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

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 were extracted using the RNasey 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.

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.