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Review article
Pregledni članak

GENE EXPRESSION STUDIES: HOW TO OBTAIN ACCURATE AND RELIABLE DATA BY QUANTITATIVE REAL-TIME RT PCR

IZUČAVANJE EKSPRESIJE GENA: KAKO DOBITI TAČNE I POUZDANE PODATKE KVANTITATIVNIM RT PCR-OM U REALNOM VREMENU

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Summary: Real-time RT PCR has been recognized as an accurate, reliable and sensitive method for quantifying gene transcription. However, several steps preceding PCR represent critical points and source of inaccuracies. These steps include cell processing, RNA extraction, RNA storage, assessment of RNA concentration and cDNA synthesis. To compensate for potential variability introduced by the procedure, normalization of target gene expression has been established. Accurate normalization has become an absolute prerequisite for the correct quantification of gene expression. Several strategies are in use for the normalization of data, including normalization to sample size, to total RNA or to an internal reference. Among these, the use of housekeeping genes as an internal (endogenous) control is the most common approach. Given the increased sensitivity, reproducibility and large dynamic range of this methodology, the requirements for a proper reference gene for normalization have become increasingly stringent. The aim of this paper is to discuss the concept of normalization in mRNA quantification, as well as to discuss several statistical algorithms developed to help the validation of potential reference genes. By showing that the use of inappropriate endogenous control might lead to incorrect results and misinterpretation of experimental data, we are joining the creators of Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) in an attempt to convince scientists that proper validation of potential reference genes is an absolute

Kratak sadržaj: RT-PCR je prepoznat kao precizna, pouzdana i osetljiva metoda za kvantifikaciju transkripcije gena. Međutim, ovoj metodi prethodi nekoliko koraka koji predstavljaju kritične tačke i izvor potencijalnih grešaka. Ovi koraci uključuju obradu ćelijskog materijala, ekstrakciju i čuvanje RNK, određivanje koncentracije RNK i sintezu cDNK. Da bi se kompenzovala potencijalna varijabilnost nastala tokom procedure, uvedena je normalizacija ekspresije ciljnih gena. Precizna normalizacija je postala apsolutni preduslov za tačnu kvantifikaciju ekspresije gena. Postoji nekoliko strategija za normalizaciju eksperimentalnih podataka, uključujući normalizaciju u odnosu na veličinu uzorka, ukupnu RNK ili internu kontrolu (referencu). Kao interna (endogena) kontrola najčešće se koriste geni sa stabilnom ekspresijom. Imajući u vidu veliku osetljivost, reproducibilnost i veliki dinamički opseg PCR metode, zahtevi za odgovarajućim referentnim genima koji će se koristiti za normalizaciju podataka postali su veoma restriktivni. Cilj ovog rada je da razjasni koncept normalizacije i prokomentariše nekoliko statističkih algoritama koji su razvijeni kako bi pomogli u validaciji potencijalnih referentnih gena. Pokazujući da korišćenje neodgovarajućih referentnih gena (endogenih kontrola) može da dovede do netačnih rezultata i pogrešne interpretacije eksperimentalnih podataka, mi se priključujemo tvorcima uputstva MIQE (eng. *Minimum Information for Publication of Quantitative Real-Time PCR Experiments*) u pokušaju da ubedimo naučnu javnost da je ispravna validaci-

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List of abbreviations: B2M, β_2-microglobulin; BA, β-actin, FRET, Fluorescence Resonance Energy Transfer; GAPDH, Glyceraldehydes-3-phosphate dehydrogenase; HPRT, Hypoxanthine phosphoribosyltransferase 1; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; PCR, Polymerase Chain Reaction; RT, reverse transcription.

prerequisite for correct normalization and, therefore, for providing accurate and reliable data by quantitative real-time RT PCR gene expression analyses.

Keywords: real-time PCR, reference gene, normalization, validation, GeNorm, NormFinder, MIQE

ja potencijalnih referentnih gena apsolutni preduslov za tačnu normalizaciju i, shodno tome, preduslov za dobijanje tačnih i pouzdanih podataka u analizi ekspresije gena metodom kvantitativnog PCR-a u realnom vremenu.

Ključne reči: PCR u realnom vremenu, referentni gen, normalizacija, validacija, GeNorm, NormFinder, MIQE

Introduction to real-time RT PCR

In 2013, the year of DNA anniversaries, we are celebrating 60 years of Watson and Crick's discovery of the DNA structure, 40 years of the genetic modification of bacteria using recombinant DNA, 30 years of the invention of Polymerase Chain Reaction (PCR) and 10 years of the announcement of the completion of human genome sequencing. As for the PCR, in addition to 30 years from its invention, this year it has been 20 years since Mullis won the Nobel Prize for PCR.

Ever since its development, PCR is considered an essential tool in molecular biology, allowing amplification of nucleic acid sequences (DNA and RNA) through repetitive cycles *in vitro*. The mechanisms underlying this methodology are similar to those occurring *in vivo* during DNA replication. There are three main sequentially repeating steps of PCR: *denaturation*, *annealing* and *elongation*. ***Denaturation***, as the first step that proceeds at high temperatures, serves to separate DNA strands. In the ***annealing*** step, at a lower temperature, each strand is used as a template for DNA synthesis. The selectivity of PCR results is achieved in this step by using the primers complementary to the sequences outlining the targeted DNA region. During the ***elongation*** step, DNA polymerase creates two double strand target regions, each of which can again be denatured and ready for a second cycle of annealing (hybridization) and elongation (*Figure 1*).

If the reaction runs with 100% efficiency there will be a two-fold increase in target amplicons after each cycle of PCR. Therefore, after **n** cycles of reaction, the copy number of the target sequence will be $\mathbf{2^n}$. In practice, however, reactions do not work with perfect efficiency, as reactants within the PCR mixture are depleted after many cycles and the reaction reaches a plateau phase, in which there is no change in the amount of the product. Plateau phase is preceded by a linear ground phase, an exponential phase and a log-linear phase (*Figure 2*). Only in the exponential phase the quantity of PCR products is proportional to the quantity of initial template. The main disadvantage of conventional PCR, which is also called end-point PCR, regarding the quantification, is the fact that the results of amplification can be visualized only after n cycles of amplification at the end of the reaction.

In recent years, modifications of the conventional PCR method have been developed in order to

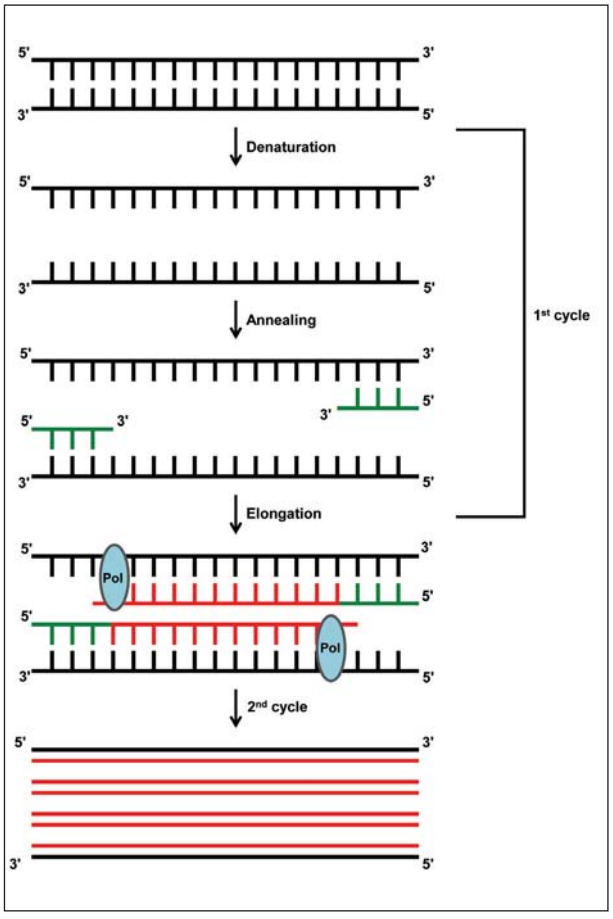


Figure 1 Phases of the Polymerase Chain Reaction (PCR).

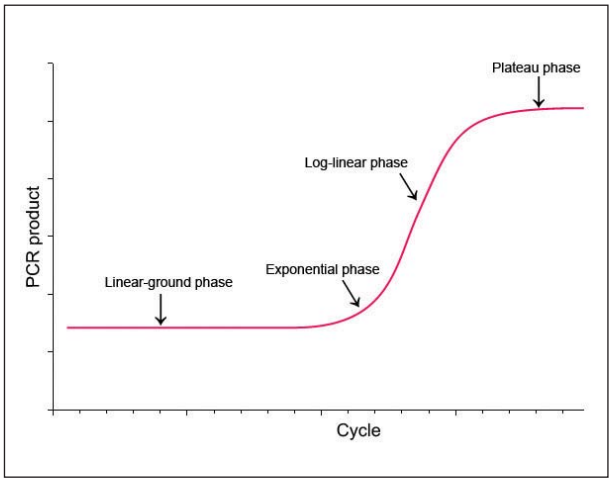


Figure 2 PCR Amplification Plot.

Table I End-point PCR vs Real-time PCR.

End-point PCR	Real-time PCR
Semiquantitative	Quantitative
Nonautomated	Automated
Needs post-PCR processing	No post-PCR steps
Time-consuming	Fast
Low technical sensitivity	High technical sensitivity
Low precision	High precision
Short dynamic range	Wide dynamic range
Low resolution (detection of 10-fold change)	High resolution (detection of 2-fold change)
Possible cross contamination	Minimized cross contamination
No possibility for a multiplex approach	Multiplex approach possible
	Incorporated specialized software for data analysis
	High throughput

improve its performance and specificity. One of them, called real-time PCR or fluorescence based PCR, enables us to collect data throughout the PCR process, enabling quantification at a point in which every sample is in the exponential phase of PCR reaction, when reagents are in excess, product is doubling at every cycle, and the product quantity positively correlates with starting template quantity. Real-time PCR allows us to quantitate nucleic acids from various sources, to compare the variable states of infection, to detect chromosomal translocations, to genotype single nucleotide polymorphisms, to determine the gene expression level. It is mostly used for two reasons: either as a primary investigative tool to determine the gene expression level, or as a secondary tool to validate the results of DNA microarrays (1).

There are many advantages of using real-time RT PCR instead of end-point PCR in gene expression studies (*Table I*). First of all, it is a quantitative method for the determination of gene expression, while end-point PCR is semiquantitative. Real-time RT PCR collects data during the exponential phase of the PCR amplification process, in which the PCR reaction is not limited by enzymatic activity or substrate concentration, while in end-point PCR data are obtained at the end of the reaction using usually agarose gels for detection. Due to the ability of detection of fluorescent signals in »real-time« during each subsequent PCR cycle, real-time RT PCR data can be obtained in a short period of time and no post-PCR processing is needed. Since no postamplification steps are required, the risk of PCR product contamination is drastically reduced (2) and reliability and reproducibility of the assay are increased (3, 4). In contrast to end-point PCR, real-time RT PCR is automated, and data analyses, including standard curve generation and copy number calculation, are performed automatically. In general, it is less time- and labor-intensive, and can be high throughput when using the proper equipment. The major disadvantage of real-time RT PCR is the expensive equipment and reagents relative to those used in end-point PCR. In addition, due to its extremely high sensitivity, the understanding and proper implementation of normalization strategies are imperative for accurate conclusions.

Detection chemistries

As mentioned before, in real-time RT PCR the reaction products are quantitatively measured in »real-time« during each PCR cycle (5). The method is based on the detection and quantification of a fluorescent signal, which increases proportionally to PCR product accumulation. There are two types of detection chemistries that are used in real-time RT PCR, designated specific and nonspecific. Specific sequence detection distinguishes the sequence of interest from primer dimers or nonspecific amplification, whereas nonspecific detection registers all double-stranded DNA produced during the reaction.

Nonspecific detection chemistry

SYBR Green. SYBR Green represents the simplest and the most economical choice for real-time RT PCR product detection (6). This fluorogenic intercalating dye emits a strong fluorescent signal upon binding to double-stranded DNA while unbound dye in solution exhibits little (undetectable) fluorescence (*Figure 3*). There are several advantages of using SYBR Green: it is the least expensive, simple and easy to use. It can be used with any pair of primers, for any target, with no need for any additional fluorescence-labeled oligonucleotide. Therefore, it can be easily applied to already established PCR assays, but for the same reason it is not possible to perform multiplexing reactions. The major disadvantage of using SYBER Green is that both specific and nonspecific PCR products are detected. Namely, SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other nonspecific reaction prod-

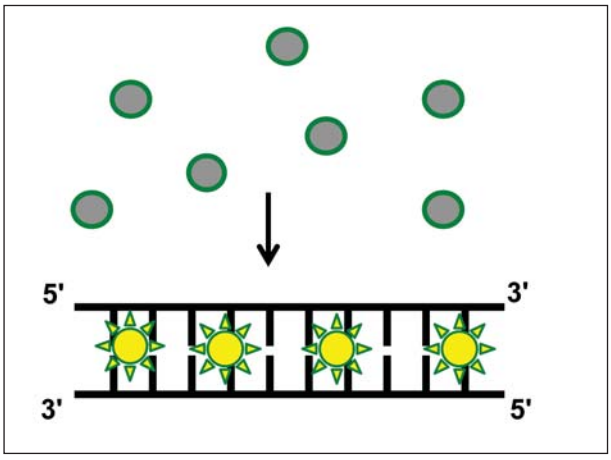
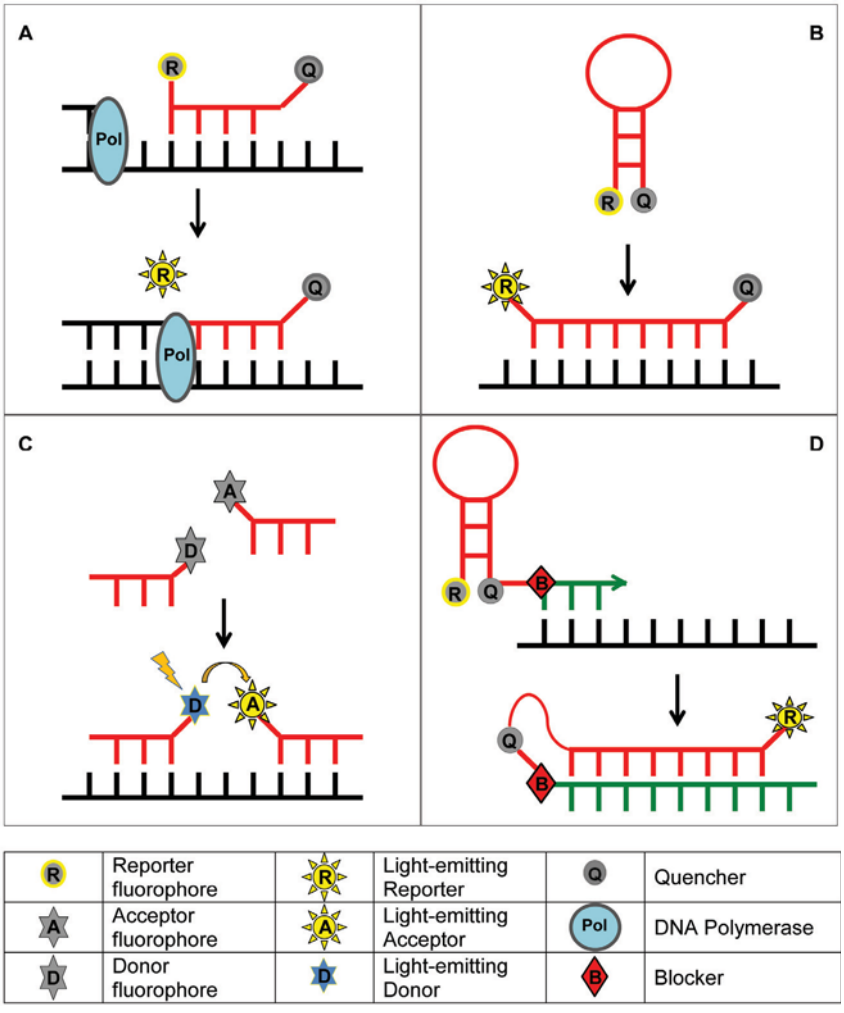


Figure 3 Nonspecific detection chemistry: DNA-binding dye – SYBR Green.

ucts, leading to overestimation of the target sequence concentration (7). Therefore, this method requires extensive optimization of the PCR conditions and a clear differentiation between specific and nonspecific PCR products using melting-curve analysis (8).

Specific sequence detection chemistry

In order to avoid major disadvantages of SYBR Green, sequence specific, fluorescent primer/probe-based chemistries have been developed. These chemistries are based on the introduction of an additional fluorescence-labeled oligonucleotide – the probe, and depend on Fluorescence Resonance Energy Transfer (FRET) (9). The most frequently used sequence specific detection chemistries are: the TaqMan hydrolysis probes, Molecular Beacons, dual Hybridization Probes, and Scorpions (*Figure 4*).



R	Reporter fluorophore	R	Light-emitting Reporter	Q	Quencher
A	Acceptor fluorophore	A	Light-emitting Acceptor	Pol	DNA Polymerase
D	Donor fluorophore	D	Light-emitting Donor	B	Blocker

Figure 4 Specific sequence detection chemistries: TaqMan hydrolysis probes (A), Molecular Beacons (B), dual Hybridization Probes (C), and Scorpions (D).

In most cases, primer/probe chemistries are designed to exploit FRET in quenching fluorescence in order to ensure that specific fluorescence is detected only when the product of interest is amplified. However, in some cases FRET is used to enhance the signal, such as in the case of dual hybridization probes, when fluorescence of donor dye excites the acceptor dye, resulting in emission of detectable fluorescence only when two fluorochromes are in close proximity (10–14).

The main advantage of primer/probe-based chemistries is increased specificity, which no longer depends only on primer binding (15). Nonspecific amplification due to mispriming or primer–dimer artifacts does not generate a signal and is ignored by the fluorescence detector. Another advantage of these chemistries over intercalating dyes includes a possibility to perform multiplexing reactions (16). Namely, using a different fluorophore on each gene-specific probe allows the detection of amplification products from several distinct sequences in a single PCR reaction. The major advantage of primer/probe-based chemistries is increased accuracy and specificity of PCR product detection, achieved by precise, gene-specific matching of usually three independent nucleotide sequences, which practically eliminates false positive results. However, these chemistries are far more expensive in comparison to SYBR Green, since each target requires its own specific probe.

Hydrolysis or TaqMan Probes. Hydrolysis assays include three sequence-specific oligonucleotides: forward primer, reverse primer, and a probe (17, 18). The probe is labeled with a fluorescent reporter dye at the 5′ end and a quencher dye at the 3′ end (*Figure 4A*). The assay exploits the 5′ → 3′ exonuclease activity of certain thermostabile enzymes, usually Taq or Tht polymerase. When the probe is intact, the quencher dye absorbs the fluorescence of the reporter dye due to the proximity between them. Upon amplification of the target sequence, the probe is displaced and hydrolyzed by the 5′ → 3′ exonuclease activity of the polymerase. Consequently, the reporter is separated from the quencher, resulting in a fluorescence signal that is proportional to the amount of amplified product. During each PCR cycle, fluorescence will further increase due to progressive and exponential accumulation of free reporter.

Molecular Beacons. Real-time PCR assays with molecular beacons also use three sequence-specific oligonucleotides: forward primer, reverse primer, and a probe (19). The probe is a »molecular beacon« – an oligonucleotide labeled with a fluorescent reporter dye at the 5′ end and a quencher dye at the 3′ end, which forms a hairpin structure, thus bringing the reporter and quencher together (*Figure 4B*). When molecular beacon is free in solution, it forms a hairpin structure, so that the reporter and the quencher are in close proximity and no fluorescence is emitted. During the annealing step the probe undergoes a conformational change and binds to target sequence. The reporter and quencher are separated, and consequently, quenching is abolished and the fluorescence of reporter dye is emitted and detected. Unlike hydrolysis probes, molecular beacons are displaced, but not destroyed during the PCR amplification.

Hybridization Probes. In real-time PCR analysis with dual hybridization probes, four oligonucleotides are used: two primers and two juxtaposed probes (20). First probe is labeled with donor fluorophore at the 3′ end, while the acceptor fluorophore is attached to the 5′ end of the second probe (*Figure 4C*). The probes hybridize in a head-to-tail orientation in close vicinity to the target sequences, bringing the two fluorophores into close proximity and allowing FRET. Excited donor fluorophore emits light that excites acceptor dye, which dissipates fluorescence at a different wavelength. The reaction is monitored at the emission wavelength of the acceptor fluorophore. Since probes are not destroyed during the reaction, after each PCR cycle more probes can anneal to target sequences, which results in higher fluorescence signals. The amount of fluorescence is directly proportional to the amount of target DNA generated during the PCR process.

Scorpions. These reactions use two oligonucleotides: a primer and a bi-functional Scorpion that combines the upstream primer with a hairpin–loop probe labeled with a reporter dye at the 5′ end and a quencher dye at the 3′ end (21). This configuration brings the fluorophore in close proximity with the quencher and avoids fluorescence (*Figure 4D*). During PCR, the Scorpion primer is extended at its 3′ end; the loop sequence of Scorpion hybridizes to newly synthesized target within the same strand of the PCR product. The fluorophore and the quencher are separated, leading to emission of fluorescence. Scorpion probe contains a PCR blocker, which prevents DNA polymerase to read-through Scorpion primer and copy the probe region during the extension of the opposite strand. Moreover, in comparison with molecular beacons and TaqMan probes, Scorpions are faster and are able to produce a much stronger fluorescence signal, since they are based on kinetically more favorable unimolecular rearrangements (22).

In addition, many other fluorescence based chemistries are developed (LUX fluorogenic primers, amplifluor assays, QZyme primers, Light-Up probes, eclipse probes, Pleiades), and new designs are still being proposed (23–30). These assays are expected to be more adopted in the future.

All previously discussed chemistries can deliver excellent results, and each has its advantages and disadvantages (31). They all require less RNA than endpoint assays and are particularly suitable when working with small amounts of starting material; also, they

are more precise and more resistant to nonspecific amplification. The choice of detection chemistry is highly dependent on the characteristics of each individual experiment. Among many available primer/probe-based chemistries, in our opinion, TaqMan probes have proven to be well established and at this moment may be the best choice for gene expression studies. This opinion is supported by an exponentially increasing number of publications, showing that the results obtained by TaqMan chemistry are very specific and sensitive, which appears to be particularly important when analyzing target genes with very low expression levels. Moreover, this system has very well written guidelines and protocols, and is fairly error proof when designed and run according to protocol. On the other hand, Molecular Beacons and Scorpions are especially suitable for identifying point mutations.

Normalization of real-time RT PCR data

Real-time RT PCR has become a method of choice for the investigation of gene expression in biomedical research (32, 33). Nevertheless, a number of problems are still associated with its use (34–36). Importantly, the improper use of this technique as a clinical tool might have significant public health implications (37). Therefore, it is important to clearly point to some disadvantages of the current methods and to the critical steps that need to be carefully considered in experimental design. The reliability of real-time RT PCR results depends on the precision of many steps during the experimental procedure, from sample acquisition, preparation, handling and storage (38, 39), to reverse transcription (40), specific amplification (35), and data analysis (34, 35, 39, 41, 42). Nevertheless, variations in the amount of starting material, together with other potential experimental inaccuracies can be corrected by the normalization of target gene expression (43). Therefore, one of the most important steps in real-time RT PCR is the choice of an appropriate normalization strategy. There are several strategies that should be considered for the normalization of real-time RT PCR data (36, 43, 44).

The easiest, yet most intuitive, method for normalization is equalizing initial sample size by using a similar cell number, tissue volume or weight. Although it is a good practice to always use similar sized samples (e.g. a similar number of cultured cells, or similar sized biopsy), applying this method as an exclusive normalization strategy is insufficient because it does not account for the cumulative errors that can occur in cDNA preparation. Moreover, in some cases similar tissue volumes (blood, for example), or weights (such as adipose tissue) do not contain the same cellular material (types of blood cells or cell number in case of adipocytes). Therefore, using solely this approach for normalization can be misleading.

The second method is normalization to total RNA content after the extraction. Such normalization has also been advocated as unreliable since the total RNA content is usually determined spectrophotometrically. An alternative, more sensitive and accurate determination can be achieved by using the dye RiboGreen. RNA quantification using a Bioanalyzer (Agilent) represents a useful, but time–consuming step. This analysis provides useful information about the quality of the RNA, but again does not account for the cumulative errors that can occur in cDNA preparation. Namely, normalization to total RNA does not take into account reverse transcription efficiency. Finally, total RNA consists predominantly of rRNA, and hence is not always representative of the mRNA fraction.

Normalizing to genomic DNA can be an effective strategy in some cases, but it is generally impractical since most RNA preparation protocols deliberately eliminate the presence of DNA.

Normalizing to an artificial RNA molecule has many advantages over commonly used methods (45). Since this synthetic RNA should be included at a known concentration during the extraction stage, it will be affected and prone to the same experimental errors as RNA of interest. Spiking RNA with an artificial RNA molecule at a known concentration has been suggested as a method to normalize the errors that occur during cDNA preparation. However, this approach does not provide normalization for the actual concentration of sample cDNA. Stability of the spiked nucleic acid can also emerge as a problem. Finally, generating artificial RNAs might be impractical for small laboratories, while commercially available standards increase the costs of the experiments.

Normalizing to a stably expressed reference (housekeeping) gene that is representative of the cDNA concentration in a sample is the most commonly used normalization approach. The reference (housekeeping) gene is subject to the same errors in cDNA preparation as the gene of interest, thus being an excellent normalizing control. However, careful and strategic selection of the most stably expressed reference gene is essential (46–48). Random selection of a reference gene can add large and unpredictable errors to the analysis (49, 50).

There is no universally accepted strategy for normalization, as there is no error-free procedure. Since the mentioned approaches do not exclude each other, the best way to ensure the precision, repeatability and the reliability of the results (*Figure 5*) is to equalize sample sizes, ensure similar input of RNA for reverse transcription and measure internal control (reference gene or artificial molecule).

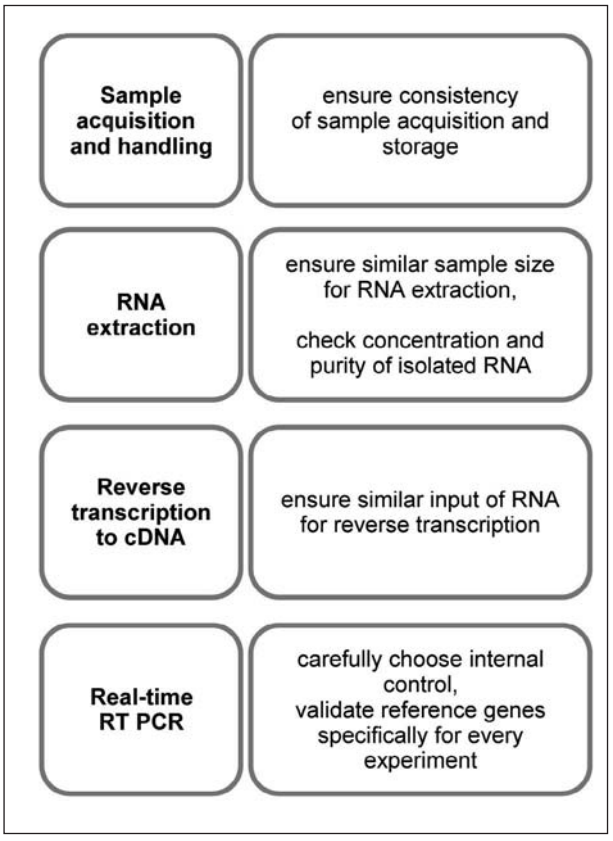


Figure 5 Real-time PCR workflow: critical steps and normalization strategies.

Housekeeping genes as reference genes

Although several alternative normalization procedures were proposed (43), the most commonly used strategy is normalization to an internal reference or a housekeeping gene. The term housekeeping gene was initially used for all genes that are essential for the function of each cell. Housekeeping genes are widely used as reference genes since their expression is assumed to be stable. However, the presumption of their invariable expression has been clearly discredited by a number of papers. Namely, numerous studies imply that the expression of housekeeping genes varies in response to treatment (47, 51), pathological (52), or environmental conditions (53), nutritional status (54), ageing (55, 56) and developmental stages (57, 58), as well as between sexes (59, 60), tissue types (48, 61, 62) and cell lines (63). So the search for an ideal reference gene began.

The ideal reference gene was supposed to fulfill several requirements: it must be constitutively expressed and unregulated regarding the experimental conditions, treatment, stage of the disease, age, gender etc. Preferably, it should be expressed at a similar level as the target gene (36, 47). It appeared that the search for such a gene was a search for the Holy Grail. Numerous studies were performed in order to test potential candidates, either by comparing their expression in various tissues and organs, in both sexes, by applying different drugs and other substances, or by examining their expression in various diseases. All these results together made a giant step forward in solving the problem of the perfect reference gene and pointed to a crucial finding: there is no single ideal reference that can be universally applied in all experimental designs. On the other hand, they pointed to a simple solution of the problem: reference genes must be chosen specifically for each experiment. Thus, the goal of the researchers became to identify the most reliable reference gene or a set of genes for every particular experiment.

The 18S RNA, β-Actin (BA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), beta-glucuronidase (GUSB), RNA polymerase II (RPII or POLR2A), tyrosine-3 monooxygenase/tryptophan-5 monooxygenase activation protein, zeta polypeptide (YWHAZ) and ubiquitin C (UBC) are some of the most commonly used reference genes in real-time RT PCR studies, although the issue of using these genes for normalization is a matter of constant debate. Namely, apart from their basic cellular roles, these proteins also participate in other cellular functions. Consequently, numerous studies demonstrated variability in the level of expression of these genes under various experimental conditions. Nevertheless, there is also evidence favoring their use, either separately or in various combinations, as appropriate internal standards in a number of carefully defined conditions, thus supporting the concept of proper validation for every single experimental design. Worryingly, this is still not a widely appreciated or acknowledged instruction. Unfortunately, the majority of studies applied reference genes without previous evaluation of their suitability for the specific experimental model.

GAPDH is one of the most commonly used reference genes, but its use as a reference gene for quantitative PCR analysis has been extensively debated (36, 46). GAPDH has been well known as a glycolytic enzyme. However, its role beyond glycolysis is increasingly elucidated, as it appeared to be involved in many other cellular processes, including DNA repair, nuclear RNA export, transcriptional and post-transcriptional regulation of gene expression, vesicular transport, receptor mediated cell signaling, membrane fusion and transport, cytoskeletal dynamics and cell death (64, 65). Participation in multiple pathways of homeostatic regulation indicates that GAPDH may have a fundamental role in a variety of pathologies including diabetes, cancer, malaria and neurodegenerative disorders, such as Huntington's, Parkinson's and Alzheimer's disease. Therefore, GAPDH should be considered as a reference gene only after proper validation in every given experimental design.

Similarly to GAPDH, BA is one of the most commonly used reference genes whose reliability is frequently questioned based on the new experimental data. BA is a member of a multigene family. It is one of the major components of cytoplasmic microfilaments and it plays an important role in cytoplasmic steaming, cell motility, cell division, phagocytosis, changing the cell shape, contraction of muscle cells, etc. (66, 67). Although there are cases when BA can be used as a reference gene (68, 69), it is not advisable to use it in situations when a tissue undergoes extensive morphological changes, as expected in different developmental stages, or in rapidly growing tissues, such as cancers.

Phosphoglycerate kinase (PGK) 1 is an ATP-generating glycolytic enzyme that forms part of the glycolytic pathway. Though it is often included as an endogenous control in commercially available kits for relative quantification of gene expression, this gene should be widely avoided when cancer tissues are analyzed because it is known to be involved in the onset and development of different malignancies (70).

By definition, a good internal control has a constant expression level across the set of samples being studied. The 18S rRNA has a low turnover rate, while the large 18S rRNA pool is less prone to substantial changes elicited by physiological perturbations. Therefore, the 18S rRNA gene may be a useful internal control in gene expression studies. Its use has been validated by numerous studies that showed its invariant expression across various organisms, tissues, developmental stages, and treatments (36, 71). However, there are two practical disadvantages in using 18S rRNA as a reference gene. First, having no poly(A) tail, 18S rRNA is absent from purified mRNA samples and is traditionally reverse transcribed with either specific primers or random hexamers (72, 73). Second, 18S rRNA is much more abundant than any typical mRNA transcript and therefore must be diluted to obtain a threshold value within the dynamic range of real-time PCR instruments, which inevitably introduces variability to the measurement. However, our results have shown that 18S rRNA can be successfully reverse transcribed using poly(dT)$_{18}$ with lower efficiency in comparison to specific or random primers, providing a way for its potential use as a reference gene (74).

B2M is a small subunit of the MHC class I molecule, and it has been successfully used as the reference gene in various experimental designs. However, its altered expression has been demonstrated in various pathophysiologies including different types of cancers. More importantly, B2M is considered a discriminatory biomarker and a good predictor, as well as a potential therapeutic target in numerous pathophysiological states, such as chronic kidney disease, peripheral and coronary artery disease, ovarian cancer, multiple myeloma (75–77). Therefore, this gene could be used as a reference gene with caution; in other words, its use should be experimentally evaluated.

HPRT is an enzyme involved in nucleotide metabolism and it represents one of the housekeeping genes expressed at a low level. It is advisable, therefore, to consider it as a reference gene in the experiments with low abundance target genes.

In order to find the best possible reference gene, some new, not commonly used genes should be included in the evaluation studies. A good example is importin 8 (IPO8), which exhibits excellent expression stability and shows no differences between normal and malignant lung samples (78). At this time point, several laboratories are in search for novel candidate reference genes that will meet the criteria of an invariant reference gene as closely as possible (79).

Statistical algorithms used for validation of reference genes

Validation of reference genes represents a time-consuming and expensive procedure, yet the use of nonvalidated genes may result in incorrect data. Several groups have developed statistical models and software programs for the analysis of candidate gene stability in order to help identifying the best reference genes (62, 80, 81).

GeNorm, developed by Vandesompele and coworkers, is among the most popular softwares (62). This software makes pairwise comparison between one and all other potential reference genes, in all samples, regardless of the sample groups and experimental conditions. The software ranks reference gene stability by the average expression stability value. It also analyzes pairwise variation values between two sequential normalization factors (geometric means of the best reference genes). Normalization factors are calculated by stepwise inclusion of an extra, less stable reference gene, to determine how many reference genes should be used. The authors strongly recommend using at least three reference genes for normalization, as a way to increase the accuracy of the results and to reach the sensitivity needed for detection of subtle changes in the target gene expression. Nevertheless, performing normalization by using the geometric mean of several reference genes increases the costs and the quantity of starting biological material and, therefore, is not always the most convenient solution.

Although the pairwise comparison approach employed by GeNorm represents an authoritative method for the analysis of potential reference genes, it is important to be aware that it ranks genes according to the similarity of their expression profiles, rather than minimal variation. Another frequently used soft-

ware is NormFinder (80). It is an application for Microsoft Excel, which provides information on intra- and inter-group variability and chooses the best reference gene, as well as the best combination of two reference genes. NormFinder, with its account of sample groups and its direct estimation of expression variation, provides even more precise and robust measurement of gene expression stability and, most importantly, candidate coregulation does not significantly affect the approach. Therefore, the best way to identify the most stable reference gene/s is to use both softwares. Besides these two, BestKeeper is also commonly used software for selection of reference genes. It employs pairwise correlation analysis of candidate reference genes and calculates the geometric mean of the best suited ones (81).

Inaccurate interpretation of data

The time-consuming process of data analysis during the selection of suitable reference genes can be significantly simplified by using the algorithms like geNorm, NormFinder and BestKeeper. Surprisingly, these approaches are still underutilized. Instead, the majority of reported research on gene expression analysis uses traditional housekeeping genes as references without any validation. Although considerable efforts were made to encourage the use of validated references in real-time RT PCR experiments, so far their use and justification are still not obligatory for publication in all journals.

Even though it is well documented that using unstable reference genes can lead to incorrect results (49, 50, 82, 83), the importance of this issue does not seem to be fully realized. Namely, continuous use of inappropriate reference genes increases the risk of reporting erroneous and conflicting results that might further affect data interpretation in basic and biomedical research, with particularly appalling implications in diagnostics, disease monitoring or drug development (37).

In 2009, Guenin et al. (50) elegantly illustrated how easily misinterpretation of the results could arise from the use of an inappropriate reference gene, by reevaluation of the expression stability of a set of 14 reference genes in 2 different experimental setups. In line with these results, our previous studies also showed that the use of inappropriate reference for normalization can lead to under- or overestimation of the target gene expression level and to misinterpretation of the results (55, 84).

In order to highlight the importance of endogenous control selection, and point to the extent to which the use of an invalid reference gene could affect the results of real-time RT PCR analysis, herein we assessed the expression of gap43 as a target gene 6, 12 and 18 months after treatment. The expression of gap43 was normalized to a stable reference gene

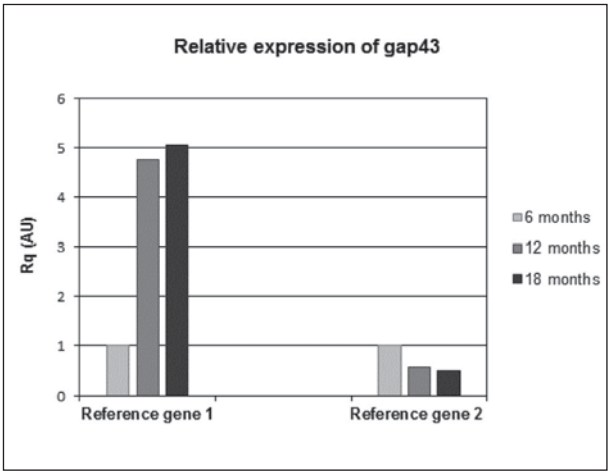


Figure 6 Relative expression of gap43 measured 6, 12 and 18 months after a treatment and normalized to reference gene 1 and reference gene 2.

(reference gene 1, GAPDH) and to an unsuitable reference gene (reference gene 2, 18S rRNA). As shown in *Figure 6*, strikingly different patterns of the gap43 expression level were noticed after normalization to GAPDH, as compared to normalization to 18S rRNA. Consequently, an erroneous conclusion that the gap43 expression level 12 and 18 months after treatment decreases by 50% as compared to the 6-month time point could be drawn based on incorrect normalization, while in reality, a 5-fold increase in the gap43 expression level occurs.

MIQE guidelines

In order to minimize possible future misinterpretations, inconsistencies and discrepancies in the published results, as well as to bring order into the terminology and in the manner of describing procedures and reagents, several leading scientists published MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (85). It is a set of guidelines that provides the information necessary for evaluation of real-time RT PCR experiments, aiming to contribute to the accuracy and reproducibility of published articles. MIQE guidelines address some of the crucial steps in real-time RT PCR, such as sample acquisition, RNA isolation, reverse transcription and PCR reaction. It points to critical information that should be incorporated in the Material and Methods section, thus helping editors and reviewers to evaluate the technical quality of the submitted manuscripts (85–88).

For researchers, the MIQE checklist represents a good starting point. It helps investigators to wisely plan and conduct experiments, as well as to clearly write and present the results (*Figure 7*) (89, 90). Namely, it is important to provide complete technical information in a manuscript, to validate protocols and

Figure 7 Information necessary for evaluation of real-time RT PCR experiments.

to present results and conclusions on the basis of appropriate methods of analysis.

In brief, to minimize possible misinterpretations and to assure good biological reproducibility for the published data, it is important to clearly define experimental conditions and experimental groups (controls vs. treatment/disease, etc), to accurately describe the sample (tissue/organ, biopsy, cell line), as well as to provide information on the type and number of replicates, experimental procedures and chemistry. When working with human samples, special care should be taken during the preanalytical phase. A detailed description of the possible errors during sample collection, transportation, reception, handling and storage in the laboratory is reviewed by Majkić-Singh and Šumarac (91). Samples should be stored frozen at a temperature not higher than –70 °C until use. It is important to report in detail where the sample was obtained and whether it was processed immediately or preserved (how long and under what conditions it was stored). The RNA extraction method should be stated and if a DNase treatment step is included, it is essential to report the type of DNase used and the reaction conditions. The extracted RNA should be highly pure and undegraded. The use of degraded RNA increases variability and can generate false results, while impurities may lead to inhibition of the reverse transcription and PCR reactions, which also leads to varying and incorrect quantification of the results. Therefore, the amount, quality and integrity of RNA must be recorded. Since the RNases are highly abundant in the environment, it is good to perform the reverse transcription of total RNA to a more stable molecule – cDNA, immediately after the quality check. The reverse transcription is probably the most variable step (92). Therefore, it is essential to provide a detailed description of the protocol and reagents used, including the amount of RNA reverse transcribed, priming strategy, enzyme type, volume, temperature, and duration of the reverse transcription step. It is advisable to use the same amount of total RNA for reverse transcription for all samples, in order to minimize variability between biological replicates. cDNA should be stored frozen at a temperature not higher than –20 °C until use. For description of real-time PCR, target accession numbers, amplicon locations and sizes, primer and probe (if used) sequences (or commercial assay catalogue numbers), experimental conditions and the manufacturer of a PCR instrument should be listed together with the information regarding melting curve analyses (for DNA bind-

ing dye assays), PCR efficiency, linear dynamic range, limits of detection and precision. It is a good practice to include »inter-run calibrators« e.g. to run one identical sample on different plates, in order to allow plate-to-plate comparison as well as measurement of inter-run variation. »No template controls« should be included on each plate for each gene, since they provide information about PCR contamination. Finally, it is important to justify the choice of the used reference gene and provide detailed information on the methods of data analysis and confidence estimation, together with specification of the software used.

In general, it is important to work under tightly controlled and well-defined conditions, since the variability of results obtained from identical samples assayed in different laboratories continues to be a problem (93). Also, it is important to report all measured parameters and to list the chemistry used, as well as the conditions for all reactions.

By following the precise MIQE guidelines, with a well-designed and carefully performed experiment and a good normalization strategy, real-time RT PCR becomes a sensitive, efficient and reproducible method for measuring gene expression that guarantees reliable data.

Conclusion

Quantitative real-time RT PCR significantly simplifies and accelerates the process of producing reproducible and reliable quantification of target genes transcription. Proper normalization is an absolute prerequisite for reliable mRNA quantification. There are several strategies that can be used for normalizing real-time RT PCR data. These strategies are not mutually exclusive, but at the moment, using reference genes represents the most acceptable strategy, because it is simple to use and can provide control for every stage of the real-time PCR. However, it must be used with caution. The key to normalization when using this strategy is to be able to demonstrate that it is valid. Therefore, the selection of suitable reference gene(s) is one of the most important steps. We strongly recommend using validated reference genes rather than relying on traditional housekeeping genes. Realizing the full potential of real-time RT PCR and its advantages over related conventional techniques, together with adopting the systematic validation of reference genes as a prerequisite, would greatly improve the accuracy and consistency of the published results. Finally, to ensure the relevance, accuracy and correctness in the interpretation of data, we encourage precise following of the MIQE guidelines. Adopting MIQE guidelines and including a detailed description of sample acquisition and handling, together with a full description of the PCR conditions, chemistry and data analysis, will promote experimental transparency, repeatability, accuracy and relevance, and consequently help in ensuring consistency of the results between laboratories.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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