

RqPCRAnalysis: Analysis of Quantitative Real-time PCR Data

Frédérique Hilliou¹ and Trang Tran²

¹UMR-IBSV-INRA-CNRS, Université de Nice Sophia Antipolis, Nice, France

²INGENOMIX, Lanaud, 87220 Boisseuil, France

Keywords: Quantitative Real-time PCR, Normalization, Biological Replicates, Statistics, R.

Abstract: We propose the statistical RqPCRAnalysis tool for quantitative real-time PCR data analysis which includes the use of several normalization genes, biological as well as technical replicates and provides statistically validated results. This RqPCRAnalysis tool improved methods developed by Genorm and qBASE programs. The algorithm was developed in R language and is freely available. The main contributions of RqPCRAnalysis tool are: (1) determining the most stable reference genes (REF)--housekeeping genes-- across biological replicates and technical replicates; (2) computing the normalization factor based on REF; (3) computing the normalized expression of the genes of interest (GOI), as well as rescaling the normalized expression across biological replicates; (4) comparing the level expression between samples across biological replicates via the test of statistical significance. In this paper we describe and demonstrate the available statistical functions for practical analysis of quantitative real-time PCR data. Our statistical RqPCRAnalysis tool is user-friendly and should help biologist with no prior formation in R programming to analyze their quantitative PCR data.

1 INTRODUCTION

In molecular biology the real-time quantitative polymerase chain reaction (RT-qPCR) has become the most powerful method for the detection and quantification of nucleic acid sequences including gene expressions. The technique is widely used for example for the validation of genes differentially expressed in microarray experiments. Several programs have been developed to extract quantification cycle values (Cq) from recorded fluorescence measurements and are specific for each qPCR machine manufacturers. The process of these raw data is however not always adequate for the biologist because they lack for example normalization steps and are often dedicated to a single instrument. Recently Bustin *et al.* provided standard for qPCR technique: MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (Bustin et al., 2010); (Bustin et al., 2002). Design of qPCR data experiment should include technical replicates, biological replicates as well as several reference genes used for data normalisation.

Base of the MIQE we propose an R application that should allow users whatever instruments they

used to determine reference genes in their set of data and to quantify expression levels of their genes of interests. R is a widely used open source language and environment for statistical computing and graphical representation which has become a standard in statistical modeling, data analysis, biostatistics and machine learning. An important feature of the R environment is that it integrates generic data analysis and visualization functionalities with the latest advances in computational statistics.

This paper introduces the new R package RqPCRAnalysis, where the acronym stands for analysis of real-time quantitative PCR data. The purpose of the package RqPCRAnalysis is to provide a comprehensive, simple and easy to use tool for real-time quantitative PCR data analysis. More importantly, functions are provided for biologists who have little statistical and R programming background. Our R-script was developed base on Genorm algorithm (Vandesompele et al., 2002) to search for reference genes and on qBASE algorithm for calculation of gene relative expression levels (Hellemans et al., 2007). We developed a statistical method for the validation of differential expressions for genes of

interest (GOI) and we compared the expression level between samples via the test of statistical significance. A graphic representation of these results is also available.

2 METHODS

2.1 Biological Data

2.1.1 Plant Growth Conditions and Wounding Treatment

Arabidopsis thaliana wild-type (ecotype Columbia 0, Col0) and *CYP74A1-OverExpressed* mutant (M) seeds were cold-treated at 4°C for two days. After cold treatment, seeds were sown on soil previously autoclaved 1h at 130°C and then placed in a controlled growth room under long-day conditions: -12h of light with a minimum intensity of 100mmolm⁻²s⁻¹ at 21°C. -12h of dark at 21°C.

Plants were watered once a week. Wounding was done on 4-week-old plants (8 to 10 mature leaves) as described in Park *et al.*, 2002. Wounded leaves were harvested 2h and 4h after wounding and were immediately frozen in liquid nitrogen. Undamaged leaves were also harvested as control. This control is described at t=0h. Twenty plants of each genotype were pooled for each time point. Three biologically independent experiments were done and used as biological replicates.

2.1.2 RNA Extraction

RNA was extracted using Trizol reagent (Invitrogen

Life technologies) according to manufacturers' instructions. Three independent extractions were performed on three independent biological replicates for each time point of the time course and for each genotype.

2.1.3 Real Time Quantitative PCR

For each RNA template we reverse-transcribed 2µg of total RNA during 2h at 42°C using 200 units of SuperScript™ II (Invitrogen), 5µM oligo(dT)₁₈, 500µM dNTP's, 40 units of Ribonuclease inhibitor (RNasin, Promega). Fifty nanograms of cDNA synthesis were then used in qPCR experiments using qPCR™ Mastermix Plus for SYBR Green I (Eurogentec) and 3.6µM of each gene-specific primers in a final volume of 15µl. qPCR reactions were carried out on an Opticon monitor 2 (BioRad). The PCR conditions were as follows: 95°C for 15 min to activate the hot-start DNA polymerase, followed by 40 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s. Each reaction was performed in technical duplicates and the mean of three independent biological replicates was calculated. For each gene, primers efficiency was assessed and new set of primers were designed until efficiency between 85-105% was obtained. Czechowski (2004) proposed several "stable" genes for *Arabidopsis* obtained using plant growth in several conditions (Czechowski *et al.*, 2004). We chose three most stable genes At5g46630, At1g58050, and At1g62930 and their respective primers among these to be used as reference genes in our experiment. The stability of these three genes was also tested using GeNorm (Vandesompele *et al.*, 2002) and our R package RqPCRAnalysis.

Table 1: Primer characteristics.

gene name	accession number	primer sequences	amplicon size	PCR efficiency	correlation coefficient
HKG1	AT5G46630	TCGATTGCTTGGTTTGGAAAGAT	60	96%	0,991
		GCACTTAGCGTGGACTCTGTTTGATC			
HKG2	AT1G58050	CCATTCTACTTTTTGGCGGCT	60	80%	0,99
		TCAATGGTAACTGATCCACTCTGATG			
HKG3	AT1G62930	GAGTTGCGGGTTTGTGGAG	98	105%	0,994
		CAAGACAGCATTTCAGATAGCAT			
cyp74a	At5g42650	GGAAGCTCCGTTAATTTCTCG	92	93%	0,996
		GGACTACACAGGTGCGAACA			
cyp76c5	At1g33730	GATGGCGAAAGGAGATGAGA	105	83%	0,984
		CCCTATGACATGAAGGGACTG			
pdf1.2a	At5g44420	GAAGGAGCCAAACATGGATC	108	87%	0,978
		AATACACACGATTTAGCACC			

GOI primers were designed using Primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi>) and absence of secondary structures was checked using Netprimer software (<http://www.premierbiosoft.com/netprimer/netprlaunch/\netprlaunch.html>). Primers characteristics are summarized in Table 1 for REF genes and GOI.

2.2 Statistical Methods

Let us note m , n respectively the number of biological replicates and number of technical replicates; r , number of reference genes; E_j is the PCR amplification efficiency coefficient of gene j ; Cq , quantitative cycle; QCq , relative quantity; SD , standard deviation; SE , standard error; NF , normalization factor.

2.2.1 Identification of Reference Genes

The normalization of relative quantities with the reference genes (REF), the most stable genes, can be calculated on the assumption that the REFs are known (i.e. provided by the biologist). When the REFs are not know, we can determine the most stably expressed genes across all tested samples and all biological replicated experiments based on the stability parameter and coefficient of variation. Here, we use the algorithm described in (Helleman et al., 2007) to identify the reference genes.

2.2.2 Computation of Relative Quantities

For each biological replicate i , the average of Cq is computed for n technical replicates of the same gene j :

$$\begin{aligned} \overline{Cq}_{ij} &= \frac{\sum_{k=1}^n Cq_{ijk}}{n} \\ SD(Cq_{ij}) &= \sqrt{\frac{1}{n-1} \sum_{k=1}^n (Cq_{ijk} - \overline{Cq}_{ij})^2} \end{aligned} \quad (1)$$

The relative quantity associated with \overline{Cq}_{ij} is expressed as follows where the highest expression level set to one,

$$QCq_{ij} = E_j^{\min(\overline{Cq}_{ij}) - Cq_j} \quad (2)$$

and the standard deviation of QCq_{ij} is obtained by taking the derivative of (2):

$$SD(QCq_{ij}) = SD(Cq_{ij}) * QCq_{ij} * \ln(E_j) \quad (3)$$

2.2.3 Computation of Normalization Factor

The normalization factor of sample k in biological replicate i across r reference genes, noted NF_{ik} , is given by the geometric mean

$$NF_{ik} = \sqrt[r]{\prod_{j=1}^r QCq_{ij}} \quad (4)$$

And the standard deviation of this normalization factor is

$$SD(NF_{ik}) = NF_{ik} \sqrt{\sum_{j=1}^r \left(\frac{SD(QCq_{ij})}{r QCq_{ij}} \right)^2} \quad (5)$$

with r corresponding to the number of reference genes.

2.2.4 Normalization of Relative Quantities

For each biological replicate i , the normalization of relative quantity of GOI j for each sample k can be now calculated by dividing the raw GOI quantity (2) by the normalization factor (4) as follows

$$QCq_{ijk}^* = \frac{QCq_{ij}}{NF_{ik}} \quad (6)$$

and the standard deviation of this normalized GOI expression is given by

$$\begin{aligned} SD(QCq_{ijk}^*) &= QCq_{ijk}^* \sqrt{\left(\frac{SD(QCq_{ijk})}{QCq_{ij}} \right)^2 + \left(\frac{SD(NF_{ik})}{NF_{ik}} \right)^2} \end{aligned} \quad (7)$$

Here, we are also interesting in the error on the mean by using the standard error (SE) values instead of standard deviation (the error on a single measured value) as the following. Using this SE, the true mean has a 95% chance of lying between the measured mean ± 1.96 times the SE.

$$SE(QCq_{ijk}^*) = \frac{SD(QCq_{ijk}^*)}{\sqrt{n}} \quad (8)$$

Now, the average of expression level of GOI j for each sample k across m biological replicates is calculated by

$$\overline{QCq}_{jk}^* = \frac{\sum_{i=1}^m QCq_{ijk}^*}{m} \quad (9)$$

and the SD and SE are basically given by

$$SD(QCq_{jk}^*) = \sqrt{\frac{1}{m-1} \sum_{i=1}^m (QCq_{ijk}^* - \overline{QCq_{jk}^*})^2}$$

$$SE(QCq_{jk}^*) = \frac{SD(QCq_{jk}^*)}{\sqrt{m}} \quad (10)$$

Finally, the user can use the reference sample k , the logarithm transformation or the smallest sample for rescaling the expression level

$$\overline{QCq_{jk}^*}^{(k)} = \frac{\overline{QCq_{jk}^*}}{\overline{QCq_{jk}^*}}$$

$$\overline{QCq_{jk}^*}^{(min)} = \frac{\overline{QCq_{jk}^*}}{\min_k \overline{QCq_{jk}^*}} \quad (11)$$

The SD and SE corresponding to the transformation in (11) are

$$SD(\overline{QCq_{jk}^*}^{(k)}) = \frac{SD(QCq_{jk}^*)}{\overline{QCq_{jk}^*}}$$

$$SE(\overline{QCq_{jk}^*}^{(k)}) = \frac{SD(QCq_{jk}^*)}{\sqrt{m} \overline{QCq_{jk}^*}}$$

$$SD(\overline{QCq_{jk}^*}^{(min)}) = \frac{SD(QCq_{jk}^*)}{\min_k \overline{QCq_{jk}^*}}$$

$$SE(\overline{QCq_{jk}^*}^{(min)}) = \frac{SD(QCq_{jk}^*)}{\sqrt{m} \min_k \overline{QCq_{jk}^*}} \quad (12)$$

2.2.5 Sample Comparison

For each GOI, all pairs of samples across all biological replicates are compared. Here we used the classical method of pairwise t -test with pooled standard deviation. We assumed that the two sample sizes are equal and the two distributions have the same variance. In order to apply the t -test, we assume data follow normal distribution. Here we use a simple logarithm transformation to transform the normalized relative expression,

$$\overline{QCq_{jk}^*}^{(log)} = \log_2(\overline{QCq_{jk}^*}) \quad \text{for all } k \quad (13)$$

Now, the t statistic to test whether the means are different between the samples S_i and S_j follows a Student's t -distribution with $M-1$ degrees of freedom and can be calculated as follows:

$$t = \frac{|\bar{S}_i - \bar{S}_j|}{SD(S_i - S_j)/\sqrt{M}} \quad (14)$$

where $SD(S_i - S_j)$ is the standard deviation of the differences between the S_i and the S_j :

Once a t value is determined, the p-value of the test can be calculated from Student's t -distribution with $M-1$ degrees of freedom. The user can choose a threshold for the statistical significance to reject or accept the null hypothesis ($H_0: \mu_i = \mu_j$) in favor of the alternative hypothesis ($H_a: \mu_i \neq \mu_j$).

3 RESULTS

3.1 Determination of Relative Expression

An example of raw data is available in Table 3 (APPENDIX). This example consists of three biological replicates and two technical replicates. We compared expression levels of six genes (3 REF genes and 3 GOIs namely *CYP74A*, *CYP76C5*, and *PDF1.2a*) in *Col* versus the mutant M *CYP74A1-OE*. We performed our qPCR analysis in six different biological conditions. In this example, the REF genes were obtained using GeNorm software and were confirmed using our R-script. Figure 1 presents results of rescaled expression of *GO11*, *GO2* and *GO3* where all six biological conditions and their respective standard deviations are plotted. We observed that the *GO11* (*CYP74A*) is over-expressed in all mutant conditions when compared to *Arabidopsis thaliana* wild type (*Col*). This was an expected observation since the mutant is over-expressing this gene. This gene is also induced 2h and 4h after wounding in *Col* plants. The *GO12* is induced 4h after wounding in both *Col* and mutant M plants. The *GO13* is induced mainly 4h after wounding treatment in both *Col* and M plants and its expression is globally higher in *Col* compared to M plants. However the late induction (4h) is not statistically validated at a threshold value of 0.05. All these results are provided with pair-wise t -tests (Table 2). Figure 1 consists of graphs plotting expression data for each biological condition with their respective standard deviation. Within our R-script we can choose to compare conditions to a chosen one that will take the value one. By default the condition with the minimal value is given the value of 1.



Figure 1: Expression levels of GOI1, GOI2 and GOI3, respectively, in control (Col0) and mutant (M) and after treatments (0h, 2h, 4h). Error bars represent the standard deviation for each biological condition.

3.2 Comparison between Samples

Figure 2 plots respectively all three *GOI* in the biological conditions Col0h1, Col2h1 and Col4h1 with their associated standard deviations. Similarly,

Figure 3 plots the *GOIs* in the biological conditions M0h1, M2h1 and M4h1, respectively. An example of pairwise comparisons statistical analysis is provided in Table 2. We have highlighted all significantly different pair-wise comparisons in bold of Table 2.



Figure 2: Expression of GOI in each treatments. Expression levels of GOI1, GOI2 and GOI3, respectively, in each condition treatment col0h1, col2h1, col4h1, respectively. Error bars represent the standard deviation for each condition.

Table 2: Presentation of statistical results for GOI1, GOI2 and GOI3, respectively. pvalue inferior to 5% is in bold.

GOI1						
Sample	col0h1	col2h1	col4h1	M0h1	M2h1	M4h1
col0h1	1,000	0,002	0,004	0,002	0,002	0,004
col2h1	NA	1,000	0,024	0,109	0,068	0,154
col4h1	NA	NA	1,000	0,030	0,024	0,054
M0h1	NA	NA	NA	1,000	0,559	0,818
M2h1	NA	NA	NA	NA	1,000	0,772
M4h1	NA	NA	NA	NA	NA	1,000
GOI2						
Sample	col0h1	col2h1	col4h1	M0h1	M2h1	M4h1
col0h1	1,000	0,584	0,015	0,557	0,228	0,053
col2h1	NA	1,000	0,018	0,917	0,115	0,080
col4h1	NA	NA	1,000	0,005	0,008	0,150
M0h1	NA	NA	NA	1,000	0,089	0,026
M2h1	NA	NA	NA	NA	1,000	0,018
M4h1	NA	NA	NA	NA	NA	1,000
GOI3						
Sample	col0h1	col2h1	col4h1	M0h1	M2h1	M4h1
col0h1	1,000	0,820	0,239	0,998	0,841	0,909
col2h1	NA	1,000	0,334	0,790	0,905	0,853
col4h1	NA	NA	1,000	0,122	0,020	0,062
M0h1	NA	NA	NA	1,000	0,768	0,878
M2h1	NA	NA	NA	NA	1,000	0,846
M4h1	NA	NA	NA	NA	NA	1,000

GOI1 is induced in Col plants by a factor of forty 2h after wounding and by a factor of thirty 4h after wounding (pvalue= $3.9 \cdot 10^{-5}$ and $2 \cdot 10^{-4}$, respectively). We also observed a 4-fold induction of *GOI2* (*CYP76C5*) in Col plants 4h after wounding (pvalue=0.0015). *GOI2* is also significantly differentially expressed between Col and M 4h after wounding (pvalue=0.0288).

In Figures 2 and 3 we have summarized the results of *GOI1* compared to the two other *GOIs* including statistical pair-wise comparisons using *t*-tests in all conditions studied. Expression of *GOI1* in Col0h is statistically different from all the other conditions studied: wounding in both Col and mutant as well as control mutant (t=0h). *GOI2* is induced more than 3-times 4h after wounding in Col plants (pvalue=0.0015) compared to Col plants without wounding.

4 DISCUSSION

The data analyzed here as an example include only a small set of genes and biological conditions but the R-script does not have a limit on the number of samples and genes that can be included. Our method includes the option for biological data in addition to technical replicates. The biological replicates were not taken in account in the qBASE software however they are very important in the design of meaningful biological experiments. The second difference in our script compared to qBASE is the absence of inter-run control in our script but it can be implemented for diagnostic validated by qPCR. The use of normalization was also available in the functions “relQuantPCR” and “normPCR” in the SLqPCR package as we propose it in formula 2 and 4. Another very important feature included in our R-

script is the statistical validation of the results for the biologist. The principle statistical validation of the results we describe in formula 14 was available in the HTqPCR package but done on Cq values (Dvinge and Bertone, 2009) and in the web-based QPCR (Pabinger et al., 2009).

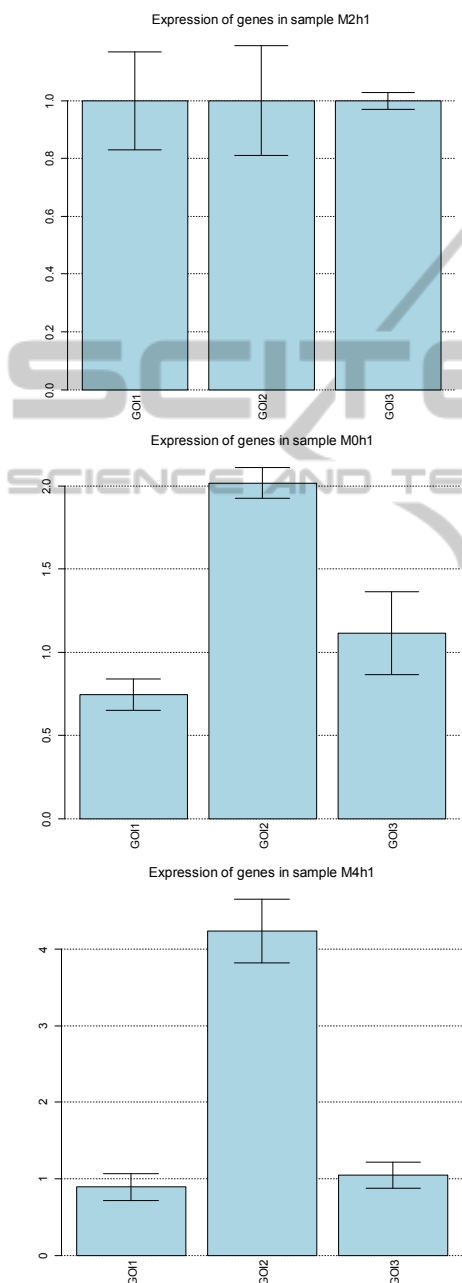


Figure 3: Expression of GOI in each treatment. Expression levels of GOI1, GOI2 and GOI3, respectively, in each condition treatment M0h1, M2h1, M4h1, respectively. Error bars represent the standard deviation for each condition.

Finally our R-script program can be used with any qPCR machine softwares since a simple text file is necessary as the input file. The qpcR package is also using the error propagation formula we use in formula 7 however the “ratiocalc” function is used on raw Cq value and on efficiency values rather than on normalized expression value as we do in our package.

The biological condition determines as the reference condition (value 1) is chosen by the biologist. Our program also allows the modification of the figures in any graphic software in order to personalize the colour, legends, x- or y-scale for instance.

The R-script described in this paper implements features for qPCR analysis that are scattered in several packages, or available in commercial packages.

Our data demonstrated the over-expression of *GOI1* in our mutant plants as well as induction of its expression in Col and mutant plants after mechanical wounding. The expression of *GOI2* is also modulated according to plant genotypes in the time course of the wounding. The last gene studied in our experiment, *GOI3*, did not show any statistically valid repression or induction in all conditions described in our experiment.

5 CONCLUSIONS

We have presented in this paper a free R-script program that can be easily used by biologists and that is independent of the qPCR instrument software. Our script includes:

- Biological replicates in the design of the experiment as well as technical replicates
- The choice of reference genes using Genorm formulas (Vandesompele et al., 2002),
- The normalization and rescaling of expression levels for each GOI using qBASE software formulas (Hellemans et al., 2007),
- A flexible graphic representation of gene expression levels including standard deviations
- Statistical tests to validate the differentially expressed genes compared to controls.

This software is user-friendly for biologists and a step by step guide is available. It was already used in two publications (Brun et al., 2010); (del Giudice et al., 2011); (Giraud et al., 2011).

ACKNOWLEDGEMENTS

This work was supported by an Agence Nationale de la Recherche Grant 06 BLAN 0346 given to R. Feyereisen, UMR IBSV INRA-CNRS-Université de Nice Sophia Antipolis. The authors thank R. Feyereisen and E. Wanjberg for useful discussion and correction of the manuscript. Trang Tran was financially supported by an Agence Nationale de la Recherche Grant 06 BLAN 0346 given to R. Feyereisen.

REFERENCES

- Brun-Barale, A., Héma, O., Martin, T., Suraporn, S., Audant, P., Sezutsu, H., Feyereisen, R., 2010 Multiple P450 genes overexpressed in deltamethrin-resistant strains of *Helicoverpa armigera*, *Pest Management Science*, 66: 900-909.
- Bustin, S. A., 2002. Quantification of mRNA using real time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology*, 29: 23-39.
- Bustin, S. A., 2004. *A-Z of Quantitative PCR*. La Jolla California: International University Line.
- Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, 55 (4): 611-622.
- Bustin, S. A., Beaulieu, J.-F., Huggett, J., Jaggi, R., Kibenge, F., Olsvik, P., Penning, L. and Toegel, S., 2010. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology*, 11 (74).
- Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.-R. and Udvardi, M.K., 2004. Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant Journal*, 38: 366-379.
- del Giudice, J., Cam, Y., Damiani, I., Fung-Chat, F., Meilhoc, E., Bruand, C., Brouquisse, R., Puppo, A. and Boscari, A., 2011. Nitric oxide is required for an optimal establishment of the *Medicago truncatula*-*Sinorhizobium meliloti* symbiosis. *New Phytologist*, 190.
- Giraud, M., Califano, J., Hilliou, F., Tran, T., Taquet, N., Feyereisen, René, Le Goff, G., 2011. Effects of Hormone Agonists on Sf9 Cells, Proliferation and Cell Cycle Arrest. *PLoS ONE*, 6, 10, e25708.
- Dvinge, H. and Bertone, P., 2009. HTqPCR: High-throughput analysis and visualization of quantitative real-time PCR data in R. *Bioinformatics*, 25, 3325-3326.
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. and Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, 8 (2):R19.
- Pabinger, S., Thallinger, G., Snajder, R., Eichhorn, H., Rader, R. and Trajanoski, Z., 2009. QPCR: Application for real-time PCR data management and analysis. *BMC Bioinformatic*, 10, 268.
- Park, J.-H., Halitschke, R., Kim, H. B., Baldwin, I. T., Feldmann, K.A. and Feyereisen, R., 2002. A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. *Plant Journal*, 31: 1-12.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3.

APPENDIX

Demonstration of RqPCRAnalysis Package R-script

The package RqPCRAnalysis will be submitted to Bioconductor (<http://www.bioconductor.org>). RqPCRAnalysis is one of a default set of packages installed by biocLite in the Bioconductor project. The core Bioconductor packages can be installed from the command line of R.

We assume that an experiment has been conducted with one or more biological replications and each sample has one or more technical replicates. The data are stored in ".txt" file and have the following table structure (recommended). Note that the biological replicates must be ranked successively by different blocks. The suffix *_j* is recommended to distinguish the biological replicate. In each block of biological replicates, the technical replicates of each sample are also ranked successively in sub-block "pair by pair" (Table 2). "Efficiency" is the PCR amplification efficiency coefficient established for each qPCR array (i.e. each primer couple) by means of calibration curves. This coefficient should be determined from the slope of the log-linear portion of the calibration curve. Specifically,

$$PCR\ efficiency = 10^{-1/slope} - 1 \quad (16)$$

when the logarithm of the initial template concentration is plotted on the x-axis and C_q is plotted on the y-axis (4)

In the beginning, one computes all parameters for each gene in each biological block: Mean.Cq (Mean of Cq), SD.Cq (Standard Deviation of Cq), QCq (Relative Quantity) and SD.QCq (Standard Deviation of QCq). See Section *Statistical methods* for detail.

Validation Reference Genes

The reference genes (REF), previously referred as housekeeping gene is then selected. By default a function of the script [select.ref.genes()] provides a list of REF by finding the most stable genes (at least 2) across all biological replicates. Otherwise REF can be chosen manually (not recommended). The [select.ref.genes()] function ranked the genes from most stable to least stable as done in (5).

qPCR Data Analysis and Graph Representations

The normalization factor based on these REFs for each gene of interest (GOI) is then computed. The normalised expression consists of the expression level, as well as the standard deviation and the standard error associated for each biological replicates. As a convention, this function applies for each biological replicate. Rescaling the normalized expression for each GOI is done according to qBASE (5) and our script is able to run with missing values at this step.

Statistical Studies of Gene Expressions

Our script then provides sample comparisons which were not included in qBASE. Here we use the method of pair-wise t-tests. For each GOI, all pairs of samples across biological replicates are compared. The normalized expression output or the rescaled expression output can be tested. Graphs of the results are available to export, they contain normalized expression or rescaled expression and the GOI to be plotted can be chosen. By default all the GOI will be represented on the graph with their standard deviations but representation with standard errors can be plotted as well. In complement to the graphs, a table is given containing the rescaled expression values as well as their corresponding standard deviation. An additional table with the results of pair-wise comparison using t-tests is also provided.

Data Import

We assume that an experiment has been conducted with one or more *biological replications* and each sample has one or more *technical replicates*. The data are stored in .TXT file and have the following Table 3 (recommended). Note that the biological replicates must be ranked successively by different

blocks. The suffix *_j* is recommended to distinguish the biological replicate. In each block of biological replicates, the technical replicates of each sample are also ranked successively in sub-block "pair by pair" (see the example file "example.txt"). "Efficiency" is the PCR amplification efficiency coefficient established for each qPCR array (i.e. each primer couple) by means of calibration curves. This coefficient should be determined from the slope of the log-linear portion of the calibration curve (2).

Table 3: Data storage format.

	Sample	Gene1	Gene2
	efficiency	2	1.8
Biological replicate 1	Sample1_1	23	25
	Sample1_1	24	30

	Sample1_i	21	10
Biological replicate j	Sample1_j	11	12
	Sample1_j	12	12
	Sample1_j	14	15

Biological replicate m	Sample1_j	21	22
	Sample1_j	25	23
	Sample1_m	14	17
	Sample1_m	21	25

	Sample1_m	14	15
	Sample1_m	12	14

Data Analysis

- Reading data into workspace:

```
> data
read.data(filename="example.txt", path=path, bio.rep=3)
```

Computing parameters for each biological replicates:

```
> parameter<-
pcr.processing(data,num.rep=2,na.rm=TRUE)
```

- Computing normalisation factor: Finding most stability genes (REF) and normalization factor based on REF:

```
> stability.value<-
reference.genes(data, num.ref = 3, na.rm = TRUE)
> ref.gene<-
as.character(stability.value$order[1:3])
```

```

(or > ref.gene<-
c("REF1","REF2","REF3")
> ref.factor <-
normalization.factor(parameter,ref.
gene=ref.gene)
- Computing expression level (normalized
expression) of genes of interest (GOI) in each
biological replicate based on normalization factor:
>normalized.exp<-
normalized.expression(parameter,ref
.factor,num.rep=2,
ref.gene=ref.gene,goi.gene=NULL)
- Comparing the difference
between samples across biological
replicates:
> comparison<-
test.sign(normalized.exp,bio.rep=3,
path=path)
- Computing the expression
across all biological replacates
for each gene:
> average<-
average.expression(normalized.exp,b
io.rep=3,na.rm = TRUE)
- Rescaling the normalized
expression for each GOI:
> final.result<-
rescaled.expression(average,na.rm =
TRUE)
- Plotting and saving results:
>pcr.plot(final.result,path=path
,goi.gene=NULL,sample.name=NULL,typ
e="gene",error="SE")
>qPCR.plot(final.result,path=pat
h,goi.gene=NULL,sample.name=NULL,ty
pe="sample",error="SE")
>
save.expression(final.result,path=p
ath,gene.names=NULL)

```