Review Article

A survey of tools for the analysis of quantitative PCR (qPCR) data

Stephan Pabinger [a,*,1], Stefan Rödiger [b,1], Albert Kriegner [a], Klemens Vierlinger [a], Andreas Weinhäusel [a]

[a] Health & Environment Department, Molecular Diagnostics, AIT – Austrian Institute of Technology, Muthgasse 11, 1190 Vienna, Austria
[b] Faculty of Natural Sciences, InnoProfile Group "Image-based Assays", Brandenburg University of Technology Cottbus – Senftenberg, Großenhainer Straße 57, 01968 Senftenberg, Germany

ARTICLE INFO

ABSTRACT

Real-time quantitative polymerase-chain-reaction (qPCR) is a standard technique in most laboratories used for various applications in basic research. Analysis of qPCR data is a crucial part of the entire experiment, which has led to the development of a plethora of methods. The released tools either cover specific parts of the workflow or provide complete analysis solutions.

Here, we surveyed 27 open-access software packages and tools for the analysis of qPCR data. The survey includes 8 Microsoft Windows, 5 web-based, 9 R-based and 5 tools from other platforms. Reviewed packages and tools support the analysis of different qPCR applications, such as RNA quantification, DNA methylation, genotyping, identification of copy number variations, and digital PCR. We report an overview of the functionality, features and specific requirements of the individual software tools, such as data exchange formats, availability of a graphical user interface, included procedures for graphical data presentation, and offered statistical methods. In addition, we provide an overview about quantification strategies, and report various applications of qPCR.

Our comprehensive survey showed that most tools use their own file format and only a fraction of the currently existing tools support the standardized data exchange format RDML. To allow a more streamlined and comparable analysis of qPCR data, more vendors and tools need to adapt the standardized format to encourage the exchange of data between instrument software, analysis tools, and researchers.

© 2014 Published by Elsevier GmbH.

Contents

* Corresponding author. Tel.: +43 50550 4409.
  E-mail addresses: stephan.pabinger@ait.ac.at (S. Pabinger), Stefan.Roediger@HS-Lausitz.de (S. Rödiger), albert.kriegner@ait.ac.at (A. Kriegner), klemens.vierlinger@ait.ac.at (K. Vierlinger), andreas.weinhaeusel@ait.ac.at (A. Weinhäusel).
[1] These authors contributed equally to this work.

1. Introduction

Since its commercial introduction almost 2 decades ago, real-time quantitative polymerase-chain-reaction (qPCR) has come to play a prominent role in the life sciences. It provides the base for a plethora of applications in basic research, pathogen detection, and biomedical diagnostics. Furthermore, it is widely accepted as the *gold standard* for the analysis of gene expression. qPCR is a molecular biology technique, which allows amplification and simultaneous quantification of a targeted DNA molecule. The advancement compared to the original PCR method is the ability to measure the amplification of DNA as the reaction progresses in real time [1]. This allows quantifying initial amounts of template molecules by comparing the number of amplification cycles required for the response curves to reach a particular quantification threshold fluorescence signal level [2]. The more copies of a DNA template are present at the beginning of an experiment, the fewer PCR cycles are needed to make enough material for detection. In the past years, isothermal amplification strategies emerged as alternatives to PCR. Examples include the helicase-dependent isothermal DNA amplification (HDA), and the recombinase polymerase amplification (RPA). In contrast to traditional PCR, these methods do not require changing the reaction temperature and therefore use time-based measurements instead of cycle based measurements. However, isothermal amplification methods have less clinical use than conventional PCR [3,4].

Since the commercialization of qPCR in 1996, the number of publications referring to qPCR has increased exponentially (see Fig. 1). To ensure quality of results and allow potential reproduction, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have been published [5]. They provide a basic set of quality criteria and detail 85 parameters ranging from experimental design over sample processing and assay validation to data analysis.

After successfully generating a high-quality qPCR run, the data needs to be correctly analyzed to get biological meaningful results. To facilitate sharing and exchanging experimental data, the Real-time PCR Data Markup Language (RDML) has been developed [6]. The data standard is based on XML and contains details about the experimental setup, information about the samples and targets, and all measured data. As the data analysis step is an essential part of the qPCR workflow, it should be performed in a standardized and reproducible way [7]. As a consequence, dedicated software
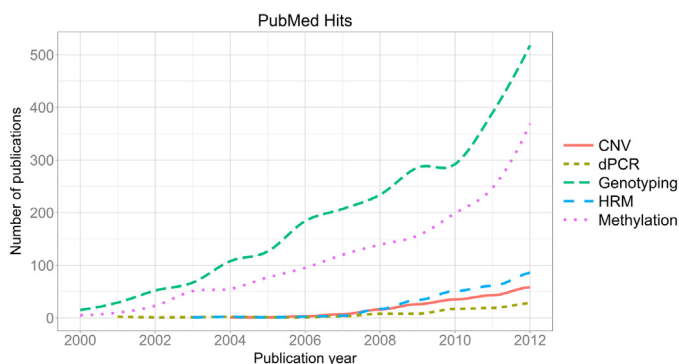
packages and data analysis suites were created targeting different aspects of the qPCR analysis workflow. Usually, commercial qPCR systems are equipped with software for data analysis and visualization. However, in most of the cases these closed source software modules are black boxes and do not provide full control over the entire process. Recently, Nature [8] and Science [9] reminded the scientific community about the importance of open computer programs.

In this survey we reviewed 27 software packages and tools for the analysis of qPCR data. We targeted all available open-access tools capable of analyzing raw fluorescence or Cq (quantification cycle) values. First we provide an overview about the main application fields for qPCR. Next, we report an overview of the functionality, features and specific requirements of the individual tools. Finally, we briefly discuss the surveyed applications and provide a report on their provided functionalities.

2. DNA quantification strategies

The design of a qPCR experiment depends, amongst others, on biological (e.g., studied organism, given biological question) and technical (e.g., available platforms, choice of chemistry, primers) parameters. A thorough guideline and detailed procedures covering the technical setup of a qPCR experiment has been published elsewhere [10].

The quantification of target DNA in each cycle of a qPCR experiment is based on measuring the emission of a fluorescent reporter dye. Dyes that bind to double-stranded DNA and upon excitation emit light (e.g., SYBR Green) are the most widely used DNA dyes due to ease of use, cost efficiency, and generic detection [11]. Their disadvantage is that they bind to any double-stranded DNA, including non-specific reaction products, which might result in an overestimation of the target concentration. Probe-based methods, such as TaqMan probes, molecular beacons, or scorpion primers rely on the sequence-specific detection of a desired PCR product resulting in increased specificity and sensitivity [12,13].

Furthermore, different probes can be used for multiplex PCR, in which multiple targets are amplified and detected in a single reaction tube. Unique probes or specific melting temperatures distinguish each PCR target amplified by a different set of primers [14]. Multiplex PCR reduces reagent consumption and allows studying more combinations of samples and target genes on one chip, but might reduce sensitivity as targets compete with each other for reaction resources. Currently, up to five different reactions can be detected simultaneously in one tube.

Based on the detected fluorescent signal and the chosen experimental setup several analysis steps are needed to obtain biological meaningful results (see Fig. 2). First, based on the raw fluorescence values a baseline is subtracted from the raw data, which is a crucial step in qPCR data analysis [15]. Next, the quantification cycle (Cq) value – previously known as the threshold cycle (Ct), crossing point (Cp), or take-off point (TOP) – can be calculated. In general, the Cq value represents the number of cycles needed to reach a set threshold fluorescence signal level. In addition, the raw fluorescence values can also be used for inferring the amplification efficiencies. Using the determined Cq value, quantification of nucleic acids can be performed by absolute quantification (via standard curve or digital PCR) or relative quantification (delta Cq)
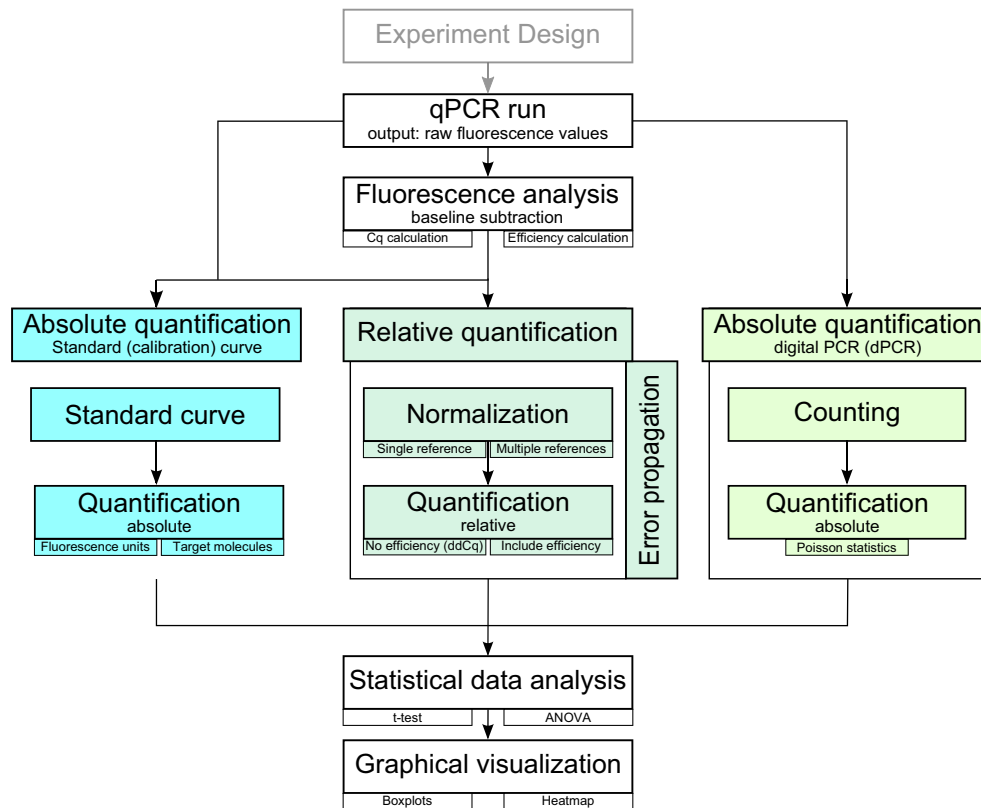

Fig. 1. Number of publications in PubMed related to qPCR. This plot shows the number of publications in PubMed related to specific qPCR applications.

Fig. 2. Flowchart of qPCR data analysis. This flowchart displays the different steps of qPCR data analysis. After a successful qPCR run, the raw fluorescence values can be used to calculate Cq and amplification efficiency values. Next either absolute or relative quantification is performed. Finally, statistical analysis can be performed on the generated quantification results and data can be displayed graphically.

[16]. Finally, the quantification results can be tested for statistical significant differences and presented in a graphical way.

2.1. Absolute quantification (standard curve)

Absolute quantification yields the exact number of target DNA molecules by comparison with DNA standards using a calibration curve. The curve is generated by using serially diluted standards of know concentrations and produces a linear relationship between Cq and the logarithm of the initial amount of total template DNA [17]. The reliability of the absolute quantification method depends on the amplification efficiencies for the target and the calibration curve, which needs to be considered in the analysis workflow.

2.2. Absolute quantification (digital PCR)

Digital PCR (dPCR) is a novel method for precise quantification of nucleic acids [18]. Prior to PCR, the reaction mixture is divided into a very large number of separate tiny volumes, such that there is either zero or one target molecule present in any individual reaction. Currently available commercial systems can generate up to ten thousands (Bio-Rad, Life Technologies), fifty thousand (Fluidigm), and ten million (RainDance) droplets per experiment [19]. In effect, each reaction becomes binary and the discrete signals can be counted. After applying a Poisson correction to account for wells with more than one copy, the counts can be used to quantify the absolute number of DNA molecules in the sample [20]. dPCR can be used for absolute quantification without the need for standard curves [21], and has been reported to have a higher accuracy [21] as well as robustness to amplification efficiency [22] compared to qPCR.

Due to the rising interest in dPCR, separate digital MIQE guidelines have been published to assist researchers in designing, conducting, and analyzing their experiments [23]. Recently, a study systematically compared the performance of qPCR with that of dPCR. The authors could show by using a variety of targets in different sample backgrounds, that dPCR with droplets showed greater precision and more reproducible findings [24] than qPCR [25].

2.3. Relative quantification

Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. It avoids the need for a standard curve as the amount of the studied gene is compared to the amount of a reference gene. Various mathematical models are established to calculate the expression of a target gene in relation to an adequate reference gene [26]. Based on the method, they can perform normalization using single or multiple reference genes, include PCR efficiency, and allow calibration across multiple plates. The final calculated relative expression level relates the target gene in a sample relative to a calibrator sample. The generated relative quantities can be compared across multiple real-time RT-PCR experiments. Appropriate normalization strategies and selection of reference genes have been reviewed elsewhere [27,28]. For large scale expression profiling studies it has been shown that mean expression value normalization outperforms the normalization strategy based on a few reference genes [29,30]. As relative quantities are determined by computations based on several observables (e.g., replicates, amplification efficiency), it has been shown that the random error of the final result is influenced by error propagation. Consequently, methods have been developed to include error propagation into relative quantification calculations [31].

2.4. Data handling, statistical analysis, and result presentation

To calculate the Cq value from raw fluorescence data, selected software packages and tools perform preprocessing steps. These may include methods for the reduction of noise (e.g., introduced by technical components) curve smoothing, removal of outliers, normalization, curve fitting, and background subtraction [32]. In contrast to closed source software, open source software platforms provide full control over such tasks. A further important aspect is the handling of missing values (NA). There are in principle two levels to handle NAs.

The first level is on the raw fluorescence data of the amplification curves, which may occur randomly in developmental qPCR technologies, such as the VideoScan platform [33], but also in existing data sets. A common approach of handling missing values is by imputation. Methods include for example cubic spline interpolation or imputation of location parameters such as the mean or median [34].

The second level of NA handling is after initial Cq calculation. It might be the case that certain reactions are missing corresponding values. This might be due to different factors, such as technical difficulties, detector problems, or low quantity genes. Currently, several methods have been presented to handle missing values. First, they can be simply excluded from any downstream analysis. As long as enough technical replicates are available and not too many reactions are marked as excluded this is a legitimate approach. Second, they can be assigned the Cq value of the maximum number of cycles (often 40), which might deviate the mean of the analyzed samples due to outlier creation. Third, they can be imputed by using the Cq values of technical or biological replicates. Methods range from very simple median calculations to more sophisticated statistical models (e.g., hierarchical models) [35,36]. These methods generally yield good results, but may lead to inflated stability values for reference genes [37].

After performing relative or absolute quantification, usually different groups (e.g., healthy vs. control, time series) are tested for their statistical significant difference. To allow generation of meaningful results, an experiment ideally should encompass at least three independent biological replicates of each treatment [38]. As budgetary considerations often hamper the use of multiple replicates, more focus should be given to biological replicates than technical replicates [39]. Various statistical methods can be used to test for significant differences. The analysis of variance (ANOVA) can be used to compare treatments using the previously calculated quantification results. If only two conditions are compared, a standard *t*-test can be applied [40].

Final results are often presented in a graphical way allowing easier interpretation and understanding of the obtained findings. The data may be presented as a heatmap and clustered similar to the presentation of microarray data [41]. Instead of creating the widely popular bar plots for reporting findings, we recommend the use of box plots as they represent both the summary statistics and the distribution of the primary data [42]. Bar plots with error bars are difficult to compare and encourages the perception that the mean is related to the height of the bar rather than the position of its top. They are best used for visualizing counts or proportions. Box plots require a sample size of only 5 and provide more information about samples than conventional error bars. Results obtained from very small sample sizes should be displayed using traditional mean-and-error or column scatter plots [43].

3. Applications of qPCR

qPCR can be applied for several applications spanning a wide range of use cases. The most commonly used application is gene expression analysis. In addition to quantification experiments, qPCR can be used for the analysis of copy number variations, DNA methylation, and genomic variants.

3.1. Quantification of DNA, RNA, and DNA methylation

qPCR was originally designed for DNA quantification and has been extensively developed in clinical microbiology laboratories for routine diagnosis of infectious diseases. It is applied for detecting bacterial, fungal, and viral pathogens, especially when testing to discover the source of an unknown infection [44].

In addition to DNA quantification, it has been widely used to quantify the expression of messenger RNA (mRNA) [45]. First, RNA is reverse transcribed into a cDNA template, which is then used in qPCR reactions to detect and quantify gene expression products. Amongst others, it has been successfully applied for the analysis of transcriptional biomarkers [46,47], cancer [48], and Mendelian diseases [49].

Since their discovery in 1993, microRNAs (miRNAs) have been a valuable research target for scientist worldwide. miRNAs are small, regulatory, noncoding single-stranded RNAs, which are usually 20–25 nucleotides long [50]. The first qPCR assay to quantify miRNAs was developed almost a decade ago [51], and subsequent protocols for using relative and absolute quantification have been developed [52]. miRNAs have been successfully identified as possible biomarkers for cancer [53], autologous transfusion [54], diabetes [51] and cardiomyopathies [55]. In addition to miRNAs, a large number of different non-coding RNAs have been described, where many show regulatory functions. Amongst others, long non-coding RNAs (lncRNAs), small inhibiting RNA (siRNA), and small nuclear RNA (snRNA) can be quantified by qPCR methods.

DNA methylation is a heritable epigenetic modification in genomes which is known to be involved in biological processes such as regulation of gene expression, cell differentiation, DNA structure and disease states [58]. To date, several protocols for determining the methylation state have been developed such as real-time Quantitative Methylation-Specific PCR (QMSP) [59], methylation-sensitive restriction enzyme digestion PCR (MSRE-PCR) [60], MethyLight [61], and methylation-sensitive high resolution melting (MS-HRM) [62]. The analysis of methylation qPCR data depends on the selected protocol and involves absolute or relative quantification as well as high resolution melting (HRM) analysis.

3.2. Copy number variations (CNV)

The gene copy number is the number of copies of a particular gene in the genotype of an individual, e.g., diploid genomes usually possess two copies of each gene. Copy number variations (CNVs) in the genome are genetic variations where segments of DNA are present in a variable number of copies in comparison to the reference (e.g., diploid human) genome. CNVs can arise by a variety of processes, such as deletions, duplications and translocations during meiosis. They may influence both mRNA and protein levels and have recently been associated with several complex and common diseases [56]. The experimental setup and statistical methods to calculate copy number variations have been made accessible and were widely applied for studying CNVs in various diseases [57–59]. By using qPCR, the Cq values of the target gene can be compared to unrelated reference sequences. It has been shown that including multiple reference samples results in more accurate results. Furthermore, it allows combining references with a normal copy number and references with a known CNV, which can serve as reference point and as positive control for the detection of CNVs [60]. The generated ΔCq values are then used for CNV calculation. As
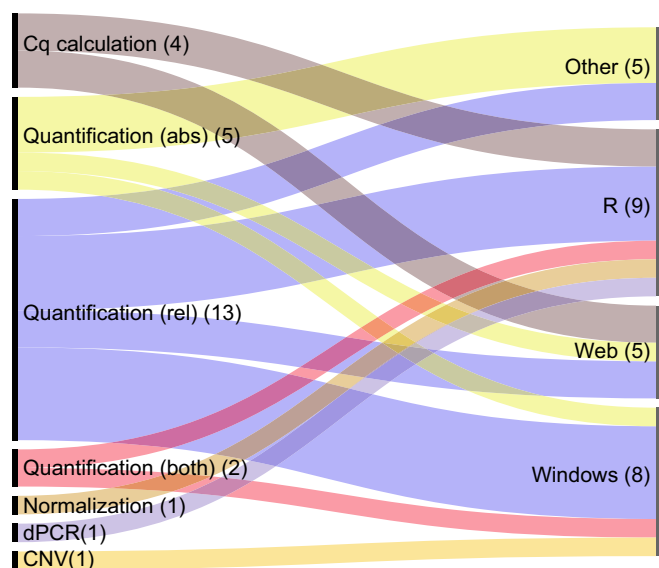
Fig. 3. Quantification tools. This figure displays the relationship between analysis type (left side) and platform (right side). It can, for instance, be seen that tools for Cq calculation analysis are only available as R packages or Web-based applications.

a consequence, methods that support quantification analysis are applicable for CNV calculations [60].

3.3. Genotyping

Several methods have been developed that involve different PCR preparation techniques to discover and genotype small DNA sequence variations such as single nucleotide polymorphisms (SNPs) [61]. The created assays vary in sample preparation techniques, turnaround time, costs, and multiplexing capabilities [62]. Depending on the used protocol, different normalization and quantification strategies need to be applied after Cq value calculation.

4. Software packages and tools for the analysis of qPCR data

In this review, we comprehensively surveyed 27 open-access packages, software tools, and web applications performing analysis on either raw fluorescence values (amplification curve data either before or after baseline subtraction) or predetermined Cq values (see Table 1). Tools and packages had to be accessible or available at the time of the survey. We excluded methods for designing PCR primers and determining the most stable reference genes. Furthermore, tools for advanced statistical calculation of quantified qPCR data were not included in this survey. Commercial tools such as GenEX (MultiD, Gothenburg, Sweden), Genevestigator (Nebion, Zürich, Switzerland), qbase⁺ (Biogazelle, Zwijnaarde, Belgium), and the various software suites distributed by the vendors of thermo-cycler machines were not part of this review.

The evaluated tools for qPCR data analysis were divided into five different types (see Fig. 3): quantification analysis (20 tools), Cq calculation (4 tools), normalization analysis (1 tool), CNV analysis (1 tool), and dPCR analysis (1 tool). Several tools assigned to quantification analysis are also capable of calculating Cq values and allow performing CNV analysis. In addition, we grouped the surveyed tools according to their platform: Microsoft Windows (8 tools), web-based (5 tools), R-based (9 tools), and other platforms (5 tools).

We provide an overview about supported steps of the analysis workflow (see Fig. 2), accepted input files, used file formats, availability of a graphical user interface (GUI), date of last update, and number of citations (see Table 1). Based on the identified analysis

workflow for qPCR data, six of the surveyed tools support the calculation of Cq values and eight applications allow determining the amplification efficiency values in addition to either absolute or relative quantification. Amongst the reviewed tools, eight tools allow performing of absolute and 15 tools support relative quantification. Only two tools are able to carry out relative as well as absolute quantification. Error propagation for relative quantification is not widely applied and only available in five tools. Many applications (15 tools) support some kind of graphical quantification result presentation; bar plots are the most common format. If downstream statistical analysis is provided (9 tools) it usually includes testing for significant quantification differences between groups.

In the next section, we briefly describe the tools and provide a report on their provided functionalities.

4.1. Windows tools

The standalone software **CopyCaller** performs relative quantification analysis of genomic DNA targets from copy number assay experiments. It can be applied to detect and measure copy number variations of specific genomic sequences without a known calibrator sample. It returns the calculated copy number and estimates the confidence of copy number calls.

DART-PCR, released over a decade ago, is a Microsoft Excel based tool for analyzing raw fluorescence data (before baseline subtraction). It calculates the Cq values and performs subsequent analysis for relative quantification and assay variability. Amplification efficiency is determined by the application and tested for outliers. Next, a user set efficiency value is used for normalization and calculation of relative quantities, which can be graphically displayed as bar plots. DART-PCR does not support normalization to reference genes.

LinRegPCR is a standalone program for analyzing raw fluorescence data (before baseline subtraction) using a graphical user interface. The recently updated application is RDML compliant and additionally accepts Excel as input format. LinRegPCR determines and subtracts baseline fluorescence, sets a Window-of-Linearity and calculates PCR efficiencies per sample. Next Cq value and the starting concentration per sample (reported in an arbitrary unit) are calculated. The program provides methods to visualize raw fluorescence value curves and is able to plot graphs for comparing efficiency values of different samples. The determined Cq and efficiency values can later be used for quantification analysis.

qBase is a Microsoft Excel based program for the relative quantification of qPCR data. Its analysis is based on Cq values and it supports the direct import of export files from various systems (ABI, Bio-Rad, Rotor-Gene, Stratagene). Furthermore, qBase supports the RDML format, and applies a normalization strategy that features error propagation and multiple reference genes. The standard curve, used for efficiency calculation, can be graphically displayed and quantification results can be visualized as bar plots. Since February 2008 the application has been superseded by qbase⁺, a commercially available application, and no updates for the Excel based version have been published.

qCalculator is a Microsoft Excel based Visual Basic application for the calculation of relative quantification values. The tool takes Cq values as input and uses multiple sheets for data manipulation and result presentation. qCalculator is able to calculate amplification efficiency from standard curves, which is used in subsequent analysis steps. It supports normalization to reference genes, where multiple reference genes are not combined but treated separately.

qPCR-DAMS is a database tool for qPCR based on Microsoft Access 2003. It is designed to analyze, manage, and store relative as well as absolute quantitative real-time PCR data. The analysis module of the system allows user to perform absolute and relative quantification including normalization to (multiple) reference

Table 1

qPCR data analysis software packages and tools. Software packages and tools for the analysis of qPCR data are listed. For each tool its corresponding application area is specified, divided into: Cq calculation, normalization, quantification, CNV, and dPCR. The input type can either be precalculated Cq values (Cq) or raw fluorescence values (Raw). For each tool the supported operating system or the underlying framework is specified. Frameworks are often available on different operating systems allowing the package to run on several platforms. GUI specifies the existence of a graphical user interface for data input and output. ABI, Applied Biosystems format; ABT, Lightcycler export format; CSV, comma separates values, FLO, Lightcycler export format; REX, Rotor Gene export format; R format, encompasses all import and export formats provided by the default R installation and auxiliary R packages (e.g., PDF, SVG, HTML, and XLS).

Tool	Web	Feature	Cq/Raw	Input	Output	OS/Framework	GUI	Last update	Ref
CAmpER	[76]	Cq calculation, Normalization, Quantification	Raw	FLO, ABT, CSV, REX, TXT	CSV, TXT	Web based	Yes	2009-06-01	[77]
chipPCR	[34]	Cq calculation	Raw	Native R format	Native R format	R based	Yes	2014-06-25	[34]
CopyCaller	[78]	CNV	Cq	ABI	CSV, TXT, XLS	Windows	Yes	2009-02-01	[79]
Cy0 Method	[80]	Cq calculation	Raw	XLS, TXT, DOC	XLS	Web based	Yes	2010-01-01	[81,82]
DART-PCR	[83]	Cq calculation, Normalization, Quantification	Raw	XLS	XLS	Windows, Excel based	Yes	2002-12-16	[84]
ddCT	[85]	Normalization, Quantification	Cq	TXT, native R format	TXT, PDF, native R format	R based	No	2013-10-14	[86]
Deconvolution	[87]	Quantification	Raw	TXT	TXT	Perl based	No	2010-04-29	[88]
dpcR	[89]	dPCR, Quantification, CNV, Genotyping	Cq, Raw	TXT, CSV, native R format	TXT, native R format	R based	No	2013-09-08	[90]
EasyqpcR	[91]	Normalization, Quantification	Cq	TXT, CSV	TXT	R based	Yes	2013-11-24	[92]
FPK-PCR	[93]	Cq Calculation	Raw	CSV, TXT	TXT	R based	No	2012-01-20	[94]
HTqPCR	[95]	Normalization, Quantification, Statistics	Cq	TXT, native R format	TXT, PDF, native R format	R based	No	2013-10-14	[96]
LinRegPCR	[97]	Cq calculation, Quantification	Raw	XLS, RDML	XLS, RDML	Windows	Yes	2014-02-19	[98]
LRE Analysis	[99]	Quantification	Raw	XLS	XLS	MATLAB based	Yes	2012-02-21	[100]
LRE Analyzer	[101]	Quantification	Raw	XLS	XLS	Java based	Yes	2014-01-07	[102]
MAKERGAUL	[103]	Cq calculation, Quantification	Raw	CSV	HTML	Web based	Yes	2013-08-27	[104]
NormqPCR	[105]	Normalization, Quantification	Cq	TXT	TXT	R based	No	2013-03-23	[73]
PCR-Miner	[106]	Cq calculation	Raw	TXT	TXT	Web based	Yes	2011-10-21	[107]
pyQPCR	[108]	Normalization, Quantification	Cq	TXT, CSV	TXT, PDF	Python based	Yes	2012-01-03	[109]
qBase	[110]	Normalization, Quantification	Cq	XLS, RDML	XLS, RDML	Windows, Excel based	Yes	2007	[26]
qCalculator	[111]	Normalization, Quantification	Cq	XLS	XLS	Windows, Excel based	Yes	2004-01-26	[112]
QPCR	[113]	Cq calculation, Normalization, Quantification, Statistics	Raw	CSV, RDML	CSV, RDML, XLS, SVG, PNG	Web based	Yes	2013-06-10	[114]
qpcR	[115]	Cq calculation, Normalization, Quantification, Melting curve analysis	Cq, Raw	CSV, native R format	TXT, PDF, native R format	R based	No	2014-06-02	[116]
qPCR-DAMS	[117]	Normalization, Quantification	Cq	XLS	XLS	Windows	Yes	2006-02-18	[118]
qpcrNorm	[119]	Normalization, Statistics	Cq	CSV	TXT	R based	No	2013-10-14	[120]
REST	[121]	Normalization, Quantification, Statistics	Cq	TXT	TXT	Windows 32 Bit	Yes	2009	[122]
SARS	[123]	Normalization, Statistics	Cq	XLS, TXT	TXT	Windows	Yes	2011-05-01	[124]
SASqPCR	[125]	Normalization, Quantification, Statistics	Cq	XLS, CSV	TXT	SAS based	No	2011-06-01	[126]

genes. qPCR-DAMS takes predetermined Cq values as input and provides three quality control steps and a system to monitor data variation.

The **Relative Expression Software Tool (REST, version 2009)** is a GUI application for the relative quantification of qPCR data. It operates on Cq values (direct support for Rotor-Gene), includes different PCR efficiencies of the genes, and uses multiple reference genes for normalization. The tool provides methods to visualize raw fluorescence value curves and displays quantification results as box plots. Furthermore, expression ratios are calculated based on a pairwise reallocation (resampling) approach and can be tested for statistical significance.

SARS is a Python and R-based Windows application for relative quantification of qPCR data providing a graphical user interface. The standalone software suite needs to be installed and takes Cq and efficiency values in Excel or TXT format. In addition to normalization and quantification, SARS is able to run statistical tests on the generated results.

4.2. Web based tools

CAmpER is a web-based tool for the basic analysis of qPCR data. The system has been last updated in 2008 and consequently its user interface is not meeting current standards. CAmpER allows uploading raw fluorescence data in a generic CSV format and supports file formats from different vendors (ABI, Bio-Rad, Mastercycler, Opticon, Roche, Rotor-Gene, Stratagene). Reaction efficiency and Cq value can be calculated using two different methods: DART and FPLM. After relative quantification, which does not offer to normalize the data to reference genes, data can be exported in CSV format. Furthermore, the tool supports the display of quantification results as bar plots.

The **Cy0 method** is provided as a free web interface (requiring registration) that takes raw fluorescence data (before baseline subtraction) as input. The method uses nonlinear regression to obtain the best fit estimators of reaction parameters. Similar to traditional Cq methods it is a threshold-based method, but the returned Cy0 value depends on the threshold and the amplification kinetics and thus compensates for small variations among samples. Calculated results are not directly returned to the user, but sent to a specified e-mail address.

The web-application **MAKERGAUL** uses raw fluorescence data (before baseline subtraction) to calculate quantification data based on MAK2 [63]. Using a simple web interface, quantification based on the MAK2 method can be performed without the requirement for standard curves or normalization with reference genes. As the tool is not available online, it needs to be installed and configured on a local server, which is described as challenging.

PCR-Miner is a web-based application for calculating Cq and efficiency values. It takes raw fluorescence data (before baseline subtraction) as input and requires converting the values into a specific text input format. The tool can be used without registration and either returns the calculated values directly on the website or sends them to a provided e-mail address.

QPCR is a web-based application supporting storage, management, and analysis of qPCR data. It comprises a parser to import data from qPCR instruments (ABI, Bio-Rad, Roche) and supports the RDML format. QPCR incorporates a variety of analysis methods to calculate Cq and amplification efficiency values. The analysis pipeline includes replicate handling, normalization using multiple reference genes, inter-run calibration, error propagation and fold change calculation. Calculated quantification results can be displayed as bar plots. Installation and configuration requires several steps when the application is used on a private server.

4.3. R-based tools

R is a free, open source cross-platform (e.g., UNIX platforms, Windows, and MacOS) software environment for statistical computing, visualization, and report generation. This scripting language is considered to be the *lingua franca* in the academic sector and in business applications. R is distributed with many statistical routines, but most of the functionality is provided by "R packages". R packages are collections of functions, data, documentation, and occasionally compiled code in a structured format. Most of the packages can be installed from the Comprehensive R Archive Network (CRAN) or Bioconductor [64]. By default R is command-line driven but tools have been published to provide a graphical user interface (GUI) or Integrated Development Environment (IDE) on top of the underlying framework [65,66].

ddCT is an R package that collects, analyzes, and visualizes qPCR data using an improved ddCT [67] method. It takes predetermined Cq and optional efficiency values to perform relative quantification using (multiple) reference genes. Results are returned in text format and the package offers methods to generate quantification results as bar plots. The methods can be either invoked by a script or through a provided application programming interface (API). The package is well documented and sample R-code is provided.

The R package **dpcR** is the first open source package for the statistical analysis of digital PCR experiments. It is a comprehensive collection of functions for dPCR data analysis and simulation based on Poisson statistics. The package can be used for chamber based real-time digital PCR systems as well as droplet based digital PCR systems and contains methods derived from peer-review publications for quantification and zygosity determination. In addition, novel uncertainty calculations have been introduced for the analysis of dPCR experiments. Absolute quantification is performed by counting the number of positive compartments and relating it to the total number of compartments by means of Poisson statistics. Furthermore, dpcR is able to perform analysis of CNVs and rare mutations. The package includes many published statistical approaches, summary functions, and data sets for dPCR. In addition, it supports the generation of bar plot for quantification results, amplitude plots and density plots. dpcR uses the shiny [68] R packages to provide an interactive web or off-line graphical user interface application, which can be used by both R novices and experts.

The recently released R package **EasyqpcR** is based on the qBase algorithms [26] and calculates relative quantities using Cq values. It is able to determine amplification efficiencies using dilution series, and calculates normalization factors based on multiple reference genes. In general, the provided functionality is very similar to qBase. A graphical user interface is built upon *R GUI Studio* and the *gWidgets* package.

FPK-PCR is an R-based tool for the analysis of raw fluorescence data (before baseline subtraction). It calculates reaction efficiencies and Cq values by reconstructing the entire chain of cycle efficiencies. The R-code is released with a short documentation illustrating a general example.

HTqPCR is a newly published R package designed for the analysis of Cq values. It accepts tab-delimited text files and performs quality assessment, normalization (quantile normalization, rank-invariant, scale rank-invariant, housekeeping – deltaCq, geometric mean), and testing for statistical significance. Individual samples can be flagged as undetermined or unreliable, which is used throughout the analysis. The package supports principal component analysis (PCA) and clustering of analysis results. It offers multiple methods for data visualization, such as bar/box plots of quantification results, or Cq values as heatmap/scatter plot. HTqPCR is released with an extensive documentation illustrating several use cases.

The R package **NormqPCR** provides methods for the normalization and quantification of qPCR data. Its documentation contains several examples helping novice R-users to get used with the package. It supports technical replicate handling and offers methods for selecting the best genes for normalization. Furthermore, it uses the R-package ReadqPCR for data import and supports normalization using multiple reference genes. Relative quantification can be performed by assuming perfect amplification or by including predetermined reaction efficiencies.

The R-based package **qpcrNorm** includes three (quantile normalization, rank-invariant, housekeeping – deltaCq) algorithms for qPCR data normalization. It uses Cq values as input but can also deal with any measure of transcript abundance. The included normalization methods are especially applicable to any high-throughput qPCR setup or experiments where the stability of reference genes has not been validated. In addition to textual output, the package provides functionality to generate diagnostic plots (e.g., scatter plot for comparing the effects of normalization methods). Calculated quantification results can be displayed as box plots. Documentation explains the basic functionality but does not include sample code.

The **qpcR** library is an R-based package that assists researchers in the modeling and analysis of quantitative real-time PCR data. It includes many published methods to perform a variety of qPCR data analysis steps including different methods for replicate handling, Cq value calculation, normalization, and relative quantification. qpcR is able to perform melting curve analysis and allows visualization of the raw fluorescence curve (before as well as after baseline subtraction). Furthermore, it features customizable import functionality and plotting quantification results as bar plots, box plots, or Cq heatmaps. The package is well documented but does not provide much sample code for novice R-users. To provide a graphical user interface for the qpcR library, a plug-in for the RKWard [66,69] system has been created. RKWard is a GUI and IDE for statistical analysis with R. The RKWard qPCR plugin uses core features (e.g., Cq value calculation) of the qpcR package while other functionality for preprocessing (e.g., removal of missing values, data transformation) and melting curve analysis is derived from the chipPCR [34] and the MBmca [70] packages. The **chipPCR** package contains functions to perform background subtraction, to simulate qPCRs, and to detect the start of an amplification reaction. Furthermore, it includes a Cq value quantification method based on the first and second derivative maximum method and supports calculation of the amplification efficiency of dilution experiments.

4.4. Other platforms

Deconvolution is a Perl based program for the efficiency-independent absolute quantification of qPCR data. The method depends on a computer-assisted deconvolution that determines amplification behavior between the unknown template and an amplicon standard. Deconvolution has been released in 2010, uses raw fluorescence values (before baseline subtraction) and outputs quantification results in text format.

LRE Analyzer is a Java-based, standalone, platform independent desktop application offering automated analysis and data storage capabilities. It presents a state-of-the art graphical interface and uses Microsoft Excel for import of raw fluorescence data (before baseline subtraction). The application allows users to conduct absolute quantification without construction of target-specific standard curves. Quantification results are presented in fluorescence units or in target molecules if a known quantity is amplified and used as a reference. The application allows users to visualize the raw fluorescence curve.

The MATLAB based tool **LRE Analysis** is based on the published LRE method [71]. It reads raw fluorescence values (after baseline

subtraction) from an Excel file that needs to comply with a specific format. Currently, it supports one ABI system and performs data processing in a semi-automated way. Quantification results are exported in a new Excel file containing calculated values.

pyQPCR is a Python based application that provides a modern graphical user interface. It takes Cq values as input and supports data import from a few Eppendorf and ABI systems file formats. The methods implemented in pyQPCR are based on qBase [26], and the application is able to perform relative quantification and determination of efficiencies using standard curve calculation. Quantification results can be displayed as bar plots.

SASqPCR is a program that requires the commercial SAS software suite, but according to the authors extensive SAS programming knowledge is not required. The tool performs calculations on Cq values and incorporates functions for assessment of PCR efficiencies, validation of reference genes, and normalization using multiple reference genes. Furthermore, SASqPCR provides methods for the statistical comparisons of target gene expression of parallel samples.

5. Discussion

In our review, we provide a comprehensive survey of software packages and tools for the analysis of qPCR data, including Cq calculation, efficiency determination, normalization, and quantification. In addition, we report tools dedicated for the analysis of dPCR experiments. The information we provide represents a valuable resource for researchers working with qPCR data and supports users with the selection of the appropriate analysis tool for a specific application.

Surveyed software packages and tools are manifold and vary in the supported methods and usability. The choice of the correct method to analyze the qPCR data at hand strongly depends on the experimental setup and assay. Therefore, a number of review articles were recently published [15,72] to facilitate the choice of the most suitable analysis method for a particular application.

Windows tools for quantification analysis include standalone applications and tools based on Microsoft Excel. "LRE analyzer" is a recently developed, platform independent tool with an intuitive interface that can be used for absolute quantification without the need for construction of target-specific standard curves. REST is a popular tool available on the Microsoft Windows platform, which has been released in four versions so far. Amongst the reviewed tools, three applications are built on top of Microsoft Excel, where qBase has been cited the most. CopyCaller is the only tool dedicated to the analysis of CNVs.

Web-based tools include CAmpER, Cy0 Method, MAKERGAUL, PCR-Miner, and QPCR. QPCR offers the most comprehensive functionality and supports, in addition to storing and sharing of raw and processed data, relative quantification of qPCR data. MAKERGAUL and QPCR can also be installed on a private server where configuration and management may be challenging. PCR-Miner is a widely used tool for calculating Cq and efficiency values. The user interfaces of CAmpER, Cy0 Method, and PCR-Miner do not comply with current standards and thus the tools may be difficult to use.

Several **R-packages** are available based on the statistical computing and graphics environment R, which usually do not offer a graphical user interface and need to be used on command line. They provide an excellent choice for users who wish to keep the pipeline for the analysis transparent and highly customizable. The currently available packages enable the seamless integration of analysis strategies for experimental qPCR platforms and commercial platforms. Furthermore, it has been demonstrated in several occasions that custom-made cross-platform GUIs and report generators can be created with little programming effort. In order to

Table 2

Quantification software packages and tools for qPCR. Listed are software packages and tools capable of performing quantification analysis. The Cq column was ticked if the tool is able to perform Cq calculation from raw fluorescence data. If the tool is able to calculate amplification efficiencies (based on dilution series or on raw fluorescence data) the appropriate column was ticked. Tools are marked for absolute quantification (Abs quant) if they are able to output absolute quantification values. In order to qualify for relative quantification (Rel quant) fold change values after relative quantification need to be returned. Error propagation denotes that ability of the tool to propagate the error throughout the various analysis steps. The normalization column was ticked if the tool implements one or several normalization techniques (not limited to normalization to reference genes). NA handling describes the possibility to deal with missing values after initial Cq calculation. Graphs were checked if the tools included methods for graphical (quantification) result presentation. Statistics was ticked, if the tool includes methods for statistical analysis or direct calls to underlying statistical frameworks after obtaining quantification or normalization results.

Tool	Cq	Efficiency	Abs quant	Rel quant	Error propagation	Normalization	NA handling	Graphs	Statistics	Compliant with MIQE recommendations[a]
CAmpER	+	+	–	+	–	–	–	+	–	–
chipPCR	+	+	–	–	–	–	+	+	–	+
Cy0 Method	+	–	–	–	–	–	–	–	–	+
DART-PCR	+	–	–	+	–	+	–	+	–	–
ddCT	–	–	–	+	–	+[b,c]	–	+	+	+
Deconvolution	–	–	+	–	–	–	–	–	–	+
dpcR	–	–	+	–	–	–	+	+	+	+
EasyqpcR	–	+	–	+	–	+[b,c]	+	–	–	+
HTqPCR	–	–	–	+	–	+[b]	+	+	+	–
FPK-PCR	+	+	–	–	–	–	–	–	–	–
LinRegPCR	+	+	+	–	–	–	–	+	–	+
LRE Analysis	–	–	+	–	–	–	–	–	–	+
LRE Analyzer (lreqcpr)	–	–	+	–	–	–	–	+	–	+
MAKERGAUL	+	–	+[d]	–	–	–	–	–	–	+
NormqPCR	–	–	–	+	–	+[b,c]	+	–	–	+
PCR-Miner	+	+	–	–	–	–	–	–	–	+
pyQPCR	–	+	–	+	+	+[b,c]	+	+	–	+
qBase	–	–	–	+	+	+[b,c]	+	+	+	+
qCalculator	–	+	–	+	–	+[c]	+	+	–	–
QPCR	+	+	–	+	+	+[b,c]	+	+	+	+
qpcR	+	+	+	+	+	+[b,c]	+	+	–	+
qPCR-DAMS	–	–	+	+	–	+[b,c]	+	–	–	+
qpcrNorm	–	–	–	–	–	+[b]	–	+	+	–
REST	–	–	–	+	+	+[b,c]	–	+	+	+
SARS	–	–	–	+	–	+[b,c]	–	–	+	+
SASqPCR	–	+	–	+	–	+[b,c]	–	–	+	+

[a] MIQE compliant: rel quant → includes PCR efficiency, normalization against multiple reference genes.

[b] Supports multiple reference genes.

[c] Includes efficiency in normalization.

[d] Based on MAK2.

facilitate data input, the package ReadqPCR has been developed to parse raw qPCR data from different platforms [73]. Moreover, most cyclers are able to export raw data in various forms, which can be imported by custom-made parsers via the powerful import and export facilities of R. Since all described R packages inherit the properties of the R computing environment, we would like to emphasize that parallelization of analysis steps and distribution as service (e.g., shiny) are easy to implement. Among the surveyed R-packages that support normalization and quantification, the script based *qpcR library* currently provides the most comprehensive functionality ranging from different methods for Cq and efficiency calculation to quantification and data visualization. HTqPCR provides different normalization techniques that can be used when reference genes are not present or not reliably expressed. dPCR is an R-package for the analysis of dPCR data providing complete functionality to analyze the respective experiment.

In addition to R-based packages, tools for **other platforms** using MATLAB, Perl, Python, and SAS have been published where the Python based tool pyQPCR offers the most extensive functionality. The tool offers a state-of-the art user interface and is based on the highly cited methods from [26]. A recent survey of publications mentioning qPCR revealed that methods used for analyzing the data were poorly reported [74]. Consequently, the MIQE guidelines specify that in addition to the analysis results detailed information about the applied methods need to be published. Furthermore, to allow repeatable and reproducible research the computer code used to analyze the data has to be made available to others [75]. Amongst others, the MIQE guidelines list essential information that has to be submitted with the manuscript: qPCR

analysis program, Cq method, normalization methods, and statistical methods for results significance. Moreover, the exact software version and source code of newly developed methods need to be published as well. The use of specific qPCR analysis software allows providing detailed information on the methods of data analysis and confidence estimation. We therefore encourage using published, peer-reviewed software that proved to be suitable for qPCR data analysis.

In addition, the MIQE guidelines provide specific recommendations, which need be considered when performing qPCR data analysis. Emphasis is placed on the importance of PCR efficiency, especially when reporting mRNA concentrations for target genes relative to those of reference genes. Researchers need to either ensure that the genes were amplified with comparable efficiencies or include the specific amplification efficiencies in the analysis. Furthermore, when data is normalized against reference genes at least two reference genes have to be included in the analysis, unless data is presented that shows that a single reference gene is invariantly expressed under the experimental conditions described. Table 2 includes information about tools that adhere to these MIQE recommendations.

Over the years, different qPCR platforms and many analysis tools have been made available, usually supporting their individual file format. As a consequence, the input file for each analysis tool needs to be created either manually or semi-automatically. Only a very limited number of tools support the direct use of export files from various qPCR systems. The introduction of the RDML file format aimed to encourage the exchange of qPCR data between instrument software, data analysis tools, and researchers. Unfortunately,

RDML is not widely spread amongst the surveyed tools and software packages – so far only three applications are able to work with RDML data. In order to allow a more streamlined and comparable analysis of qPCR data, more vendors and tools need to support the standardized data exchange format RDML.

Acknowledgments

References

[1] Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (NY) 1992;10(4):413–7.
[2] Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, et al. The real-time polymerase chain reaction. Mol Aspects Med 2006;27(2–3):95–125.
[3] Rödiger S, Liebsch C, Schmidt C, Lehmann W, Resch-Genger U, Schedler U, et al. Nucleic acid detection based on the use of microbeads: a review. Microchim Acta 2014.
[4] Khodakov DA, Ellis AV. Recent developments in nucleic acid identification using solid-phase enzymatic assays. Microchim Acta 2014:1–14. Available from: URL: http://link.springer.com/article/10.1007/s00604-014-1167-z [cited 08.04.14].
[5] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611–22.
[6] Lefever S, Hellemans J, Pattyn F, Przybylski DR, Taylor C, Geurts R, et al. RDML: structured language and reporting guidelines for real-time quantitative PCR data. Nucleic Acids Res 2009;37(7):2065–9.
[7] Bustin S, Bergkvist A, Nolan T. In silico tools for qPCR assay design and data analysis. Methods Mol Biol 2011;760:283–306.
[8] Ince DC, Hatton L, Graham-Cumming J. The case for open computer programs. Nature 2012;482(7386):485–8 [cited 03.04.14].
[9] Morin A, Urban J, Adams PD, Foster I, Sali A, Baker D, et al. Shining light into black boxes. Science 2012;336(6078):159–60. Available from: URL: http://www.sciencemag.org/content/336/6078/159 [cited 08.04.14].
[10] Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc 2006;1(3):1559–82.
[11] Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997;22(1):130–1, 134–8.
[12] Gudnason H, Dufva M, Bang DD, Wolff A. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Res 2007;35(19):e127.
[13] Ruijter JM, Lorenz P, Tuomi JM, Hecker M, van den Hoff MJB. Fluorescent-increase kinetics of different fluorescent reporters used for qPCR depend on monitoring chemistry, targeted sequence, type of DNA input and PCR efficiency. Microchim Acta 2014:1–8. Available from: URL: http://link.springer.com/article/10.1007/s00604-013-1155-8 [cited 08.04.14].
[14] Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KS. Real-time multiplex PCR assays. Methods 2001;25(4):430–42.
[15] Ruijter JM, Pfaffl MW, Zhao S, Spiess AN, Boggy G, Blom J, et al. Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. Methods 2013;59(1):32–46.
[16] Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. Biotechniques 2005;39(1):75–85.
[17] Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6(10):986–94.
[18] Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96(16):9236–41.
[19] Baker M. Digital PCR hits its stride. Nat Methods 2012;9(6):541–4.
[20] Kalinina O, Lebedeva I, Brown J, Silver J. Nanoliter scale PCR with TaqMan detection. Nucleic Acids Res 1997;25(10):1999–2004.
[21] White RA, Blainey PC, Fan HC, Quake SR. Digital PCR. provides sensitive and absolute calibration for high throughput sequencing. BMC Genomics 2009;10:116.
[22] Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 2004;15(3):155–66.
[23] Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin Chem 2013;59(6):892–902.
[24] Marx V. PCR:paths to sensitivity. Nat Methods 2014;11(3):241–5.
[25] Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 2013;10(10):1003–5.
[26] Hellemans J, Mortier G, de Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007;8(2):R19.
[27] Cikos S, Bukovská A, Koppel J. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 2007;8:113.
[28] Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet 2013;54(4):391–406.
[29] D'haene B, Mestdagh P, Hellemans J, Vandesompele J. miRNA expression profiling: from reference genes to global mean normalization. Methods Mol Biol 2012;822:261–72.
[30] Mestdagh P, van Vlierberghe P, de Weer A, Muth D, Westermann F, Speleman F, et al. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 2009;10(6):R64.
[31] Nordgård O, Kvaløy JT, Farmen RK, Heikkilä R. Error propagation in relative real-time reverse transcription polymerase chain reaction quantification models: the balance between accuracy and precision. Anal Biochem 2006;356(2):182–93.
[32] Logan J, Edwards K, Saunders NA. Real-time PCR: current technology and applications. Horizon Scientific Press; 2009.
[33] Rödiger S, Schierack P, Böhm A, Nitschke J, Berger I, Frömmel U, et al. A highly versatile microscope imaging technology platform for the multiplex real-time detection of biomolecules and autoimmune antibodies. Adv Biochem Eng Biotechnol 2013;133:35–74.
[34] Rödiger S, Burdukiewicz M. chipPCR: toolkit of helper functions to pre-process amplification data; 2013. Available from: URL: http://cran.r-project.org/web/packages/chipPCR/index.html [accessed 08.04.14].
[35] Boyer TC, Hanson T, Singer RS. Estimation of low quantity genes: a hierarchical model for analyzing censored quantitative real-time PCR data. PLOS ONE 2013;8(5):e64900.
[36] McCall MN, McMurray HR, Land H, Almudevar A. On non-detects in qPCR data. Bioinformatics 2014.
[37] Sørby LA, Andersen SN, Bukholm IR, Jacobsen MB. Evaluation of suitable reference genes for normalization of real-time reverse transcription PCR analysis in colon cancer. J Exp Clin Cancer Res 2010;29:144.
[38] Udvardi MK, Czechowski T, Scheible W. Eleven golden rules of quantitative RT-PCR. Plant Cell 2008;20(7):1736–7.
[39] Bustin SA. Why the need for qPCR publication guidelines? The case for MIQE. Methods 2010;50(4):217–26.
[40] Rieu I, Powers SJ. Real-time quantitative RT-PCR: design, calculations, and statistics. Plant Cell 2009;21(4):1031–3.
[41] Schmittgen TD, Lee EJ, Jiang J. High-throughput real-time PCR. Methods Mol Biol 2008;429:89–98.
[42] Spitzer M, Wildenhain J, Rappsilber J, Tyers M. BoxPlotR: a web tool for generation of box plots. Nat Methods 2014;11(2):121–2.
[43] Krzywinski M, Altman N. Points of significance: visualizing samples with box plots. Nat Methods 2014;11(2):119–20.
[44] Maurin M. Real-time PCR as a diagnostic tool for bacterial diseases. Expert Rev Mol Diagn 2012;12(7):731–54.
[45] VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 2008;44(5):619–26.
[46] Devonshire AS, Sanders R, Wilkes TM, Taylor MS, Foy CA, Huggett JF. Application of next generation qPCR and sequencing platforms to mRNA biomarker analysis. Methods 2013;59(1):89–100.
[47] Riedmaier I, Pfaffl MW. Transcriptional biomarkers – high throughput screening, quantitative verification, and bioinformatical validation methods. Methods 2013;59(1):3–9.
[48] Ståhlberg A, Zoric N, Aman P, Kubista M. Quantitative real-time PCR for cancer detection: the lymphoma case. Expert Rev Mol Diagn 2005;5(2):221–30.
[49] Liu Y, Gibson J, Wheeler J, Kwee LC, Santiago-Turla CM, Akafo SK, et al. GALC deletions increase the risk of primary open-angle glaucoma: the role of Mendelian variants in complex disease. PLoS ONE 2011;6(11):e27134.
[50] Kim VN, Nam J. Genomics of microRNA. Trends Genet 2006;22(3):165–73.
[51] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008;18(10):997–1006.
[52] Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton TS, et al. Real-time PCR quantification of precursor and mature microRNA. Methods 2008;44(1):31–8.
[53] Yoshizawa JM, Wong DT. Salivary microRNAs and oral cancer detection. Methods Mol Biol 2013;936:313–24.
[54] Leuenberger N, Schumacher YO, Pradervand S, Sander T, Saugy M, Pottgiesser T. Circulating microRNAs as biomarkers for detection of autologous blood transfusion. PLOS ONE 2013;8(6):e66309.
[55] Siegismund CS, Rohde M, Kühl U, Lassner D. Multiparametric diagnostics of cardiomyopathies by microRNA signatures. Microchim Acta 2014:1–7. Available from: URL: http://link.springer.com/article/10.1007/s00604-014-1249-y [cited 08.04.14].
[56] Almal SH, Padh H. Implications of gene copy-number variation in health and diseases. J Hum Genet 2012;57(1):6–13.
[57] Ma L, Chung WK. Quantitative analysis of copy number variants based on real-time LightCycler PCR. Curr Protoc Hum Genet 2014;80(7):21.1–8.
[58] Weaver S, Dube S, Mir A, Qin J, Sun G, Ramakrishnan R, et al. Taking qPCR to a higher level: analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. Methods 2010;50(4):271–6.
[59] Yuan JS, Burris J, Stewart NR, Mentewab A, Stewart CN. Statistical tools for transgene copy number estimation based on real-time PCR. BMC Bioinformatics 2007;8(Suppl. 7):S6.
[60] D'haene B, Vandesompele J, Hellemans J. Accurate and objective copy number profiling using real-time quantitative PCR. Methods 2010;50(4):262–70.

[61] Gibson NJ. The use of real-time PCR methods in DNA sequence variation analysis. Clin Chim Acta 2006;363(1–2):32–47.

[62] Wiita AP, Schrijver I. Clinical application of high throughput molecular screening techniques for pharmacogenomics. Pharmgenom Pers Med 2011;4:109–21.

[63] Boggy GJ, Woolf PJ. A mechanistic model of PCR for accurate quantification of quantitative PCR data. PLoS ONE 2010;5(8):e12355.

[64] Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5(10):R80.

[65] Valero-Mora PM, Ledesma R. Graphical user interfaces for R. J Stat Softw 2012;49(1):1–8. Available from: URL: http://www.jstatsoft.org/v49/i01

[66] Rödiger S, Friedrichsmeier T, Kapat P, Michalke M. RKWard: a comprehensive graphical user interface and integrated development environment for statistical analysis with R. J Stat Softw 2012;49(9):1–34. Available from: URL: http://www.jstatsoft.org/v49/i09

[67] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.

[68] RStudio Inc. shiny: web application framework for R; 2014. Available from: URL: http://cran.rproject.org/web/packages/shiny/index.html [cited 08.04.14].

[69] Rödiger S, Böhm A, Nitschke J, Schierack P, Lehmann W, Schimke I, et al. Analysis of data from experimental qPCR systems with RKWard. qPCR & NGS 2013 Poster Presentations; 2013. Available from: URL: http://www.gene-quantification.de/qpcr-ngs-2013/posters/P088-qPCR-NGS-2013.pdf [cited 08.04.14].

[70] Roediger S, Bohm A, Schimke I. Surface melting curve analysis with R. R J 2013;5(2):37–53. Available from: URL: http://journal.r-project.org/archive/2013-2/roediger-bohmschimke.pdf

[71] Rutledge RG, Stewart D. A kinetic-based sigmoidal model for the polymerase chain reaction and its application to high-capacity absolute quantitative real-time PCR. BMC Biotechnol 2008;8:47.

[72] Karlen Y, McNair A, Perseguers S, Mazza C, Mermod N. Statistical significance of quantitative PCR. BMC Bioinformatics 2007;8:131.

[73] Perkins JR, Dawes JM, McMahon SB, Bennett DLH, Orengo C, Kohl M. ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. BMC Genomics 2012;13:296.

[74] Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, et al. The need for transparency and good practices in the qPCR literature. Nat Methods 2013;10(11):1063–7.

[75] Peng RD. Reproducible research in computational science. Science 2011;334(6060):1226–7.

[76] CAmpER. Available from: URL: http://camper.cebitec.uni-bielefeld.de/ [cited 2014].

[77] Blom J, Rückert C, Kalinowski J, Goesmann A. CAmpER – a software for the calculation of amplification efficiencies for real-time PCR-experiments; 2007. Available from: URL: http://camper.cebitec.uni-bielefeld.de/ [cited 03.03.14].

[78] CopyCaller. Available from: URL: http://www.appliedbiosystems.com/absite/us/en/home/support/software/real-timepcr/copycaller.html [cited 2014].

[79] Applied Biosystems. CopyCaller® Software v2.0. Available from: URL: http://www.appliedbiosystems.com/absite/us/en/home/support/software/real-timepcr/copycaller.html [cited 02.03.14].

[80] Cy0 Method. Available from: URL: http://www.cy0method.org/ [cited 2014].

[81] Guescini M, Sisti D, Rocchi MBL, Stocchi L, Stocchi V. A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. BMC Bioinformatics 2008;9:326.

[82] Guescini M, Sisti D, Rocchi MBL, Panebianco R, Tibollo P, Stocchi V, et al. Accurate and precise DNA quantification in the presence of different amplification efficiencies using an improved Cy0 method. PLOS ONE 2013;8(7):e68481.

[83] DART-PCR. Available from: URL: http://www.genequantification.de/download.html#dart [cited 2014].

[84] Peirson SN, Butler JN, Foster RG. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 2003;31(14):e73.

[85] ddCt. Available from: URL: http://www.bioconductor.org/packages/release/bioc/html/ddCt.html [cited 2014].

[86] Zhang JD, Ruschhaupt M, Biczok R. ddCt method for qRT-PCR data analysis; 2013.

[87] Deconvolution. Available from: URL: http://www.biomedcentral.com/1471-2199/11/30 [cited 2014].

[88] Hirakawa Y, Medh RD, Metzenberg S. Quantitative polymerase chain reaction analysis by deconvolution of internal standard. BMC Mol Biol 2010;11:30.

[89] dPCR. Available from: URL: http://cran.r-project.org/web/packages/dpcR/ [cited 2014].

[90] Burdukiewicz M, Roediger S. dpcR: digital PCR analysis; 2013. Available from: URL: http://cran.r-project.org/web/packages/dpcR/ [cited 05.03.14].

[91] EasyqpcR. Available from: URL: http://www.bioconductor.org/packages/release/bioc/html/EasyqpcR.html [cited 2014].

[92] Le Pape S. EasyqpcR for low-throughput real-time quantitative PCR data analysis. Available from: URL: http://www.bioconductor.org/packages/release/bioc/html/EasyqpcR.html [cited 03.03.14].

[93] FPK-PCR. Available from: URL: http://www.gene-quantification.de/lievens-et-alefficiency-nar-2011.pdf [cited 2014].

[94] Lievens A, van Aelst S, Van den Bulcke M, Goetghebeur E. Enhanced analysis of real-time PCR data by using a variable efficiency model: FPK-PCR. Nucleic Acids Res 2012;40(2):e10.

[95] HTqPCR. Available from: URL: http://www.bioconductor.org/packages/release/bioc/html/HTqPCR.html [cited 2014].

[96] Dvinge H, Bertone P. HTqPCR: high-throughput analysis and visualization of quantitative real-time PCR data in R. Bioinformatics 2009;25(24):3325–6.

[97] LinRegPCR. Available from: URL: http://linregpcr.nl [cited 2014].

[98] Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 2003;339(1):62–6.

[99] LRE Analysis. Available from: URL: http://www.mathworks.com/matlabcentral/fileexchange/35174-lre-analysis-of-real-time-pcrdata [cited 2014].

[100] Leigh D. LRE analysis of real time PCR data. Available from: URL: http://www.mathworks.com/matlabcentral/fileexchange/35174-lre-analysis-of-real-time-pcrdata [cited 03.03.14].

[101] LRE Analyzer. Available from: URL: https://sites.google.com/site/lreqpcr [cited 2014].

[102] Rutledge RG. A Java program for LRE-based real-time qPCR that enables large-scale absolute quantification. PLoS ONE 2011;6(3):e17636.

[103] MAKERGAUL. Available from: URL: http://www.sciencedirect.com/science/article/pii/S0009912013004918#gr3 [cited 2014].

[104] Bultmann CA, Weiskirchen R. MAKERGAUL: an innovative MAK2-based model and software for real-time PCR quantification. Clin Biochem 2014;47(1–2):117–22.

[105] NormqPCR. Available from: URL: http://www.bioconductor.org/packages/release/bioc/html/NormqPCR.html [cited 2014].

[106] PCR-Miner. Available from: URL: http://www.miner.ewindup.info/ [cited 2014].

[107] Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 2005;12(8):1047–64.

[108] pyQPCR. Available from: URL: http://pyqpcr.sourceforge.net/ [cited 2014].

[109] Thomas G, Magali H. pyQPCR; 2014. Available from: URL: http://pyqpcr.sourceforge.net/ [cited 03.03.14].

[110] qBase. Available from: URL: http://www.gene-quantification.de/hellemans-qbase-2007.pdf [cited 2014].

[111] qCalculator. Available from: URL: http://www.genequantification.de/download.html#qcalculator [cited 2014].

[112] Gilsbach R, Kouta M, Bönisch H, Brüss M. Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. Biotechniques 2006;40(2):173–7.

[113] QPCR. Available from: URL: http://www.icbi.at/qpcr [cited 2014].

[114] Pabinger S, Thallinger GG, Snajder R, Eichhorn H, Rader R, Trajanoski Z. QPCR: application for real-time PCR data management and analysis. BMC Bioinformatics 2009;10:268.

[115] qpcR library. Available from: URL: http://www.dr-spiess.de/qpcR.html [cited 2014].

[116] Ritz C, Spiess A. qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. Bioinformatics 2008;24(13):1549–51.

[117] qPCR-DAMS. Available from: URL: http://www.genequantification.de/download.html#qpcrdams [cited 2014].

[118] Jin N, He K, Liu L. qPCR-DAMS: a database tool to analyze, manage, and store both relative and absolute quantitative real-time PCR data. Physiol Genomics 2006;25(3):525–7.

[119] qpcrNorm. Available from: URL: http://www.bioconductor.org/packages/release/bioc/html/qpcrNorm.html [cited 2014].

[120] Mar JC, Kimura Y, Schroder K, Irvine KM, Hayashizaki Y, Suzuki H, et al. Data-driven normalization strategies for high-throughput quantitative RT-PCR. BMC Bioinformatics 2009;10:110.

[121] REST. Available from: URL: http://rest.gene-quantification.info/ [cited 2014].

[122] Pfaffl MW, Horgan GW, Dempfle L. Relative Expression Software Tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30(9):e36.

[123] SARS. Available from: URL: http://people.tamu.edu/~syuan/sars/ [cited 2014].

[124] Yuan JS, Reed A, Chen F, Stewart CN. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006;7:85.

[125] SASqPCR. Available from: URL: https://code.google.com/p/sasqpcr/ [cited 2014].

[126] Ling D. SASqPCR: robust and rapid analysis of RT-qPCR data in SAS. PLOS ONE 2012;7(1):e29788.