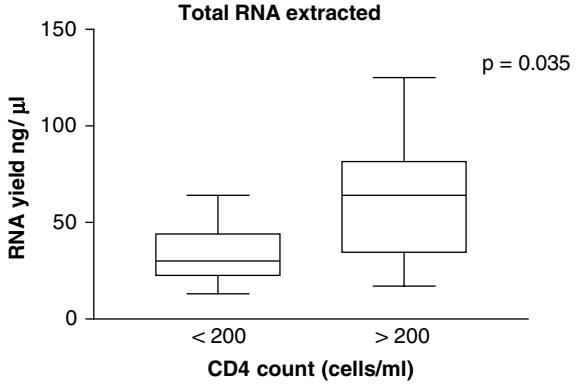


Figure 2 Total RNA yields from two different groups of HIV patients. Total RNA extracted using PAXtubes (Qiagen) as for Dheda *et al*.[5]

latter group. It would be misleading to directly compare these groups based on sample volume alone.

When dealing with *in vitro* cell culture, it can also be difficult to estimate sample size (cell number) because cells will often clump up or have different morphologies, particularly when cultured as a monolayer. Cells can be treated chemically or enzymatically to assist counting; however, this will undoubtedly effect gene expression and is likely to confuse the experimental findings. While ensuring a similar sample size is important it clearly is not sufficient on its own.

Normalisation; RNA quantification

It is essential to quantitate accurately and quality assess RNA prior to reverse transcription.[4,6] If the two HIV groups, discussed in Figure 2, are to be assessed then input RNA for the reverse transcription reaction should be similar. There are several methods for quantifying RNA, arguably the most accurate being ribogreen (molecular probes) and the LabChip (Agilent 2100). Frequently overlooked is the concomitant need for interpreting the quality of the RNA (Figure 3).

If RNA quality is not good then the measurement can be effected (Figure 4 and Bustin and Nolan[4]). Normalising a sample against total RNA has the drawback of not controlling for variation inherent in the reverse transcription[7] or PCR reactions. Normalising to total RNA also primarily measures ribosomal RNA (rRNA), which

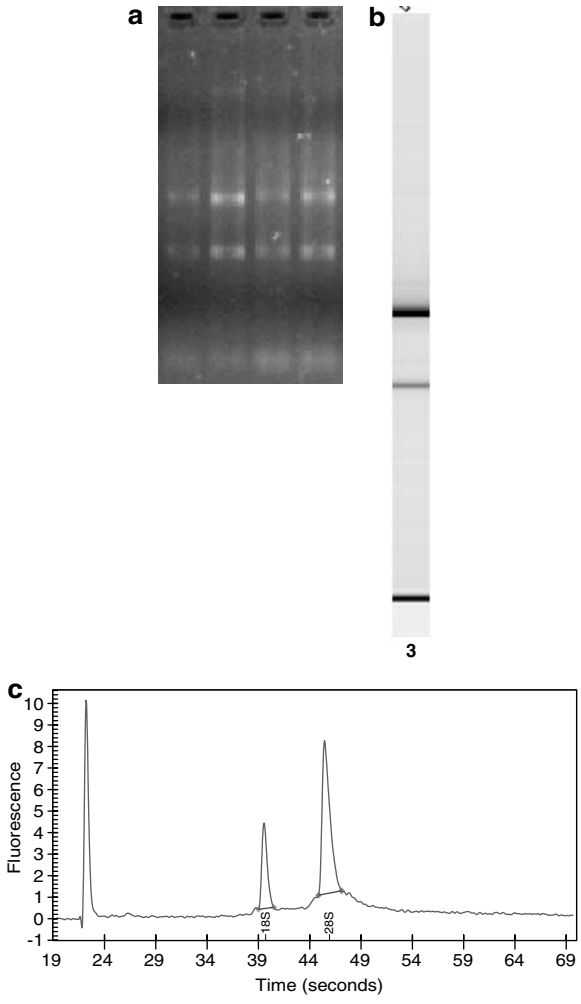


Figure 3 Example of good quality RNA assessed by (**a**) agarose gel (5 ml RNA extract), (**b** and **c**) by the Agilent Bioanalyser (1 ml RNA extract) using the RNA LabChip. Total RNA extracted using PAXtubes (Qiagen) as for Dheda *et al*.[5]

makes up ~80% of the fraction. As the majority of procedures measure protein-coding messenger RNAs (mRNA) (comprising 2–5% of the fraction), it is assumed that rRNA : mRNA ratio does not change between groups, an assumption that has been reported can be wrong.[8,9]

Normalisation; genomic DNA

Targeting genomic DNA has also been suggested for normalisation.[10] This appears to be an ideal method as it does not require reverse transcription for detection by real-time PCR. Cells that are proliferating are replicating their DNA, so contain more sets of genetic information when compared to nonproliferating cells. However, in eukaryotic organisms, this difference will usually be $\leqslant$2-fold and so unless very fine measurements are required would not cause a problem. However, tumor cells often have a variable haplotype and actively replicating bacteria can contain up to 8 $\times$ more copies of certain loci than nonreplicating cells.[11] Another major problem with using this strategy is that RNA extraction procedures are usually not designed to purify DNA, so the extraction rate may vary between different samples, with DNA yields often being low.

Normalisation; reference genes

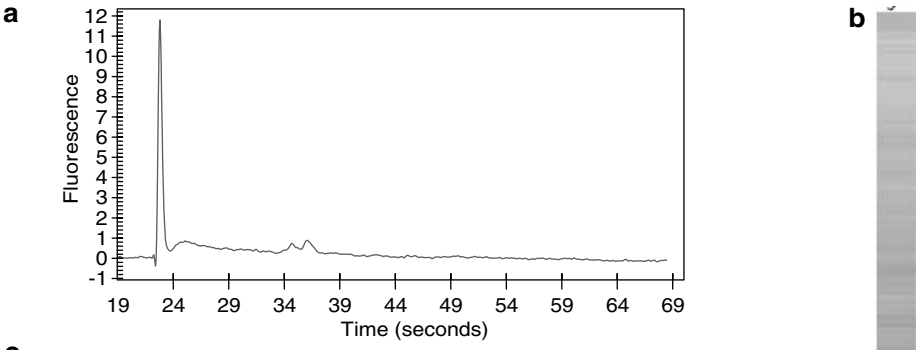
Normalising to a reference gene is a simple and popular method for internally controlling for error in real-time RT-PCR. This strategy targets RNAs encoded by genes, which have been collectively called housekeeping genes and benefits from the fact that all the steps required to obtain the final PCR measurement are controlled for. The procedure is simplified as both the gene of interest and the reference gene are measured using real-time RT-PCR. Reference genes can also control for different input RNA amounts used in the reverse transcription step (Figure 5); however, because this can vary with reverse transcriptase type[7] it must be validated.

The most commonly used reference genes include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA. They are historical carryovers and were used as references for many years in Northern blots, Rnase protection assays and conventional RT-PCR assays. Their use was acceptable for these non/semiquantitative techniques where a qualitative change was being measured. This was because these RNAs are expressed at relatively high levels in all cells and made ideal positive controls if the gene of interest was switched off. However, the advent of real-time PCR placed the emphasis on quantitative change, and should have resulted in a re-evaluation of the use of these reference genes. This was not done and studies continued to use arbitrarily chosen 'classic' reference genes for this purpose.

What is even more surprising is that these 'classic' reference genes were demonstrated to be regulated over a decade before the real-time RT-PCR was made available. As early as 1975, 18S rRNA was reported to increase in expression with cytomegalovirus infection.[12] In 1984, Piechaczyk *et al*[13] reported that while GAPDH transcription occurred at a similar rate in different rat tissues, they contained very different amounts of mRNA. In 1985, HPRT was reported to be constitutively expressed at low levels in most human tissues but was elevated in certain parts of the central nervous system[14] and in 1987 β-actin mRNA was reported to be differentially expressed in different leukaemia patient tumour samples.[15]

Despite these and other observations there are countless examples of published work that have used a particular reference gene for normalisation without any mention of a validation process. The notion that these RNAs require validation is also not new. In 1989, Barbu and Dautry[16] reported that probes to β-actin were inappropriate as a reference when comparing different mouse tissue by Northern blot.

More recently there have been a number of reports that demonstrate that the classic reference genes can vary extensively and are unsuitable for normalisation purposes due to large measurement error.[5,17–23] Some have argued that the above-mentioned factors are academic as the overall study findings will not be affected because this variability is likely to be similar in the study and



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RNA quality	Duplicate cDNA measurement of HuPO (~copies/reaction)	Duplicate cDNA measurement of IL-4 (~copies/reaction)
Good	43050	60
	43730	50
Poor	248480	410
	168600	590

Figure 4 Example of poor quality RNA assessed by the Agilent Bioanalyser using the RNA LabChip (**a**, **b**). (**c**) Different results obtained when 15 ng of good and poor quality RNA are used for reverse transcription are illustrated. (HuPO and IL-4 reactions are performed as described[5]). Total RNA extracted using PAXtubes (Qiagen) as for Dheda *et al*.[5]

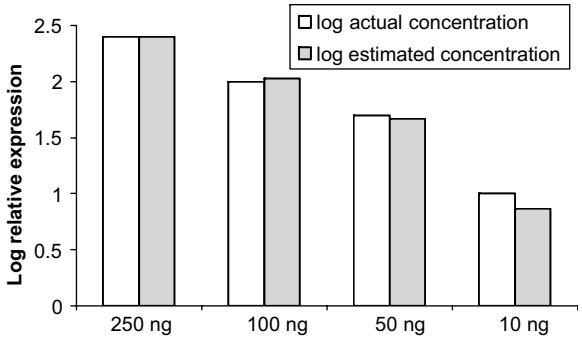

Figure 5 Comparison of the actual amount of RNA used in different reverse transcription reactions with the respective amount of HuPO cDNA measured by real time RT-PCR. There is good correlation between the RNA concentration used and the real time PCR estimation of the different amounts of HuPO cDNA (using omniscript reverse transcriptase).

control groups.[24] However, unless the variability is defined this argument simply does not hold. While genes like GAPDH have been found to be appropriate for certain experimental situations[25] they are often not.

Of particular note is the findings of Bas *et al*[17] and Tricarico *et al*,[21] who demonstrated that if the wrong reference gene is chosen it can result in altered findings. This occurs when the reference gene is regulated by the experimental conditions. This is particularly worrying and has serious implications for studies that have used unvalidated reference genes. It is no longer acceptable to choose blindly any reference gene for normalisation. Authors must be able to demonstrate that the reference gene of choice is suitable for the experiment in question.

Considerations when using a reference gene

Reference gene validation exercises are also subject to the problem of normalisation. The strategy we previously presented[5] uses total RNA to normalise the sample prior to reference gene variability assessment. The different reference genes are then measured by real-time RT-PCR and variation in the cycle threshold (Ct) or crossing point (Cp) assessed. As RNA normalisation can incorporate error and does not take into account the RT step, the measured reference gene variability represents the cumulative error of the entire process, that is, the innate variation of the reference gene under investigation and the experimental error associated with the technique.

Once this variation is defined the chosen reference gene can provide the resolution of the assay in question. Choosing the accepted level of variability will depend on the degree of resolution required. Even if the chosen gene is variable it may not matter as long as intergroup difference being measured is greater than the reference gene variation, that is, a reference gene RNA that has an error of 1 log may not be ideal, but is sufficient to measure a 2 log change in a gene of interest.

There are a number of programs based on the excel platform that allow the assessment of multiple reference genes. Gnorm allows the most appropriate reference gene to be chosen by using the geometric mean of the expression of the candidate cDNA.[26] This software is freely available (http://www.genomebiology.com/2002/3/7/research/0034/) and the underlying principles are published by Vandesompele *et al*.[26] BestKeeper also selects the least variable gene using the geometric mean but uses raw data[27] instead of data converted to copy number, it is also available at http://www.gene-quantification.de/BestKeeper-1.zip. A third program Norm-Finder,[28] freely available on request, not only

measures the variation but also ranks the potential reference genes by how much they differ between study groups, that is, the extent by which they are effected by the experimental conditions. Defining this is essential as it can generate false results as discussed above.

Vandesompele *et al* also advocate the use of multiple reference genes rather than relying on a single RNA transcript. This is a robust method for providing accurate normalisation and is consequently favourable if fine measurements are to be made. However, it is not always possible to measure multiple reference genes due to limited sample availability and cost. Furthermore, even if multiple genes are chosen the resolution of the particular assay remains dependent on the variability of the chosen reference genes.

Normalisation; artificial molecule

An alternative internal control strategy uses a molecule that is artificially incorporated into the sample.[4] This represents a potentially excellent method for normalising real-time RT-PCR data. This 'artificial' RNA molecule can be cloned and *in vitro* transcribed from another species or generated synthetically. As it can then be incorporated at the extraction stage at a defined concentration, it will be subjected to almost all the experimental error that affects the RNA of interest; furthermore, it will not suffer from the inherent biological fluctuations that effect reference genes. However, as these RNAs are spiked into the extractant, they are not extracted from within the cells, unlike the RNA of interest or reference gene RNAs. This might present a problem in certain situations (eg different histological samples when studying fibrosis) so would also require validation. The stages required to generate the alien molecule may also not be feasible for small laboratories wanting to perform limited amounts of real-time RT-PCR. Ideally, real-time RT-PCR consumables would include sets of artificial molecules that could be used for different organisms. Generation of such standards has been proposed,[29] but until they are made available commercially and more extensive research into this method is performed, artificial RNA normalisation will remain an unvalidated theoretical ideal.

Conclusion

There are a number of methods that can be used for normalising real-time RT-PCR data. These strategies are not mutually exclusive and we recommend attempting to match sample size, ensuring good quality RNA is extracted and similar quantity used for the reverse transcription reaction and finally an internal control (reference gene or alien molecule) also be measured.

Spiking the sample with a defined amount of an unique or artificial RNA may arguably be the most accurate method for internal normalisation, but at this time reference genes represent a strategy that is simple to use and can control for every stage of the real-time PCR, but they must be used with caution. The key to normalisation when using this technology is to be able to demonstrate that it is valid. Researchers should ensure that the common criticisms, discussed in this article, have been addressed before presenting real-time RT-PCR data.

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