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Suitable endogenous controls for the analysis of *C. pneumoniae* persistence induction

Eickhoff M[1,2], Wach S[1], Cramer SO[1] and Kruppa J[2]

[1]artus GmbH, Hamburg, Germany
[2]Institute of Molecular Cell Biology, UKE, Hamburg, Germany

Introduction

The obligate intracellular bacterium *Chlamydia pneumoniae* (*C. pneumoniae)* has a unique development cycle. *C. pneumoniae* infection is initiated by an environmentally resistant cell type, called elementary body (EB). The EB differentiates into a larger replicative form, the reticulate body (RB), which accumulates, converts back to an EB and is finally released from the host cell for subsequent rounds of infection. Long-term association between *C. pneumoniae* and its host cell, in which this organisms remains in a viable but culture-negative state, is described as persistence. *In vitro,* persistence can be induced by Interferon-γ (IFN-γ), suggesting an involvement of host genes, whose expression is regulated by this cytokine.

Real-time PCR is a sensitive and precise method to monitor gene expression and therefore, ideally suited for expression profiling of genes involved in *C. pneumoniae* persistence induction. To compensate for a potential variability in gene expression, the expression of endogenous control genes is commonly assessed in parallel with the genes of interest.

The aim of this study was the identification of the most suitable endogenous controls for reliable gene expression analyses in *C. pneumoniae* infected HeLa cells with and without persistence induction with IFN-γ.

Methods

The following experimental settings have been performed for the identification of the most suitable endogenous control genes:

- HeLa cells were cultivated and treated under the following conditions:
  A: 24h Mock infected
  B: 24h Mock infected with IFN-γ
  C: 24h infected with *C. pneumoniae*
  D: 24h infected with *C. pneumoniae* and persistence induced with IFN-γ
  E: 96h Mock infected with IFN-γ
  F: 96h infected with *C. pneumoniae* and persistence induced with IFN-γ
- DNA and RNA from all cell cultures was extracted using the RNeasy Mini Kit (QIAGEN, Hilden).
- The *C. pneumoniae* infection of all HeLa cell cultures was quantitatively controlled by real-time PCR using the *RealArt*™ C. pneumoniae TM PCR Kit (artus, Hamburg) on the ABI PRISM® 7000 SDS (Applied Biosystems).
- For the identification of the most suitable endogenous controls, the expression of the genes GAPDH, GUS, G6PDH, RPII, TBP, Tub and 18S RNA was determined by real-time PCR.
- Subsequently, each gene was compared with the other analysed genes and their relative quantity was set into ratio to each other. Assuming, that within and between the different cell sample types constantly regulated genes did not change their relation to each other, we selected the genes, which showed the lowest variations.

Results

C. pneumoniae infection of HeLa cells was successfully measured using the *RealArt*™ *C.* pneumoniae TM PCR Kit. The detection limit has been determined by probit analysis as 0,28 copies/µl (p = 0,05; ABI PRISM® 7000 SDS), respectively.

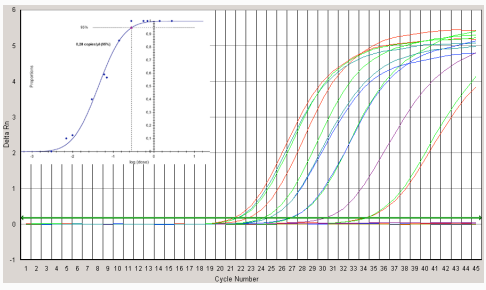

Fig. 1: Quantitative analysis of *C. pneumoniae* in infected HeLa cells using the *RealArt*™ C. pneumoniae TM PCR Kit (artus, Hamburg). Real-time PCR runs are performed with the ABI PRISM® 7000 SDS. Sensitivity was determined by probit analysis (upper left side).

By real-time PCR, significant differences in expression of some control genes were detected between the different HeLa cell samples. The largest deviations showed the gene G6PDH, whereas the genes GUS, TBP and 18S RNA have been identified as endogenous controls with the lowest deviations to each other (see Fig. 2 and Fig. 3).

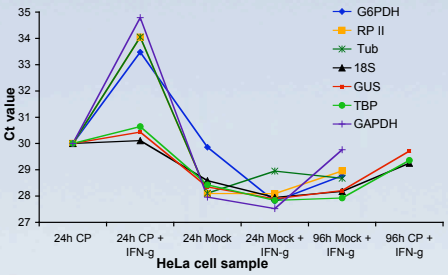

Fig. 2: Gene expression of G6PDH, RP II, Tub, 18S RNA, GUS, TBP and GAPDH in different HeLa cell samples was analysed by real-time PCR. Striking are the similar expression levels of the genes 18S RNA, GUS and TBP. In comparison, G6PDH, RP II, Tub and GAPDH show strong variations in their expression.

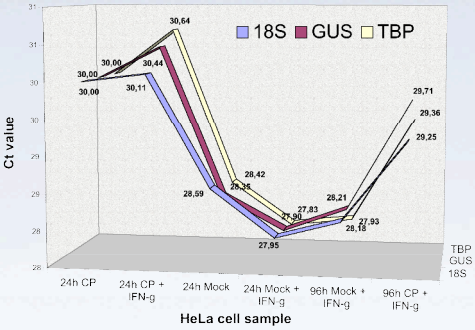

Fig. 3: Genes for 18S RNA, GUS and TBP show comparable expression levels in the respective HeLa cell samples. Marginal differences in expression levels are observed between the HeLa cell samples, which most probably are due to RNA isolation from a different cell number and unequal RNA extraction.

The similar expression levels of the genes GUS, TBP and 18S RNA could be verified in an experimental setting using dilution series of different HeLa cell samples (see Fig. 4).

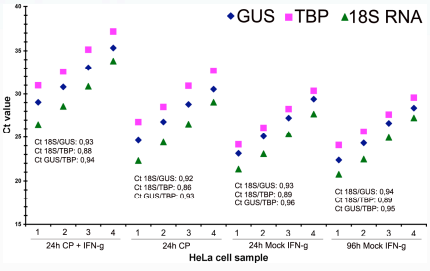

Fig. 4: The genes coding for 18S RNA, GUS and TBP show similar expression levels even in different dilutions (stock, 1:2, 1:4 and 1:16) of the particular HeLa cell samples. This was verified by determination of the mean Ct quotients.

Conclusion

Only three of seven analysed genes are suited as endogenous controls, since the other four genes are regulated by experimental conditions. The three similarly expressed genes 18S RNA, GUS and TBP are members of different gene families and underlie different transcription mechanisms. This allows the conclusion, that these genes remain unregulated after *C. pneumoniae* infection and IFN-γ induction. Little variations in gene expression levels between the different HeLa cell samples are most probably due to RNA isolation from a different cell number and an unequal RNA extraction.

Our results show that real-time PCR is an optimal method for gene expression analyses, but the selection of the endogenous control is the critical point for data interpretation. The selection must be performed for each study in order to avoid wrong conclusions. To ensure an accurate normalisation of quantitative real-time PCR data we recommend to use the geometric mean of several endogenous control genes, as normalisation with only one endogenous control gene does not compensate for variations, for example coming from the RNA isolation procedure.