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Short communication

Housekeeping genes as internal standards: use and limits

O. Thellin [a,b], W. Zorzi [a,*], B. Lakaye [c], B. De Borman [c], B. Coumans [a,b], G. Hennen [b], T. Grisar [c], A. Igout [b], E. Heinen [a]

[a] *Institute of Human Histology, University of Liège, rue de Pitteurs 20, B-4020 Liège, Belgium*
[b] *Laboratory of Biochemistry/Endocrinology, University of Liège, B-4020 Liège, Belgium*
[c] *Laboratory of Biochemistry Research group in Neurochemistry, University of Liège, place Delcour 17, B-4020 Liège, Belgium*

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Abstract

Quantitative studies are commonly realised in the biomedical research to compare RNA expression in different experimental or clinical conditions. These quantifications are performed through their comparison to the expression of the housekeeping gene transcripts like glyceraldehyde-3-phosphate dehydrogenase (G3PDH), albumin, actins, tubulins, cyclophilin, hypoxantine phosphoribosyltransferase (HRPT), L32. 28S and 18S rRNAs are also used as internal standards. In this paper, it is recalled that the commonly used internal standards can quantitatively vary in response to various factors. Possible variations are illustrated using three experimental examples. Preferred types of internal standards are then proposed for each of these samples and thereafter the general procedure concerning the choice of an internal standard and the way to manage its used are discussed. © 1999 Elsevier Science B.V. All rights reserved.

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Quantitative assays widely use housekeeping gene transcripts as β-actin, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or L32 whose presumed stable expression allows quantification of other expressions, for example those of cytokines, by comparison to this internal standard. In this paper, a series of in vivo and in vitro models are presented using housekeeping genes showing in certain cases the limits at the use of such internal standards. Different possible methods enabling the management of this problem will be discussed.

The study of biological regulations is very often correlated to quantification assays, which can be related to proteins or RNA. This paper will discuss the problem of mRNA quantification.

Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxantine phosphoribosyltransferase; PMA, phorbol 10-myristate 13-acetate.

* Corresponding author. Tel.: +32-4-366-5178; fax: +32-4-366-5173.

E-mail address: willy.zorzi@ulg.ac.be (W. Zorzi)

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Table 1
List of the most often considered housekeeping genes, used as internal standards

Internal standards	Known essential functions	Pro	Con or restrictions
G3PDH (= GAPDH)	Important glycolytic pathway enzyme	Recommended for less sensitive detection methods (Northern blot) (Petersen et al., 1990; Tang et al., 1996)	Yamada et al., 1997; Foss et al., 1998[a]
Albumin	Intravascular osmotic pressure great contributor	Goldsworthy et al., 1993	Marten et al., 1994[a]
β-, γ-actins	Essential for the structure and kinetics of the cytoskeleton	Choi et al., 1991	Marten et al., 1994; Yamada et al., 1997; Foss et al., 1998[a]
α-, β-tubulins	Essential for the structure and kinetics of the cytoskeleton	Choi et al., 1991; Serels et al., 1998	Marten et al., 1994[a]
Cyclophilin	Involved in cellular protein folding and protein interactions	Bjarnason et al., 1998; Jaschke et al., 1998	Chang et al., 1998[a]
HRPT		Recommended for sensitive detection (RT-PCR) (Marten et al., 1994; Foss et al., 1998)	Unknown
L32	Ribosomal protein	Recommended for less sensitive detection methods (Nothern blot) (Lemay et al., 1996; Wu et al., 1999)	Unknown
18S, 28S rRNA	Ribosomal subunits	Yamada et al., 1997	[b]

[a] May vary highly between different cell types, culture conditions or during cell cycle.
[b] Production highly reduced or stopped during mitosis.

Many techniques allow quantification of a given mRNA: RNase protection, Northern blot, semi-quantitative mimic RT-PCR or else. All these techniques can use internal standards, mainly housekeeping genes, so called because their synthesis occurs in all nucleated cell types since they are necessary for the cell survival. The synthesis of those molecules is often considered as being very few fluctuating in comparison to that of others and, by their commonplace use, are considered in many laboratories as constant and secure. But numerous studies showed that even these genes see their expression even highly does vary in given situations (Huitorel and Pantaloni, 1985; Zhang and Snyder, 1992; Goldsworthy et al., 1993; Hobbs et al., 1993; Bhatia et al., 1994; Bereta and Bereta, 1995; Lemay et al., 1996; Chang et al., 1998). This may partly be explained by the fact that housekeeping proteins are not only implicated in the basal cell metabolism but also participate in other functions (Petersen et al., 1990; Singh and Green, 1993; Ishitani et al., 1996).

The most often considered and used housekeeping genes are those for albumin (for hepatocytes) (Goldsworthy et al., 1993), β-, γ-actins (Choi et al., 1991; Wei et al., 1997), cyclophilin (Bjarnason et al., 1998; Jaschke et al., 1998), G3PDH (Petersen et al., 1990; Tang et al., 1996), α-, β-tubulins (Choi et al., 1991; Serels et al., 1998), hypoxantine phosphoribosyltransferase (HRPT) (Marten et al., 1994; Foss et al., 1998), L32 for other cell types (Lemay et al., 1996; Wu et al., 1999) or 18S, 28S rRNA (Finnegan et al., 1993; Bhatia et al., 1994). The essential functions of these molecules are variable (Table 1). There are few studies using two or more of these molecules at a time (Dent et al., 1997).

The application of these internal standards in three kinds of studies based on the use of nerve cells or immune cells in vivo or in vitro systems will be discussed here.

Total RNA extractions from cerebellum, brain cortex, brain stem and subcortical structures of Wistar and genetic absence epilepsy rats from

Strasbourg (GAERS) rats were performed according to the acid guanidinium thiocyanate–phenol–chlorophorm single step method. The 18S rRNA amounts were estimated by densitometry after agarose gel electrophoresis and staining by ethidium bromide. On the other hand, the G3PDH transcripts amounts were estimated by mimic RT-PCR using the Clontech PCR MIMIC Construction Kit. Obtained results showed that the G3PDH/18S rRNA ratios do not significantly vary between Wistar and GAERS rats and between different nerve tissues ($n = 6$; mean (m) and standard deviation (S.D.) (δ) respectively for cortex: $m = 149$, $\delta = 10$; for cerebellum: $m = 148$, $\delta = 14$; for subcortical structures: $m = 149$, $\delta = 12$; for brainstem: $m = 149$, $\delta = 6$). So in this in vivo case, G3PDH as well as 18S rRNA may be used as internal standards without any problem.

Another study was related to the analysis of the evolution of cytokine mRNA synthesis after in vivo immunisation of mice. RNase protection assays (Pharmingen, kit RiboQuant with mCK-1, mCK-3b and mCR-1 probes mixes) were performed on total RNA extracted from fifteen mice spleen using InstaPure LS kit (Eurogentec) at various periods of time after primary and secondary immunisation with complete Freund's adjuvant and incomplete Freund's adjuvant. In this study, L32 and G3PDH transcripts were used as internal standards in this RiboQuant RPA kit. We also checked the RNA integrity analysing the 28S rRNA/18S rRNA ratios by agarose gel electrophoresis. In these experiments, even if the mice are differently treated, receiving different immunisation protocols, L32/G3PDH ratios only slightly fluctuated ($n = 15$; $m = 1.47$; $\delta = 0.14$), appearing nearly constant, allowing thus the use of any of these housekeeping genes as internal standard, especially since during examination of the results, only variations higher than 30% were accepted as significant.

In the third study cytokine mRNA synthesis using RNase protection assay kit was also considered (Pharmingen, kit RiboQuant with hCK-1 probes) but in human peripheral blood mononuclear cells maintained in vitro and analysed after various culture conditions. Total RNA was extracted and mRNA was analysed using RNase protection RiboQuant kit as described for the mice vaccination experiments. L32 and G3PDH transcripts were used as internal standards. They showed low degrees of variations at time 0 (after preparation of blood cells) or after cultivation in absence of mitogens. However, when phorbol 10-myristate 13-acetate (PMA), ionomycin or other activators were added to the culture medium, higher variations were noted. In the mitogenic stimulated mononuclear cells, L32 and G3PDH transcripts fluctuated according the mitogen and the considered period of time. Moreover, L32 and G3PDH transcripts did not change in the same manner, rendering inadequate their use as internal standards. To circumvent this difficulty it was decided to use them in situations where they only slightly fluctuated, else 28S rRNA and 18S rRNA were referred to.

Thus, as mentioned in literature and shown in this article, housekeeping gene expressions are constant in given cell types or experimental conditions but may vary and be not useful for routine applications in laboratories. As a consequence the use of housekeeping genes as internal controls should be examined carefully in relation to the cell types and the cell metabolism, else they can be conducted to obtain eronate quantification results.

The use of 28S rRNA and 18S rRNA are recommended as internal standards for mRNA quantification study because mRNA variations are weak in comparison and cannot highly modify the total RNA level. If one wants to compare these to housekeeping gene transcripts, as often observed in papers, it is proposed to use at least two types of housekeeping gene transcripts as internal standards (Dent et al., 1997). The use of only one internal standard (Lemay et al., 1996; Tang et al., 1996) is nevertheless acceptable at the conditions where variations of housekeeping gene expression are taken as internal standard have previously been investigated in the same experimental conditions. If the mRNA ratios of the housekeeping genes used as internal standards are constant or not statistically different, it appears possible to use any of them for standard. But when these ratios are varying (housekeeping genes expressions are not generally controlled by the

same mechanisms), which can often be the case in culture conditions based on the use of mitogens, the best way is to refer to rRNA 18S, 28S as internal standards.

More generally, it is also recommended to pay extreme attention at each step of the procedure to the material, the manipulation and the handling and interpretation of the data. Here is some helpful advice when working with RNA. Check the quality of RNAs before use (on a denaturing agarose gel electrophoresis) and aliquot samples to reduce degradation to the maximum. Check regularly the accuracy of your laboratory instruments. Use preferentially non-parametric (i.e. Mann–Whitney test) than parametric statistical methods. Repeat results at least three or four times for each condition. Think about what could vary between the two result repeats (i.e. if using female blood cells, try to use cells from females in the same part of their menstrual cycle). Remember then that these recommendations will probably reduce the variations due to the experiment but will not influence the variations occurring in the cells themselves. That is why a good knowledge of the modulations of the internal standard chosen in the experimental conditions is important prior to beginning any data interpretation.

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