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Tutorial

Choosing a Normalization Strategy for RT-PCR

GenEx System Aids in the Selection of Reference Genes for Standardizing mRNA Measurements

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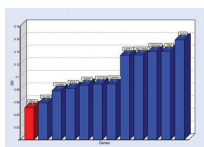


Figure 1

Quantitative real-time PCR (qPCR) is currently the prime technique to measure gene expression. When properly used it offers unprecedented sensitivity, accuracy, and reproducibility. But there are caveats. The target is mRNA, which must be extracted and converted to cDNA in a reverse transcription process that can produce highly variable yield depending on protocol. RNA is further rapidly degraded by nucleases abundant in biological samples. Generally when assaying biological subjects, there is a need to minimize confounding technical and biological variability (See Insert) while maximizing the studied effect.

To choose a normalization strategy in real-time PCR is far from trivial. Popular strategies include relating expression of marker genes to that of endogenous reference genes or to the total amount of RNA. This tutorial discusses how to select an optimum number of reference genes for normalization and also how to compare normalization with reference genes with normalization to the total amount of RNA.

GenEx, qPCR data-analysis software from **Multid Analyses** (www.multid.se), offers many options to normalize data and to standardize measurements, including: interplate calibration, efficiency correction, normalization with spike, normalization to sample amount, normalize qPCR repeats, normalization with reference genes, normalization with reference samples, and normalize RT repeats.

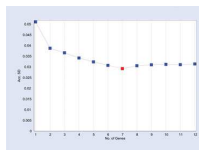
Normalization with reference genes requires identifying genes with a stable expression that is invariant of the conditions studied. This is typically done by testing a panel of candidate reference genes on representative samples. If the study compares different groups of samples such as treated and control, the panel must be tested on representative samples of each type.

The reference gene candidates are then evaluated by estimating their expression stability and any bias in their expression between the sample groups. Further, the optimum number of reference genes should be found. Often, it is also interesting to compare the effect of reference gene normalization with normalization to total RNA only.

The goal of this experiment was to identify the optimum reference genes for normalization of expression markers in a dietary study of mice. For this purpose, two groups of seven mice were studied. One group was fed a normal diet and the other group a high-fat diet. Biological material from the mouse hypothalamus was extracted, lysed, and subjected to 12 qPCR assays with primers from the Mouse Endogenous Control Gene Panel from **TATAA Biocenter** (www.tataa.com). qPCR CT values were collected and imported into GenEx for

analysis.

Ranking Candidate Reference Genes



**Figure 1
(bottom)**

NormFinder is a method for estimating the stability of genes' expression in different groups and also to estimate any bias in the expression of the genes between the groups based on two-way ANOVA. It calculates intragroup variability for the genes in each of the groups and the intergroup variability, or bias, between the groups. The optimum reference gene is the one that shows lowest total variability and the optimum pair shows the combined lowest variability.

Although unusual, the optimum pair does not have to include the single best gene, since NormFinder takes into account compensation effects. A combination of a gene that is somewhat underexpressed in one group and overexpressed in the other group combined with a gene with opposite bias may be the most stable pair. This, of course, is assuming that the biases are estimated with reasonable precision.

NormFinder was applied to the test samples, revealing that all genes in the panel have stable expression with small, if any, bias. To also find the optimum number of reference genes for normalization, NormFinder analysis was repeated, this time ignoring the groups. Assuming that the genes' expressions are uncorrelated, one can calculate the accumulated standard deviation based on any number of reference genes. For this data, the optimum number of reference genes is seven (Figure 1).

Normalization with Total RNA

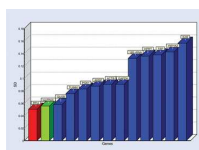


Figure 2

But does the normalization with reference genes improve the quality of the data? In essence, it depends on the options available and how stable expression of the candidate reference genes is. In some cases, no suitable reference genes are found, and other options for normalization are needed.

Using GenEx, any alternative normalizer can be included in the analysis. In Figure 2, log (total RNA) is considered the normalizer, and we find that its standard deviation is in between the best and second best reference gene. Hence for this data, normalizing with total RNA is essentially as good as normalizing with the single best reference gene (SD ~0.05). Normalizing with the seven best reference genes, however, drops SD to ~0.03 (Figure 1, bottom).

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