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Short communication

Standard curve for housekeeping and target genes: Specific criteria for selection of loading control in Northern blot analysis

Federico Herrera [a], Vanesa Martín [a], Isaac Antolín [a], Guillermo García-Santos [a,b], Jezabel Rodríguez-Blanco [a], Carmen Rodríguez [a,b,*]

[a] *Departamento de Morfología y Biología Celular, Universidad de Oviedo, Facultad de Medicina, C/Julián Clavería, 33006 Oviedo, Spain*
[b] *Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, Facultad de Medicina, C/Julián Clavería, 33006 Oviedo, Spain*

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Abstract

The election of the correct loading control in Northern blot normalization is something essential to obtain valid results. Housekeeping genes are widely used as loading control and the assumption is made that they counteract load differences between samples. We have found, however, that uneven sample load is capable to alter the results despite normalization, considering no influence of the experimental conditions on housekeeping gene regulation takes place. Normalization ratio (transcript of interest/housekeeping gene) is determined as the pattern of variation in the ratio between densitometric signals of transcripts – both target and control – and the amount of total RNA. The fact that this relationship is specific for each transcript means different ratios will exist depending on the chosen control gene. Actually, loading differences of only 2 μg may induce a 2.5-fold difference between normalized ratios, depending on the housekeeping gene selected for normalization. In order to select the appropriate loading control, it becomes essential to establish a standard curve for each transcript of interest and several housekeeping. Only the one yielding a constant ratio of normalization along the total RNA range used is to be taken into consideration.

Keywords: Northern blot; Housekeeping gene selection; Uneven RNA loading; Normalization; Standard curves

Northern hybridization became part of the standard protocols in gene expression research since the 80s. To find a suitable loading control (LC) for normalization still constitutes a main problem in Northern hybridization as the goodness of the results depends to a great extent on the correct LC election (Spanakis, 1993). To be valid, LC should (1) not be regulated by the particular experimental conditions (Goldsworthy et al., 1993), and also (2) be able to report on differences in the amount of RNA between samples (linearity

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CuZnSOD, copper–zinc superoxide dismutase; 18S and 28S Rib Sub, 18S and 28S ribosomal subunits

* Corresponding author. Tel.: +34 98 510 3057; fax: +34 98 510 3618.
E-mail address: carro@uniovi.es (C. Rodríguez).

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behaviour) (Bhatia et al., 1994; Provost and Tremblay, 2000).

If control transcript is not regulated differently for a particular experimental context but its signal varies among samples, then uneven sample loading is to be assumed to take place. Housekeeping genes are supposed to compensate for differences in the amount of RNA between samples caused by sample handling or differential transfer efficiency (Stürzenbaum and Kille, 2001). There are no studies – to our knowledge – reporting on different results induced by uneven sample load depending on the LC selected for normalization.

In order to study how housekeeping selection can influence the final results beyond normalization due to uneven sample load, Northern hybridization with cDNA probes for different transcripts was performed using increasing amounts of total RNA from rat pheocromocitoma PC12 cells. GAPDH, 18S and 28S ribosomal subunits and actin were taken as control transcripts, and CuZnSOD as target transcript (Fig. 1A).

Densitometry assays were done and regression curves were adjusted to the experimental data (Fig. 1B). Densitometry values were the dependent variable (y) and the amount of loaded RNA the independent one (x). The regression coefficients (r^2) for several ranges of total RNA loaded appear on Table 1. It is worth mentioning that actin values fit very well over the whole range of loaded RNA, while GAPDH and CuZnSOD only match the lineal regression in the interval of 5–25 μg; ribosomal subunit 18S, in the intervals of 5–15 and 10–25 μg; and 28S, in the intervals of 5–15 and 15–25 μg.

All of the transcripts studied reach a plateau at 25 μg, even showing a good r^2. This represents the top amount of RNA to be used when working with these cells. The r^2 value for 28 and 18S ribosomal subunits indicates that these transcripts must not be used as LC for Northern blot under the current experimental conditions, i.e., using total RNA from PC12 cells when more than 15 and 25 μg of total RNA, respectively, are going to be loaded.

Regression equations allow us to determine expected values from observed data. Thus, the expected densitometric data for additional theoretical amounts of RNA were estimated following the lineal fitting obtained for the various amounts of RNA loaded. From these predicted values the ratios (transcript of interest/control transcript) were calculated for each given amount of RNA (Fig. 1C). CuZnSOD signal was first triggered with 6 μg of total RNA, marking what would be the theoretical detection threshold for the CuZnSOD. This value was set as reference for the rest of the measures. We found that uneven sample loading induces dramatic changes in the final results depending on the LC selected.

CuZnSOD normalized with actin, GAPDH, or ribosomal subunits as control transcripts reach a maximum of 0.5-, 2.5-, and 8.5-fold increase versus the normalization ratio at 6 μg of total RNA (Fig. 1C). When normalizing with actin and GAPDH, the interval between 15 and 30 μg remains constant indicating that these housekeeping genes properly counteract loading differences within this interval. Any of the ribosomal subunits are adequate under our experimental conditions besides the fact they fit to a lineal regression in several intervals. Northern blot is a complex technique where RNA extraction, quantification, transfer, hybridization and exposition steps could add much variation to the final signals. Control transcripts are used to compensate for differences between samples caused by the experimental procedures, such as sample handling (i.e., pipetting or gel loading) or differential transfer efficiencies (Stürzenbaum and Kille, 2001). Nonetheless, our results indicate that uneven sample load still may dramatically influence the results if LC is not properly selected. We show that normalization ratios are not constant all along the range of RNA used for all the housekeeping tested, and therefore, uneven sample loading might alter the results.

It is generally accepted that linearity of the signal versus amount of RNA is an important feature for a loading control (Bhatia et al., 1994). We found that linearity is just not sufficient to prevent artefacts caused by an uneven load. The explanation is simple: given two linear functions ($y = ax + b$) and given the same x-value for both functions, the ratio between the correspondent y-values only depends on the slope (a) and on intercept (b) constants of the function equations, the intercept being determinant for the results obtained. If two functions have different slopes but their intercept is zero, then the ratios between y-values of both functions will remain constant for any x-value. Once the intercept was found to be different from zero, the ratios will vary more or less depending on the ratio between the constants of the function equations. Thus, if two transcripts fit to a linear model, their ratio for