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Review

Control genes in quantitative molecular biological techniques: the variability of invariance ☆

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Abstract

The measurement of transcript levels constitutes the foundation of today's molecular genetics. Independent of the techniques used, quantifications are generally normalised using invariant control genes to account for sample handling, loading and experimental variation. All of the widely used control genes are evaluated, dissecting different methodological approaches and issues regarding the experimental context (e.g. development and tissue type). Furthermore, the major sources of error are highlighted when applying these techniques. Finally, different approaches undertaken to assess the invariance of control genes are critically analysed to generate a procedure that will help to discern the best control for novel experiments. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Invariant control; Housekeeping; GAPDH; Cyclophilin; Actin; rRNA

1. Introduction

Control measurements form a fundamental tenet of all quantitative procedures. Determining the level of gene expression is no exception, whether this is performed by Northern-, southern-, slot- or dot-blotting, RNase protection-, nuclease protection- and in situ hybridization-assays, semi- and fully-quantitative PCRs all must employ appropriate controls as part of their experimental procedure. The primary control for these experiments is the parallel measurement of a control or housekeeping gene to assess differences in sample loading or reaction efficiencies, thus providing a means to evaluate and subsequently adjust for intrinsic experimental variations. This of course applies only to biological scenarios where transcription is active at all. In moribund or heavily histopathologically affected cells, for example, gene transcription may be reduced or indeed cease entirely. A similar situation exists in cells undergoing severe/acute stress (e.g. heat shock), where transcription is transiently blocked. In both of the examples mentioned, true control genes do not exist. In all other cases, pivotal control genes have to exhibit two major properties: firstly they should be essen-

Abbreviations: RT — reverse transcription; GAPDH — glyceraldehyde-3-phosphate dehydrogenase; EF — 1α-elongation factor 1-alpha; CYP — cyclophilin; HPRT — hypoxanthine phosphoribosyltransferase

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tial for the maintenance of cellular function and viability (Finnegan et al., 1993), and therefore should be ubiquitously expressed in all tissues; and secondly their transcription should not be affected within the experimental context being investigated. For example, when comparing differential exposure regimes, developmental stages or tissue distributions, the expression of invariant control genes should remain at a steady-state level, whilst the transcription of target genes may be up- or down-regulated. Therefore, the use of internal controls facilitates the direct and sound measurement of comparative gene expression.

Identifying suitable control genes has been a major challenge in the field of molecular biology. Over the last decade, numerous candidate genes have been forthcoming, some of which have now established themselves as an integral part of quantitative approaches. However, to date no single gene has been awarded the unconditional approval by the scientific community. In many cases factors may modulate the expression of control genes, resulting in a slight variability of the so-called constitutively expressed housekeeping genes. Whilst these factors are often of unidentified origin, two prevailing reasons have been highlighted, namely the cross-reactive response to pseudo-genes and the presence of differentially expressed isoforms. This short review aims to bring together some key papers on the use and abuse of control genes assumed to be invariant.

2. The choice is yours!

To ensure that all samples contain equal amounts of starting material, it may seem reasonable to assess DNA/RNA concentrations by means of standard optical spectrophotometery. However, this physical technique has serious downfalls and thus is, by itself, not a feasible option. Values obtained are notoriously unreliable due to optical interference by varying amounts of contaminating material, such as ribosomal RNAs and transfer RNAs. This also the case during cDNA synthesis when excess reaction components, including dNTPs and reverse trancription (RT) primers may also disrupt measurement. A more time consuming and experimentally

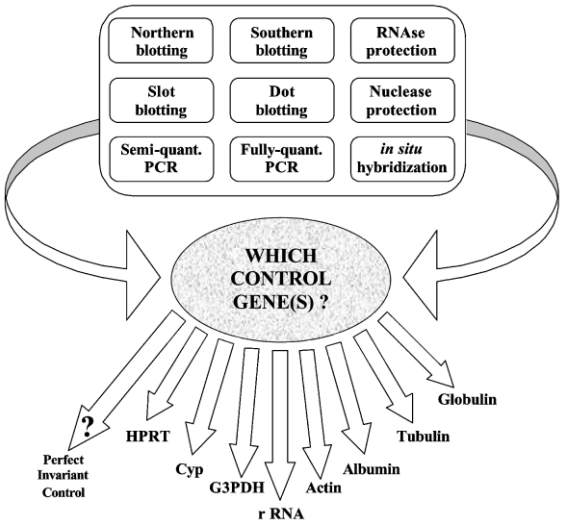

Fig. 1. Housekeeping genes in molecular biology: the choice is yours!.

more challenging approach uses poly(T) probes. Overall this is a reliable method to quantify poly(A) + mRNA as it is able to distinguish between target and contaminant. However, what both methods do not account for is differences caused by downstream experimental applications such as sample handling (pipetting and gel loading) and the variability due to differential transfer efficiencies (Goldsworthy et al., 1993). Employing control genes aims to overcome these problems. In molecular biology, there are currently at least nine housekeeping genes that are frequently used to normalise expression signals. Indeed, over 90% of signal normalisiations are performed with either of the following genes: actin; glyceraldehyde-3-phosphate dehydrogenase; the family of ribosomal genes; and to a lesser extent cyclophilin and the elongation factor 1-alpha (Fig. 1).

3. Actin

Actin is one of the major components of cytoplasmic microfilaments in eukaryotic cells. It plays an important role in diverse cellular functions, such as cyctoplasmic streaming, changes in cell shape, cell motility, phagocytosis, cell division, the distribution of plasma membrane proteins and the generation of contractile force in both muscle and non-muscle cells (Romans et al., 1995;

Kusakabe, 1997). As a member of a multigene family, every organism typically possesses three to four highly homologous actin isoforms. Including processed pseudogenes this number can rise to well over a dozen as seen in the in human and mouse genomes (based on EMBL/Genbank database entries). Although actin is one of the most widely used internal control genes, there is an increasing volume of evidence suggesting that the relative amounts of each isoform expressed and the total actin content can vary with muscle type, development, cell culture conditions, pathologically and potentially between cells within tissues (Drew and Murphy, 1997). The use of actin is principally not advisable in situations where tissues undergo extensive morphological changes, as experienced in different developmental stages. Beta-actin and to a lesser extent gamma-actin have been used successfully in a multitude of occasions, highlighting that within a defined context it can exhibit good invariable characteristics. The width of application can be enhanced by designing probes to bind specifically to the cytoplasmic β-actin and not to any of the other isoforms or pseudogenes (Raff et al., 1997).

4. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH / GAPDH)

The glycolytic tetramer glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional enzyme involved in cellular metabolism. Although it was initially believed that GAPDH is expressed as a single-copy nuclear gene (Bhatia, et al., 1994), genome sequencing data have identified at least two functionally independent GAPDH genes in *Caenorhabditis elegans*, *Drosophila melanogaster* and humans. Compared to actin, GAPDH's low copy number as well as the slightly less conserved intra-specific coding regions reduces the likelihood of isoform and pseudo-gene cross-reactivity, a distinct advantage when designing probes (Table 1). Nevertheless, the overall consensus sequences of GAPDH is sufficiently conserved (between different animal phyla) to allow the isolation of genes from unconventional or rare species with relative ease. These factors have made GAPDH the most widely used internal control gene in molecular genetic techniques. Predictably this gene also has limitations too. For example it has been reported that its use

Table 1
Intra- and inter-species sequence homologues of a *C. elegans* actin isoform (act-1, database ID: T04C12.4) and a GAPDH isoform (gpd-2, database ID: K10B3.8)

C. elegans *act-1*/T04C12.4	Sequence Homologues	% identity at aa level	% similarity at aa level
C. elegans	11	93–100% (5) 22–53% (6)	97–100% (5) 42–72% (6)
S. cerevisiae	8	89% (1) 21–47% (7)	93–100% (5) 22–53% (6)
D. melanogaster	15	94–98% (7) 22–64% (8)	98–100% (7) 40–79% (8)
M. musculus	10	95–97% (6) 26–53% (4)	98–100% (7) 46–75% (8)
H. sapiens	17	94–97% (6) 26–55% (11)	97–100% (6) 46–75% (11)
C. elegans *gpd-2*/K10B3.8	Sequence Homologues	% identity at aa level	% similarity at aa level
C. elegans	2	100% (1) 88% (1)	100% (1) 95% (1)
S. cerevisiae	3	65–67% (3)	80% (3)
D. melanogaster	3	63–75% (3)	75–87% (3)
M. musculus	2	67 + 76% (2)	81 + 88% (2)
H. sapiens	3	67 + 75% (2) 48% (1)	80 + 87% (2) 63% (1)

The number of identified related isoforms and their respective% identity/similarity scores (as defined by the BLAST algorithm) are listed. The figures in parentheses represent the number of genes within each cohort. All analyses were performed using the Proteome database and search engine (for more details, see http://www.proteome.com).

should be avoided in experimental hypoxia (Zhong and Simons, 1999), cancer cell lines (Rondinelli, et al., 1997), during ontogeny and acute pancreatitis (Calvo, et al., 1997), cell proliferation and carcinogenisis (McNutty and Toscano, 1995). In contrast, expression levels were shown to be reasonably consistent between different experimental conditions including the use of micro-dissected human renal biopsy specimens (DelPrete et al., 1998), stimulation with anti-IgE, cyclosporin-A and dexamethasone (Williams and Coleman, 1995). In a comprehensive comparative analysis of control genes, Matyas et al. (1999) conclude that GAPDH, though not perfect, is the most favourable control gene to compare normal and osteoarthritic chondrocytes during Northern analyses.

5. Cyclophilin (CYP)

Cyclophilins, or peptidyl-prolyl *cis-trans* isomerases, are enzymes belonging to the superfamily of immunophilins. Their biological significance is manifested by the catalysis of protein folding via peptide bond rotation on the amino side of proline residues (Fischer et al., 1989; Takahashi et al., 1989), the action as a chaperone for protein trafficking as well as the nucleolytic degradation of the genome (Montague et al., 1997). Bio-medical interest was triggered by the discovery that CYP shows a high affinity towards cyclosporin A, the immuno-suppressant drug used by organ transplant patients (Kunz and Hall, 1993). Every organism studied typically possesses 2–4 isoforms that differ in their molecular mass, generally ranging from 18 to 24 kDa. One distinctive difference exists between the isoforms, namely the presence or absence of an N-terminal signal sequence, which is thought to be instrumental in the translocational mediation to subcellular positions, such as the endoplasmic reticulum (Price et al., 1991) or the mitochondria (Walsh et al., 1992). Numerous reports have observed a differential induction of CYP, in general, mainly during development or exposure to certain stressors. It has been shown for example, that mercuric chloride (Martinez-Gonzalez and Hegardt, 1995), development (Marivet et al., 1992), heat shock, virus infection, ethephon and salicylic acid exposure (Marivet et al., 1994) significantly induce CYP expression in plants. Similarly, differential regulation was observed in hypoxia and heat stressed myogenic cells (Andreeva et al., 1997). All of the above studies treat CYP isoforms as a single functional unit, however, there is evidence that the expression of CYP isoforms is independent from each other. CYP-A, but not CYP-B, has been shown to be present with disease correlation in the synovial fluids from patients suffering from rheumatoid arthritis (Billich et al., 1997). Analogous observations were observed in earthworms exposed to heavy metals, where CYP-A was metal responsive, however, CYP-B remained invariant (Stürzenbaum et al., 1999). These observations have profound implications regarding cyclophilin's use as an invariant control and highlights the fact that it is essential to treat cyclophilin isoforms as separate entities, rather than one functional unit. To minimise the risk of misleading data acquisition, it is suggested that CYP probes are designed not to cross-react with different isoforms and until fully evaluated should be CYP-B specific.

6. Ribosomal subunits

The 18S and 28S ribosomal subunits are a further example of commonly used internal controls. A literature review of its uses revealed a reoccurring theme: when directly compared to other housekeeping genes they compare extremely favourably in terms of steady-state expression levels. Examples include gene expression studies conducted on normal, benign tumorigenic and highly metastatic human cell lines (Bhatia et al., 1994), rat liver tissues (Leeuw et al., 1989), analyses of transcription under hypoxia (Zhong and Simons, 1999) and the assessment of gut hormone expression (Yamada et al., 1997). In all reports the 28S ribosomal subunit outperformed other housekeeping genes including cyclophilin, actin and GAPDH. Unfortunately the ribosomal subunits show a lower degree of inter-species sequence conservation than GAPDH and actin. Therefore, the initial isolation procedure may prove more labour intensive when dealing with a new organism. Furthermore, it is vital to avoid the accidental isolation of the mitochondrial ribosomal subunit. Depending on the species examined, transcription of the mitochondrial genome is initiated either via one or two transcriptional initiation sites. It therefore, has to be

expected that the expression of the mitochondrial genes, including the mitochondrial ribosomal subunits, fluctuate according to the needs of cellular energy metabolism. Finally as the ribosomal subunits are not polyadenylated they cannot be exploited when dealing with poly(A) + RNA or cDNA derived from total RNA utilising poly T-primers in the RT reaction. It is precisely for this reason that the ribosomal subunits have failed to replace the use of other housekeeping genes. Although it possesses very promising invariant characteristics, its uses are limited, especially in the light of the fact that an increasing number of laboratories prefer the use of mRNA or polyT-primers in the RT step.

7. Other housekeeping genes

The elongation factor-1 alpha (EF-1 alpha) is an ubiquitous protein that binds aminoacyl-transfer RNA to ribosomes during protein synthesis. It has been stipulated to be a good invariant control to adjust for differences in tube-to-tube loading and/or degradation (Dostal et al., 1994). However, being an integral part of the translation apparatus, its expression can be modulated in areas of high protein turnover such as rapidly growing tissue, plant meristems and gametophytes (Ursin et al., 1991), but also in situations involving growth arrest, transformation, ageing and cell death (Duttaroy et al., 1998).

In some cases albumin has been chosen as an internal control. However, transcription may be influenced by exposure to phenobarbitone and methylclofenapate (GarciaAllan et al., 1997), the limitation of amino acid availability (Marten et al., 1994) and has been observed to change in subconfluent cultured hepatocytes (Grunnet et al., 1999). Nevertheless, a direct comparison of mRNA levels of different control genes revealed that albumin showed low inter-sample variation in proto-oncogene expression under experimental conditions that induce cell proliferation in rat liver. The study concluded that albumin is more appropriate than actin or GAPDH for normalisation purposes (Goldsworthy et al., 1993).

Alternatively, gene expression has been assessed after normalisation with tubulin. Examples include human connexin 43 (Serels et al., 1998), Ca-ATPase in fibroblasts (Reisner et al., 1997) and the growth factors PDGF and TGF-beta in glial cells (Mapstone, 1991). Whilst tubulin expression is unaffected by rapamycin (Tuhackova et al., 1999) and ethacrynic acid (Shen et al., 1995) there are reports claiming that amino acid limitation (Marten et al., 1994) and cell density (Grunnet et al., 1999) does affect tubulin transcription. Similarly, Perfetti et al. (1991) show that tubulin, but also actin and GAPDH expression is relatively constant in tonsil samples undergoing a chronic inflammatory process, but are heterogeneous in Hodgkins-disease lymph nodes.

Beta(2)-microglobulin has been successfully used in a variety of experiments, including leukemia research (Pallisgaard et al., 1999), brain pathological studies (Schramm et al., 1999), breast carcinomas (Waha et al., 1998), human corneas (Ljubimov et al., 1998) and even formalin-fixed, paraffin-embedded tissue (for a review see Krafft et al., 1997). Unfortunately, the number of publications describing the use of this gene is still relatively low and thus, beta(2)-microglobulin still awaits a full and comprehensive assessment of its general suitability as an invariant housekeeping gene.

Hypoxanthine phosphoribosyltransferase (HPRT) is involved in nucleotide metabolism and expressed at relatively low levels. Therefore, this gene is not suitable when using methodologies with low sensitivity. However, it has been shown to be invariably expressed when using both Northern protocols and PCR based approaches and in studies of alveolar macrophages, spleen cells (Foss et al., 1998) and particularly in cytokine expression studies (Konig et al., 1995). One notable exception in HPRT constant expression has been in the mammalian brain where elevated levels have been detected (Jiralerspong and Patel, 1996).

8. By chance or design

Given the wide selection of possible control genes it is difficult to identify which is the most appropriate when designing a new experiment. The choice available will initially be determined by the experimental design, for although exploitation of ribosomal RNA may be a preferred option, use of Poly(T) separation chemistry or cDNA priming will eliminate this option. If ribosomal RNA is not an option then a review of all the appropriate literature concerning the experimen-

tal exposure regime and the cells system/organism under investigation should be undertaken. If no conclusive data can be located documenting the validation of a control gene under parallel experimental design then a range of control genes must be evaluated. A number of guiding principles may help with this process, always determining the exact sequence of the target gene from the target organism and design the primers or probes used to be isoform specific, review the metabolic and physiological implications of the experimental procedure in the context of various control genes. For example, do not choose actin in studies comprising developmental biology since connective tissue physiology is under significant flux. Similarly, GAPDH should not be used when studying thyroid function as it is known to moderate the activity of this enzyme. If exploiting a control gene within a novel system it is recommended to perform parallel measurement of the nucleic acid sample using a physical technique such as a Poly(T) probe or UV/Vis/fluorescent spectroscopy. If there is an extensive discrepancy between the inter-sample ratio of the physical measurement and the selected control gene it is advisable to review your sample preparation/experimental procedure. If it is not possible to ascertain the reason for this inconsistency it is advisable to identify and measure a second or even a third control gene (Fig. 2).

The explosion of information that has been generated through chip technology will provide us with a plethora of potential control genes. It may be possible to use our understanding of biochemistry and physiology to identify an appropriate control for each experiment. Essential metabolic pathways are already well characterised and the molecular mechanisms underlying the transcriptional control of component enzymes are continuously being unravelled. This understanding may

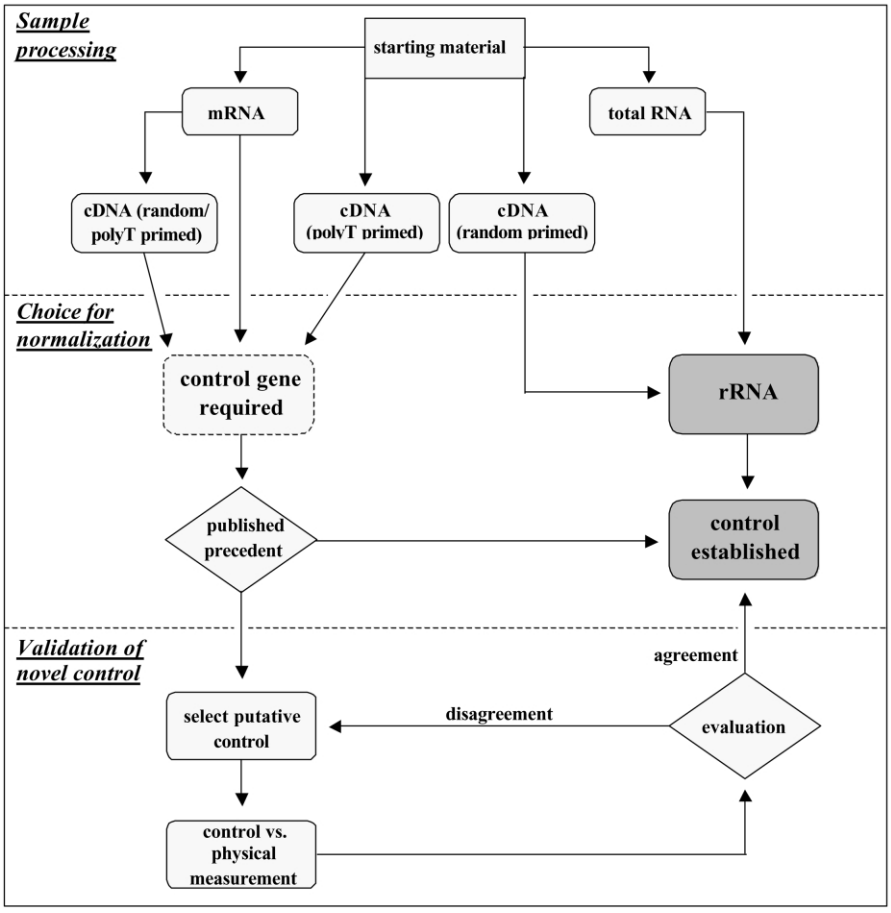

Fig. 2. Schematic flowchart depicting the suggested approach to ensure the optimal use/choice of control gene.

lead to a new generation of improved control genes identified by design rather than chance.

9. Conclusions

It may well be scientifically acceptable to use any one of the major control genes and rarely will a referee question their applicability. However, the examples brought together in this short review clearly illustrate that no single gene has been shown to be invariant per se. Therefore, it is advisable to address the issue of the 'variance of invariance' particularly when dealing with novel biological material or when applying previously untested exposure regimes. The dangers of complacency are obvious: if control signals are variable, the representation of target genes will equally be prone to be evaluated above or below their true quantity. It follows that an apparent down-regulation of a transcript may well be a true up-regulation and vice versa. Indeed, it is conceivable that because of this some valid experimental result may have been discarded. But not all is gloom: in reality these extreme situations are not likely to be a frequently encountered phenomenon. Even when deploying the most accurate quantitative technology, such as quantitative PCRs, the identification of a transcriptional response is rarely considered significant if signals fail to exceed differences in excess of a factor of two. Published data typically refer to an up- or down-regulation that ranges in the region of two to three orders in magnitude. In other words, slight differences in the expression of control genes will be masked by significant differences of the target gene expression. Overall, the methodology of exploiting housekeeping genes as invariant controls only remains scientifically robust if the differential expression of the target gene is significant and the corresponding values explicitly expressed as a figure relative to control gene transcription. This of course opens a new avenue of arguments. For example if gene expression is quantified relative to another gene the term 'fully quantitative' is misleading or even a misnomer?

In conclusion and thinking along the same lines as George Orwell one might say that 'The cellular expression profile of all housekeeping genes remains equal, however, some remain more equal than others'. The ribosomal subunits have been shown to be the least variant internal control, but their uses are limited to experiments involving total RNA or total RNA derived cDNA. When dealing with poly(A) + enriched samples alternative genes have been identified. Although none show universal invariance, they are particularly useful for specialist applications/exposures. Therefore, the choice of control gene(s) should be carefully selected and validated prior to experimentation, utilising new applications and/or organisms. A new era of gene analysis with microarray technology allows us to assess gene expression of 10 000 genes within a single experiment. Being the lowest common denominator of experimental design, the quality of the chosen internal control(s) is reciprocal to the quality of data generated and the significance of the interpretation of these global gene analysis studies!

Acknowledgements

We have tried to include the major recent findings, but have had to omit some reports due to space limitations and for this we beg indulgence from the authors. Furthermore we wish to acknowledge the continuous support from the British Natural Environmental Research Council (NERC) and the Royal Society.

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