Review Article

Reference Genes: Essential Criteria for Assessment of the Real-Time PCR Based Virus Detection in Plants Virology

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Abstract | Real-time quantitative PCR (RT-qPCR) plays an important role in the current field of virus detection and disease dynamics. The validity and the reliability of generated data can be enhanced significantly appropriate measures. As a standard for the relative expression of target genes, the selection of reference genes is crucial. This review describes the history of the RT-qPCR technology, emphasizes the importance of reference genes and enumerates several algorithms to screen reference genes to normalize the RT-qPCR data. Additionally, several possible improvements in the selection of reference genes are discussed.

Key words: RT-qPCR, Reference gene selection, Multiple, Algorithms

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Introduction

Previously, diagnostic services for virus detection were very limited. With the rapid development of modern science and technology, especially the continuous development of immunology, biochemistry and molecular biology, new diagnostic techniques and methods have been widely used for the identification of a variety of virus. Among these, enzyme-linked immunoassay (ELISA) occupied the mainstream status gradually for its convenient and accuracy (Leland et al., 2007; Zhang al., 2006). But it has disadvantages in detecting target genes quantitatively.

As a supplement, the transcriptome analyses are made up for the defect. Numerous traditional methods are used for gene expression analysis: Northern blotting, in situ hybridisation, qualitative real-time PCR, RNase protection assay, competitive RT-PCR, microarray analysis, and quantitative real-time PCR (RT-qPCR) (Radonić et al., 2006). Applied Bio-systems invented RT-qPCR technology in 1996 and has realized the PCR leap from qualitative to quantitative. Compared with these methods, the major advantages of RT-qPCR are higher sensitivity, better reproducibility and specificity, and higher throughput (Wong et al., 2005; Mafra et al., 2012; Wang et al., 2015). Therefore, the RT-qPCR has become the most frequently used approach for gene expression analysis (Tang et al., 2015), but one should be careful for its

accompanying pitfalls (Bustin SA et al., 2004). Consequently, several vital factors of RT-qPCR such as quality of RNA, PCR amplification efficiency, primers specificity, data analysis can determine the validity of the results (Garson et al., 2009). To enhance the credibility of qPCR results and ensure the correctness of the research literature, Bustin et al. proposed The Minimum Information for Publication of policy Real-Time PCR Experiments (MIQE) guidelines in 2009 (Bustin et al., 2009). The top most priority was set to be the stable reference genetic screening. Marianne Delporte et al. put forward the difference of reference genes and housekeeping genes, it appears more appropriate to describe about reference genes rather than housekeeping genes (HKGs) (Delporte et al., 2015). Selection of optimal housekeeping genes as reference genes is critical to establishing sensitive and reproducible RT-qPCR-based assays.

In theory, it should be stable for the reference genes expression level in different parts of the same species under the same conditions. However, the expression levels of several commonly used reference genes vary under certain experimental conditions, and not all 'reference' genes should be considered universally suitable for reference genes (Lin et al., 2010). The use of the unstable reference genes will lead to inaccurate results. The validation of the stability of reference genes has become the premise of fluorescence quantitative data analysis.

Various algorithms for stability analysis

Nowadays, several distinctive algorithms should be used to screen the stable reference genes, so we can carry out a comprehensive analysis and get reliable results. There are four traditional methods we commonly used: geNorm, NormFinder, BestKeeper, comparative ΔCT method (Andersen et al., 2004). The prerequisite of these methods is $2^{-\Delta\Delta CT}$ method, which was put forward by Kenneth J. Livak and Thomas D. Schmittgen in 2001 (Kenneth et al., 2001; Pfaffl MW et al., 2001). This method is able to calculate initial relative expression of genes, but it is essential that prove all genes amplification efficiency to be consistent (Garson et al., 2009). GeNorm is a Visual Basic Application (VBA) program based on Excel, which can be used to determine the stability of the internal candidate genes (accepted threshold is 1.5 usually) and the best reference gene combination (Vandesompele et al., 2002), by using the pairwise variation (PV) of two sequential normalization factors (NFs), i.e. $V_{n/n+1}$, to estimate the effect of introducing additional reference genes to the NF. The genes with low *M* value indicate more stability or strongly co-regulated (Wang et al., 2015). Normfinder, another VBA applet, a model-based variance estimation approach to calculate the expression stability of a set of candidate genes intra- and inter-group and present them visually with a box-plot (Andersen et al., 2004; Spinsanti et al., 2006). Bestkeeper, a means by comparing paired the correlation between gene and three index, including standard error (SD), correlation coefficient (r) and covariance ratio (CV) (Pfaffl et al., 2004). Comparative ΔCT method compares pairs stability of genes internal group, if two different genes within groups remain stable, it will show the two genes are stable (Chiu et al., 2001). On this basis, Radonić A. et al proposed a novel algorithm, ΔΔCT analysis. This is a method to handle genes under different treatments with a course; being advised to employ it when there has dual variables (Radonić et al., 2006; Wang et al., 2015). It is indicated that the ΔΔCT method should be applied first in virus infection experiments before the geNorm and BestKeeper for further elucidation of the acquired data. In addition, there is a relatively new algorithm, GrayNorm, which can yield the lowest level of uncertain and the highest possible accuracy (Remans et al., 2014).

There are two programs that can be applied to get a comprehensive analysis of these data. Refinder is a context-based information refinding system that allows us to refind files and web pages according to the previous access context (Deng et al., 2012). It integrates several data analysis methods mentioned above and analyzes the stability of the reference genes. Similarly, an algorithm proposed by Pihur V, rank aggregation method (RankAggreg), which can handle complex rank aggregation problems and rank them according to the stability value calculated by other algorithms (Pihur et al., 2009). Although some discrepancies exist among these algorithms, they all aimed at ideal data analysis nondiscriminativly. A flowchart for reference genes selection and RT-qPCR is supplied in Figure 1.

The necessity of stability validation in plant virology

In the field of plant pathology, genetic testing has become an important indicator to the disease of biosome.

Regardless of other provisions of MIQE guidelines, reference genes with constant expression are crucial to normalize quantitative data (Vandesompele et al., 2002). In different species, we can get the results that vary dramatically depending on the method chosen for data analysis (Skern et al., 2005). Several researchers have proved that the use of different single reference gene might influence the data interpretation, while multiple reference genes could minimize possible errors (Lin et al., 2010; Fang et al., 2015; Wang et al., 2015; Huang et al., 2016). As shown in the Table 1. Fang et al. compared the databased on Single and Multiple Reference Gene(s) in Quantitative Real-time PCR Normalization, the increasing transcript patterns of target genes were differentially lower than those normalized by multiple reference genes (Fang et al., 2015), indicating that multiple reference genes are superior to single reference gene. This is also the inevitable trend in the reference gene screening.

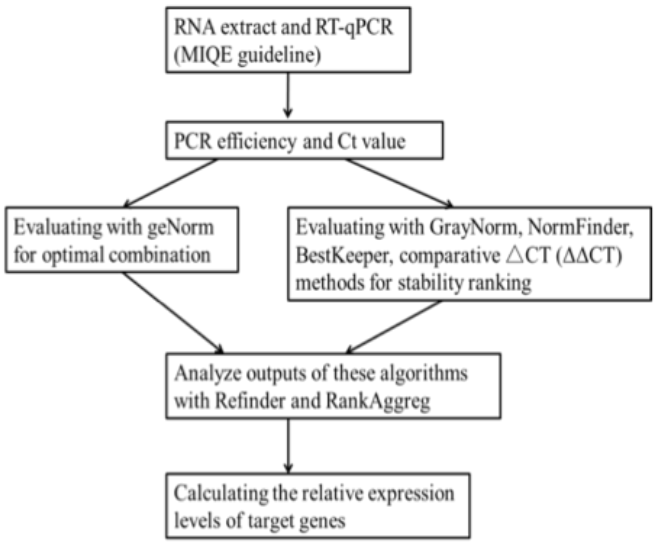

Figure 1: *A flowchart for reference genes selection and RT-qPCR. Various algorithms can be employed according we need*

Table 1: *The optimal combination of reference genes under various stresses. Each expression level of target genes ranged from 1.5- to 2-fold than normalized by single one*

Treatment	Optimal combination of Reference Genes	Research
Temperature	UBQ + Fe-SOD	Lin et al., 2010
Virus-RSV -RBSDV	UBQ 10 + GAPDH UBC + Actin1	Fang et al., 2015
Salinity	Actin + EF1α + GAPDH + RP + UBQ	Wang et al., 2015
Temperature Salinity	RPS15 + RPL17 TubB + TubA + UBQ	Huang et al., 2016

Conclusion

RT-qPCR is extremely practical and effective assay for virus detection. Selection of reference genes is an essential criterium for assessment of real-time PCR, for that the selection of it determines whether the data is stable and reliable. There are several algorithms can be employed to screen optimal reference genes. It is proposed that at least three kinds of algorithms should be selected according to our needs. Among them, geNorm is the most essential one, which can confirm the quantity of reference genes combination while the others cannot. After all, single reference gene has been proved to be applicable but not stable in most cases. The determination of the internal genes should be a criterion to narrow the difference between laboratories. In the absence of such standards for normalization, it should be questioned to the editors of journals when reviewing a paper without illustration of reference genes. For researchers, it is essential to state the reason for using these reference genes, screening or previous work. On these bases, a reference genes selection guide can be consulted when performing gene expression analysis. It normalizes the method that diagnose virus with RT-qPCR and process a better control for virus-disease.

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