Documentation
User Guide

Version 0.1.3

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Contents

1 About

qRAT is a R based standalone desktop application to automate the processing of raw Quantification Cycle (Cq) data files exported from virtually any qPCR instrument using well established and state-of-the-art statistical and graphical techniques. The purpose of this tool is to provide a comprehensive, straightforward, and easy-to-use solution for the **relative quantification** of RT-qPCR data that requires no programming knowledge or additional software installation

The current implementation allows Δ**Cq** calculation (relative to endogenous control(s)), $\Delta\Delta$**Cq** calculation (relative to endogenous control(s) and a reference sample) and **inter-plate variation correction**. Moreover, functionalities for **parsing**, **filtering** and **visualisation** of relative RT-qPCR data are included.

2 How to run qRAT?

The standalone version of qRAT is available for **Windows**. Download the latest version from the qRAT Homepage[1], double click on the downloaded installer and follow the further instructions of the installation program. If not already installed, a suitable version of R will be co-installed as part of the installation procedure of qRAT.

The application can be launched from the **Start menu** on the Windows desktop. Navigate to the **Programs** folder and search for qRAT. Alternatively, you may click on the **qRAT icon** on the Windows desktop or the file **qRAT.exe** inside the applications folder. To remove qRAT, use the **uninstaller** available in the **Start menu**.

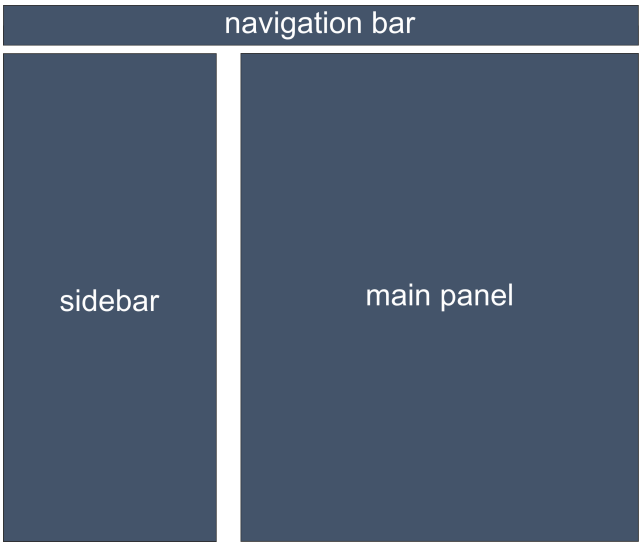
A standalone version of qRAT is not yet available for **MacOS and Linux**. Users can run the app using R console and RStudio following the steps on GitHub[2].

[1] https://uibk.ac.at/microbiology/services/qRAT.html
[2] https://github.com/DaniFlat/qRAT

3 Application Layout

The main windows of qRAT consists of 3 components:

1. Top navigation bar: allows users to browse to the information they want to open in the main panel.
2. Sidebar: elements in the sidebar allow users to set data input and different analysis settings. Sidebar elements change according to selection in main panel.
3. Main panel: allows users to navigate through tabs containing analysis outputs



4 General Data Input

qRAT processes **quantification cycle (Cq) values** measured by a qPCR instrument and exported by that instrument's software. Depending on the instrument software that generates the data, Cq values may have alternative names such as Ct, Cp, or TOP. Cq is the official abbreviation in the Real-time PCR Data Markup Language[3] which is considered as the universal data standard for exchanging quantitative PCR (qPCR) data. To start any qRAT execution, users must import **txt or csv** files containing Cq values for every well. Files can be **tab-, comma-, or semicolon**-delimited. The data import is made to be flexible and is capable of reading export files generated by most qPCR instrument software programs due to its ability to recognize synonymous column names. The import procedure does not alter the original data file.

A list of **supported qPCR instruments** and software programs is included in section *Supported Import Formats*. However, if your instrument or software is **not listed** or you experience **problems importing the data** into qRAT, please try renaming the columns in your exported file to **match the format** you see in section *Data Format*.

5 Data Format

Some qPCR machines add a header in the first rows of the file, qRAT automatically discards these header rows before parsing the content of the files. For this reason, the first column of the columns row must start by the word **"Well"**. Further requirements are that the columns **"Sample"**, **"Gene"** and **"Cq"** are present. To prevent possible errors, **avoid** using **space** and other **special characters** in any sample or column names. To ensure correct calculations, the **number of technical replications must be identical** across all samples and technical replicates of the same biological sample must have **exactly the same name**. Empty wells/blank rows (without sample name or gene target) are automatically removed. This is represented in the table below:

[3] https://rdml.org/

Well	Sample	Gene	Cq
A1	Sample1	Target1	22.2
A2	Sample1	Target1	21.6
A3	Sample1	Target1	21.2
B1	Sample1	Target2	33.3
B2	Sample1	Target2	29.9
B3	Sample1	Target2	30.1
C1	Sample2	Target1	24.1
C2	Sample2	Target1	23.8
C3	Sample2	Target1	24.3
D1	Sample2	Target2	26.2
D2	Sample2	Target2	26.6
D3	Sample2	Target2	26.4

In case your samples are spread across multiple plates, you may want to make use of inter-plate calibration. For this, the **calibrator must have exactly the same name** on all plates:

Well	Sample	Gene	Cq
A1	Sample1	Target1	22.2
A2	Sample1	Target1	21.6
A3	Sample1	Target1	21.2
B1	Sample1	Target2	33.3
B2	Sample1	Target2	29.9
B3	Sample1	Target2	30.1
C1	Sample2	Target1	24.1
C2	Sample2	Target1	23.8
C3	Sample2	Target1	24.3
D1	Sample2	Target2	26.2
D2	Sample2	Target2	26.6
D3	Sample2	Target2	26.4
E1	IPC	Target3	23.6
E2	IPC	Target3	23.4
E3	IPC	Target3	23.6

6 Supported Instruments

For most users there is no need to modify the data file as qRAT supports **exported files** from the majority of the real-time PCR instruments and accompanying data analysis software as long as they are **tab-, comma-, or semicolon**-delimited **csv or txt** files. See section *General Data Input* for more details. If your instrument or analysis software is not supported, you are advised to **modify your exported txt or csv file** so it matches the required data format as described in section *Data Format*. To prevent possible errors, **avoid** using **space** and other **special characters** in any sample or gene name. To ensure correct calculations, the **number of technical replications must be identical** across all samples and technical replicates of the same biological sample must have **exactly the same name**. Empty wells/blank rows (without sample name or gene target) are automatically removed. For *Multiple Plates Analysis* the **inter-plate calibrator** must have exactly the **same name** on all plates. **Instruments/Software:** qRAT was successfully tested with *txt/csv* files from Analytik Jena (qTower 3G), Wafergene (SmartCycler), Applied Biosystems (5700, 7000, 7300, 7500, 7900 SDS 2.4, StepOne(Plus), Viia7)

7 How to analyse data

Here we show the steps to perform a simple analysis with qRAT:

1. Choose your plate setup in top navigation bar: **Single Plate, Multiple Plates**

2. Use the **sidebar** (Data Input, Analysis Input) from top to bottom for all user inputs

   (a) Start with **Data Input** (Single Plate or Multiple Plates)

   (b) Choose the correct **formatting** of your data file (comma, tab, semicolon)

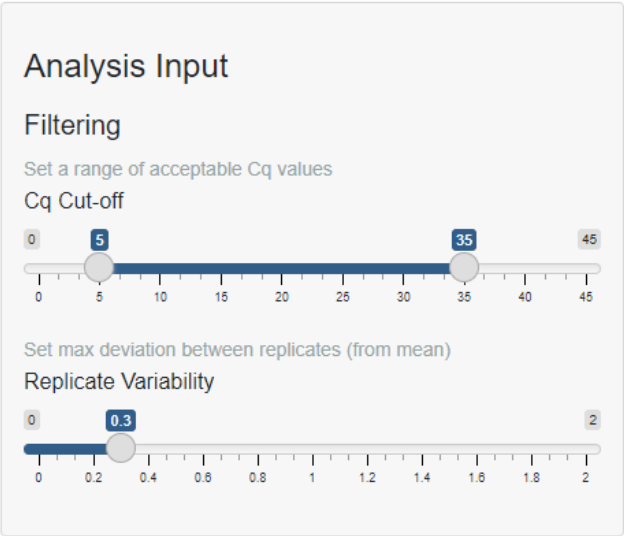
   (c) Upload the file to analyse (in Multiple Plates mode choose multiple files at once holding the [Ctrl] key). Wait until upload is complete (**main panel** will automatically update with uploaded data)

3. Navigate through the tabs in the **main panel** to show respective **Analysis Input** options and data outputs. All tables and plots are reactive and will automatically update on changes in Analysis Input.

(a) **Raw Data:** Shows the raw data input and helps to get a general overview across all samples. Spatial layout plot is inspired on the physical design of x-well plates and helps to find spatial patterns before starting to filter data.

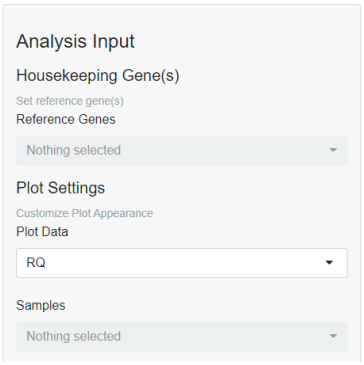
After visualizing raw data, the next step in the workflow is data filtering and quality control.

(b) **Filtering and Quality:** Analysis Input elements allow filtering of the data. Main panel shows a table of replicates that will be excluded from the analysis according to the analysis input. See chapter *Data Quality and Filtering* for more details.

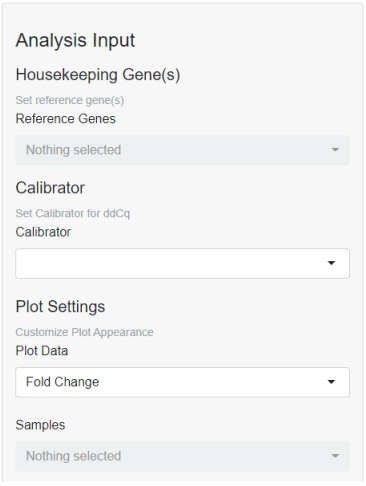


If you're happy with the data quality proceed with relative expression analysis (ΔCq, ΔΔCq).

(c) **Relative dCq:** Analysis Input elements allow the user to set reference/housekeeping gene(s) and change the settings of the plot appearance. The genes which are available for selection are displayed in a drop-down list. Main panel shows plot and table output of the ΔCq results.



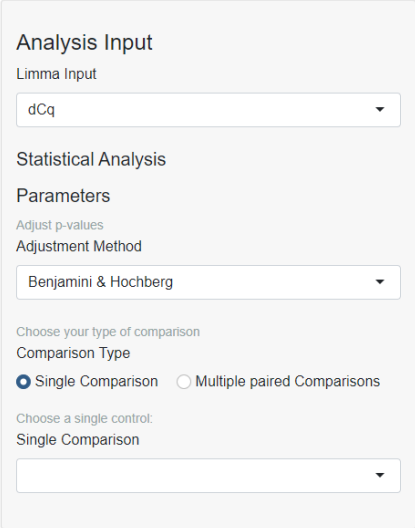
(d) **Relative ddCq:** Analysis Input elements allow the user to set reference/housekeeping gene(s) as well as a calibrator sample and to change the settings of the plot appearance. The genes and samples which are available for selection are displayed in drop-down lists. By setting a calibrator sample the relative expression levels of all the other samples are given in relation to the calibrator sample. Main panel shows plot and table output of the ΔΔCq results.



Assuming normally distributed Cq values and equal variance across sample groups being compared, fold-change significance can be assessed using methods from the limma package[4].

(e) **Statistical Analysis:** Either two or multiple groups of samples can be compared. Results comprise values of t statistics (t.test), significance of the difference (p.value), p-value adjusted by Benjamini and Hochberg's method (adj.p.value) and asterisks indicating level of statistical significance (significance).

Symbol	Meaning
-	p 0.05
*	$p \leq 0.05$
**	$p \leq 0.01$
***	$p \leq 0.001$
****	$p \leq 0.0001$



[4] https://doi.org/doi:10.18129/B9.bioc.limma

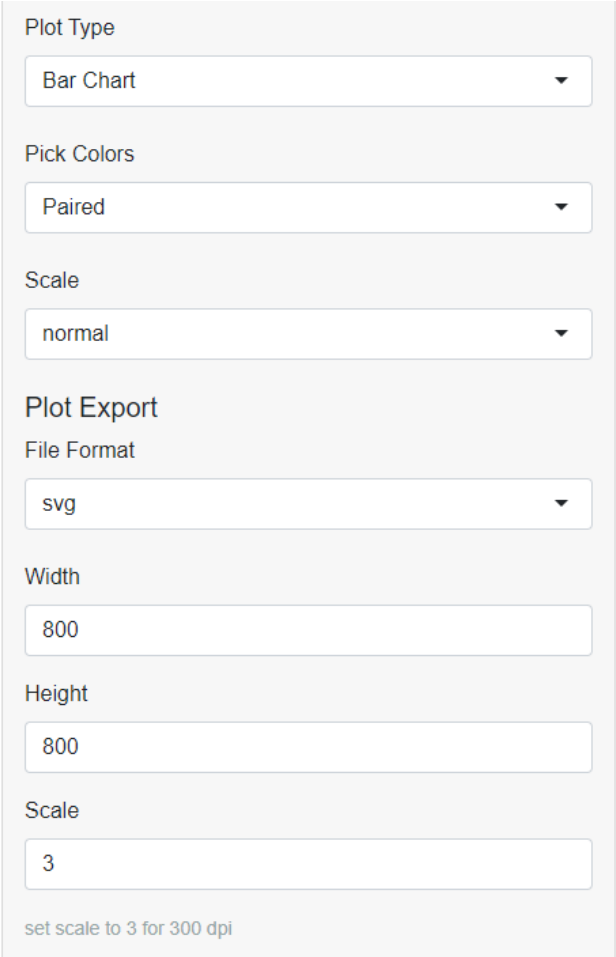
8 Results (Tables and Plots)

qRAT recalculates everything immediately (e.g. intermediate and final results, quality controls, and specific analyses) as soon as something changes in the input or analysis settings, meaning tables and plots will **automatically update** on user input.

Tables: All tables include a header with **data export** functions which copy the table data to the clipboard or exports the table data into multiple formats such as PDF or CSV that can be included as supplemental data in publications. Result tables of ΔCq and ΔΔCq analysis contain all calculated values for each gene/sample combination, e.g. ΔCq, standard errors and relative quantification (RQ) or ΔΔCq, standard errors and fold change (FC) respectively. Values in tables are rounded to two digits.

Plots: All plots are fully customizable. Changes **only affect the visual appearance** of the plot; the calculations remain the same. Plot title, axes title, legend can be edited by clicking on the respective element in the plot. Legend can be placed by drag and drop. By default, qRAT plots all genes and samples.
Genes you want to plot across all samples can be included/excluded by clicking on their symbols in the legend.
Samples you want to plot across all genes can be included/excluded via the drop-down list in the Plot Settings section.
Every plot includes a menu in the top right corner with various control options (**download plot**, zoom in, auto-scale, reset axes, etc). Clicking on the *Download Plot* icon should prompt the application to download a static file (**svg**, **png**, **jpeg** or **webp**) according to your image export settings. Images are exported with resolution of 300 dpi.
Additional parameters (**Plot Settings**) include: **Plot Data**, **Plot Type**, **change colours**, **change scale to log** and specifying **image export size and format**.
Plot Data: Specify the output data you want to plot. In ΔCq analysis you can

choose between ΔCq or RQ (relative quantity). In ΔΔCq analysis you can choose between ΔΔCq or FC (fold change).
Plot Type: Choose between bar chart and dot plot.
Colours: Specify color sets you want to use in order to color the individual bars or points. For additional information have a look at the description of the 'RColorBrewer'[5] package.



[5] https://cran.r-project.org/web/packages/RColorBrewer/index.html

9 Data Quality and Filtering

Quality control is an important aspect in qPCR data analysis. qRATs quality control parameters include a threshold for maximum replicate variability and a range of acceptable Cq values (Cq Cut-off). These quality control parameters set the criteria for automatic exclusion of certain data points. Data points that have been excluded are shown in a table in the Filtering and Quality window and will not be used for calculations. If more stringent quality control settings are used (lower replicate variability value and smaller Cq cut-off range), more replicates will fail the quality control. According to the MIQE Guidelines[6] the number of technical replicates should be $n >= 2$. In case the number of technical replicates for a given sample is below $n <= 2$, a warning message will appear in the main panel.

Replicate Variability: qRAT automatically deals with technical replicates, which are recognized as different PCR wells with an identical sample and gene name. The standard method of removing outliers from technical replicates is to remove the replicate most different from the mean. To detect outliers, the standard deviation of the technical replicates for a given sample is calculated. When the standard deviation value is above the set threshold, the divergent value will be removed. The standard threshold is set to 0.3, as suggested by the MIQE Guidelines[7].

Cq Cut-off: This defines lower and upper boundaries of a range of acceptable Cq values. Any Cq values outside the defined range will be treated as NA. The default range includes any meaningful Cq value, i.e. Cq between 5 and 35.

[6] https://rdml.org/miqe.html
[7] https://rdml.org/miqe.html

10 Computation of Relative Quantities

Relative quantities are calculated with the ddCt R package[8], which implements an improved ddCt method that requires no standard curve for each primer-target pair. For the ddCT calculation to be valid, the **amplification efficiencies** of the target and reference must be **approximately equal** and **close to 1 (100 %)**. If more than one reference gene is specified, the algorithm will use the **mean of the Cq values** of the reference genes for normalization. If more than one calibrator sample is specified, the algorithm uses the **mean of the chosen calibrator samples** as the reference line. In qRAT relative quantification is expressed either as $RQ = 2^{-\Delta Cq}$, where samples are normalized to one or multiple reference gene(s), or $FC = 2^{-\Delta\Delta Cq}$), where samples are normalized to one or multiple reference gene(s) and made relative to a given sample which is considered as a calibrator for the unknown samples. The **stability of all appointed reference genes** needs to be validated in advance. Popular algorithms to determine the most stable reference (housekeeping) genes from a set of candidate reference are geNorm, BestKeeper and NormFinder. On average 2-4 reference genes should ideally be used for final normalization in a given experiment.

11 Inter-Plate Calibration

Inter-plate calibration (IPC) is a calculation procedure to detect and remove (often underestimated) inter-plate variation. These calculations are typically needed whenever samples need to be compared that are measured in different runs. The general advice is to follow the "sample maximization" method whenever possible, stating that all samples should be analysed for a given gene in the same run. This sample maximization strategy does not suffer from inter-plate variation between samples (as all samples are measured in the same run for a particular gene), and therefore does not require inter-plate calibration to be performed. In addition, there is no need for the reference gene(s) to be measured in the same plate as the gene of interest, as the refer-

[8] https://doi.org/doi:10.18129/B9.bioc.ddCt

ence gene(s) quantify the relative concentration between samples. If "sample maximization" method is not possible, it is recommended that a few samples are repeated in all runs (so-called inter-plate calibrators; IPCs) in order to detect and remove inter-plate variation. To activate the inter-plate calibration function press *Use Inter-Plate Calibration* in the **Inter-Plate Calibration** tab in the **main panel**. The IPC sample must have the same name on all plates, as qRAT automatically uses the same set of IPCs for all plates based on identical sample and target names. **It is important to note that the inter-plate calibration function should only be used for inter-plate calibration if the same set of IPCs is used on all plates to be calibrated.**

12 Statistics

In general, there are **two approaches** in qRAT for testing the significance of differences in Cq values between samples. Both approaches are based on the framework from the limma package[9]. Either **two** or **multiple groups** of samples can be compared. The basic statistic used for significance analysis is the **moderated t-statistic**, which has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes, i.e., squeezed towards a common value, using a simple **Bayesian model**. The p.Value is the associated **p-value** and adj.P.Value is the **p-value adjusted** by **Benjamini and Hochberg's method** to control the false discovery rate. Additional p-value adjustment methods available are **Bonferroni** and **Holm**. See the limma documentation[10] for more details.

[9] https://doi.org/doi:10.18129/B9.bioc.limma
[10] https://doi.org/doi:10.18129/B9.bioc.limma

13 R Packages and Third-Party Software

qRAT is using various applications, tools and scripts originating from external sources (open-source third party software). Within this section, all tools and packages are listed. Keep in mind that most of the packages may also depend on other packages not listed here. Please follow the web links if you want to get more information on a specific package or tool.

Software	R	4.2.0
	RStudio	2022.02.3
	Electron	18.3.1
R Package	Bslib	0.3.1
	DT	0.23
	HTqPCR	1.50.0
	RInno	1.0.1
	data.table	1.14.2
	ddCt	1.52.0
	dplyr	1.0.9
	ggplot2	3.3.6
	ggpubr	0.4.0
	limma	3.52.1
	plotly	4.10.0
	reshape2	1.4.4
	scales	1.2.0
	shiny	1.7.1
	shinycssloaders	1.0.0
	shinyWidgets	0.7.0
	shinyjs	2.1.0
	thematic	0.1.2.1
	waiter	0.2.5
	xtable	1.8-4

Icons made by Freepik from www.flaticon.com.

14 System Requirements

The standalone version of qRAT supports Windows 7 and later, older operating systems are not supported. MacOS and Linux users need to have the most recent version of R[11] and RStudio[12] installed.

- Free Disk Space: more than 2 GB
- RAM: 1 GB required, 2 GB recommended

[11] https://cran.rstudio.com/
[12] https://www.rstudio.com

15 Release Notes

qRAT 0.1.3

- Public Release