

Global Normalization Algorithm for Real-Time PCR

Data is normalized using a robust global normalization algorithm that relates to an approximate copy number of the target transcript per cell, which we call Transcript Copy Number. The determined copy numbers may differ considerably from the actual copy number per cell because of the differences in the efficiency of the amplification reaction for that transcript. Nonetheless, the normalization provides a useful readout that can be used to compare levels of expression in different samples and that is relatively unaffected by regulation or errors in the determination of particular normalization transcripts.

Procedure for determining the Transcript Copy Number:

Housekeeping Genes (actin, rps11, tubulin)

1. For each housekeeping gene, determine the median threshold cycle (C_t) values for each of the samples assayed; then, determine the overall median C_t value for the gene.
2. Normalize all housekeeping genes to actin by comparing the overall gene median C_t values and subtracting the difference from the individual sample median C_t values.
3. Determine each sample's median of the normalized C_t values across the different housekeeping genes (for actin, use the overall median C_t value). This is the HKi value for each sample.

Target Genes

1. Determine the median of the triplicate C_t values for each sample
2. Determine the Transcript Copy Number:

$$\text{Transcript Copy Number} = 2500 \times 1.93^{(\text{HKi} - \text{TGi})}$$

2500: Empirical estimate of the number of actin mRNAs/cell ^[1]

1.93: Reflects a typical reaction amplification efficiency of 93%

HKi: Median C_t value of housekeeping genes for each sample

TGi: Target C_t value

* Note: **If assays are done on more than one plate, the same sample must be assayed for actin in triplicate on each following plate.** The target gene values are then adjusted by adding the sample's median C_t value on the actin assay plate minus the C_t for that sample for actin on the target gene plate. This corrects for any differences in thresholding between plates.

$$\text{Corrected Target } C_t = \text{Target } C_t + [\text{actin } C_{t(\text{actin plate})}] - [\text{actin } C_{t(\text{target plate})}]$$

^[1] Kislauskis EH, Zhu X-c, and Singer RH, *beta -Actin Messenger RNA Localization and Protein Synthesis Augment Cell Motility*. J. Cell Biol., 1997. **136**(6): p. 1263-1270.