
# qRAT

## Software Documentation & User Guide

Version 0.6.0

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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 **ΔCq** calculation (relative to endogenous control(s)), **ΔΔCq** calculation (relative to endogenous control(s) and a reference sample), **inter-plate variation correction**, and automated **reference gene stability analysis**. Moreover, functionalities for **parsing**, **filtering** and **visualisation** of relative RT-qPCR data are included.

# 2 How to run qRAT?

Since version 0.5, qRAT is no longer available as a standalone installer. You can access the application in two ways:

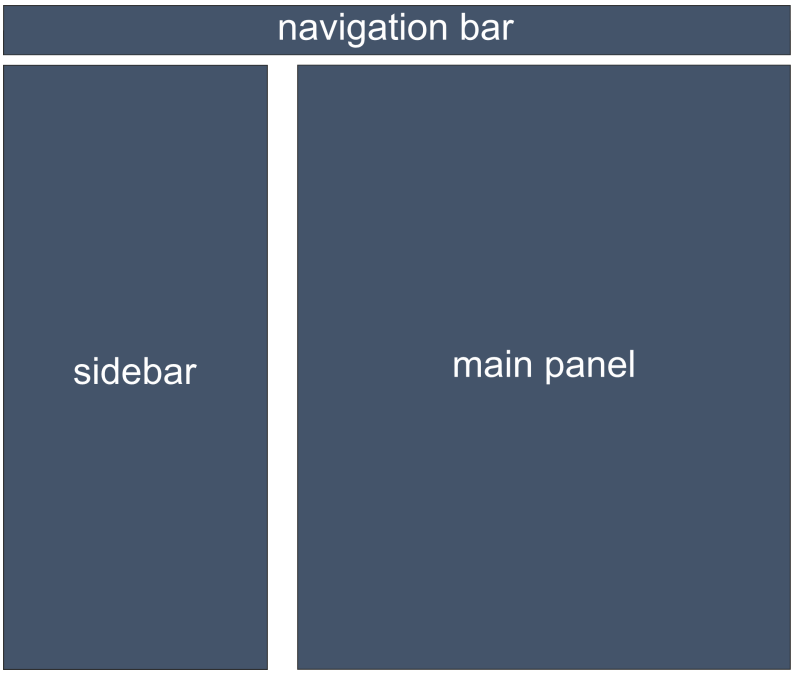
- **Web Application:** Use the hosted version at **qrat.shinyapps.io/qrat/**.
- **Local Installation (via R):** Follow the instructions on the **qRAT GitHub repository**.

To test the application, you can download a **Sample Dataset here**. Additional information and updates can be found on the **qRAT Homepage**.

# 3 Application Layout

The main windows of qRAT consists of 3 components:

1. **Top navigation bar:** Browse between Single Plate, Multiple Plates, and Help sections.
2. **Sidebar:** Dynamic settings for data input, filtering, and plot customization.
3. **Main panel:** Interactive tabs containing tables and plots.




# 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** 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 Instruments*. 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:

| 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*.

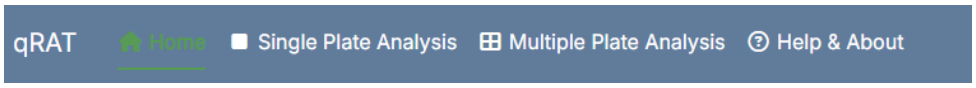
To prevent possible errors, **avoid** using **space** and other **special characters** in any sample or gene name. To ensure correct calculations, the **number of technical replications must be identical** across all samples and technical replicates of the same biological sample must have **exactly the 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

This section outlines the standard workflow for performing a relative quantification analysis in qRAT. The application is designed to guide the user from raw data import to statistical validation.

1. **Plate Setup Selection:** Begin by choosing your experimental setup in the top navigation bar: **Single Plate** for data within one file, or **Multiple Plates** for experiments spanning several runs.




2. **Sidebar Navigation (Inputs):** Use the **sidebar** (Data Input, Analysis Input) to configure your analysis. It is recommended to proceed from top to bottom:
   (a) **Data Input:** Select your plate mode.
   (b) **Formatting:** Choose the delimiter of your file (Auto, Comma, Tab, Semicolon). "Auto" is generally recommended as it detects the format automatically.
   (c) **Upload:** Upload your file(s). In "Multiple Plates" mode, select all relevant files simultaneously (e.g., by holding the [Ctrl] or [Cmd] key). The **main panel** will update as soon as the upload is complete.
3. **Analysis Workflow (Main Panel):** Navigate through the tabs to refine your analysis. All calculations are reactive and update instantly.
   (a) **Raw Data:** Provides an initial overview. The spatial layout plot helps identify plate-wide patterns or pipetting artifacts before filtering.
   (b) **Reference Finder:** If you are unsure which endogenous controls are the most stable, use this tab. It employs the **GeNorm algorithm** to rank your genes. Stable genes identified here should be selected in the subsequent analysis steps.
   (c) **Filtering and Quality:** Define your quality criteria. You can toggle between **Automatic Filtering** (SD and Cq cut-offs) and **Manual Filtering**. In the interactive table, individual wells can be excluded as outliers by simply clicking on them.

(d) **Grouping (Biological Replicates):** If your samples represent biological replicates, use this tab to assign them to common group names (e.g., "WT" or "Mutant"). This is crucial for valid statistical testing based on biological variance.

4. **Relative Expression Analysis:** Once the data is cleaned and grouped, proceed to quantification:

   (a) **Relative dCq (ΔCq):** Select your reference gene(s). The main panel displays the ΔCq values and Relative Quantities ($RQ = 2^{-\Delta Cq}$).

   (b) **Relative ddCq (ΔΔCq):** Select both reference gene(s) and a **calibrator sample** (control). This normalizes the data relative to the control group, providing Fold Change ($FC = 2^{-\Delta\Delta Cq}$) results.

5. **Statistical Validation:** qRAT utilizes the *limma* package for robust statistical analysis. You can compare two or multiple groups.

   (a) **Test Selection:** Choose between Moderated t-statistics or ANOVA-style comparisons.

   (b) **P-Value Adjustment:** To control the false discovery rate in multiple testing, select an adjustment method (Benjamini-Hochberg, Bonferroni, or Holm).

   (c) **Significance Levels:** Results are presented with t-statistics, p-values, and significance asterisks.

Table 1: Significance levels used in qRAT.

| Symbol | P-Value Range |
|---|---|
| ns | $p > 0.05$ |
| * | $p \leq 0.05$ |
| ** | $p \leq 0.01$ |
| *** | $p \leq 0.001$ |
| **** | $p \leq 0.0001$ |

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

# 8 Results (Tables and Plots)

qRAT is built on a reactive framework, meaning it recalculates all intermediate and final results (quality controls, normalization, and statistics) instantaneously. As soon as a parameter in the sidebar is modified, all tables and plots will **automatically update** to reflect the current analysis settings.

**Tables:** All data tables are equipped with an interactive header providing **data export** functions. These allow users to copy data to the clipboard or export it into various formats such as CSV, Excel, or PDF, making it easy to include raw or processed results as supplemental data in publications. Result tables for ΔCq and ΔΔCq analysis provide a comprehensive breakdown of every gene/sample combination. This includes calculated ΔCq/ΔΔCq values, standard errors (SD or SEM), and the resulting relative quantification (RQ) or fold change (FC). To ensure readability, values in the tables are rounded to two decimal places.


Figure 1: Interactive plot menu and customization options.

**Plots:** All visualizations in qRAT are fully customizable and designed for publication-ready output. It is important to note that changes in the appearance settings **only affect the visual representation**, while the underlying statistical calculations remain untouched.

By default, qRAT includes all genes and samples in the plots. However, users can precisely control the content:

- **Gene Filtering:** Select or deselect specific target genes via the "Filter Genes" drop-down menu to focus on relevant results.
- **Sample Selection:** Include or exclude specific samples or groups to simplify the comparison.
- **Sample Ordering:** Using the *Sample Ordering* feature in the Appearance section, users can reorder the samples on the x-axis via drag-and-drop to match the experimental design.

Every plot features an interactive modebar in the top right corner for quick adjustments (zoom, auto-scale, reset axes). For permanent export, qRAT provides a export method:

1. **Advanced Export:** The dedicated *Export Options* section in the sidebar allows for precise control over the output. Users can specify the **file format** (PDF, SVG, PNG), the **image dimensions** (width and height in inches), and the **resolution scale**. This ensures that exported graphics meet the strict requirements of scientific journals.

**Plot Customization (Appearance):** Additional parameters allow for deep customization of the charts:

- **Plot Data & Type:** Choose between plotting ΔCq/ΔΔCq or RQ/FC values, and toggle between bar charts and dot plots.
- **Axes & Labels:** Users can define custom titles for the plot, the legend, and both axes. To accommodate long sample names, the **rotation angle** of the x-axis labels and the overall **font size** can be adjusted via sliders.
- **Error Bars & Scales:** Toggle the display of error bars (SD or SEM) and switch the y-axis scale between linear, log2, or log10.
- **Colors:** A variety of professional color palettes from the **RColorBrewer** package are available to distinguish between samples or groups effectively.

# 9 Data Quality and Filtering

Quality control is a cornerstone of robust qPCR analysis. In the *Filtering and Quality* tab, qRAT combines automated algorithms with manual oversight to ensure data integrity. All excluded data points are summarized in a dedicated table and are omitted from all downstream calculations.

## 9.1 Automatic Filtering

Automated filtering criteria are based on two main parameters: replicate consistency and absolute Cq boundaries.

- **Replicate Variability:** qRAT identifies technical replicates by matching identical sample and gene names. To ensure precision, the standard deviation (SD) of these replicates is calculated. If the SD exceeds the user-defined threshold (default: 0.3, as per **MIQE Guidelines**), the outlier furthest from the mean is automatically removed.
- **Cq Cut-off:** This parameter defines the biological and technical detection limits. Values outside the specified range (default: 5–35) are treated as *NA*. This is essential for removing background noise or late-cycle artifacts.

## 9.2 Manual Filtering and Controls

Beyond automated rules, qRAT provides full control over the dataset through interactive elements.

- **Manual Outlier Selection:** The interactive table in the main panel allows users to manually exclude specific wells by clicking on them. This is intended for removing known technical artifacts (e.g., air bubbles or evaporation) that might not trigger the automated SD filter.
- **Experimental Controls:** Users can designate specific samples as Non-Template Controls (NTC) or Reverse Transcriptase minus (RT-) controls. Selecting these samples in the sidebar excludes them from the relative quantification process to prevent them from biasing the experimental groups.

According to **MIQE Guidelines**, the number of technical replicates should be $n \geq 2$. If filtering reduces the number of replicates for a sample below this limit, qRAT will display a warning message in the main panel to alert the user to the reduced statistical power.

# 10 Reference Gene Selection (Reference Finder)

The stability of reference genes is critical for accurate normalization. qRAT now includes an automated **Reference Finder** tool.

In the *Reference Finder* tab, qRAT implements the **GeNorm** algorithm to rank candidate reference genes based on their expression stability ($M$-value).

- **M-Value:** Measures the average pairwise variation of a gene against all other candidate genes.
- **Selection:** Lower $M$-values indicate higher stability. Genes with $M < 0.5$ in homogeneous samples are generally considered excellent.

Use the generated stability plot to identify the most robust targets before proceeding to ΔCq or ΔΔCq analysis.

# 11 Computation of Relative Quantities

The core of qRAT's analysis engine is the **ddCt R package**. It utilizes an optimized ΔΔCq method that assumes high and comparable amplification efficiencies across all targets, eliminating the need for a standard curve for every run.

## 11.1 Mathematical Framework

For the ΔΔCq method to be statistically valid, the amplification efficiencies of the target and reference primers should be approximately equal and close to 2 (representing 100 % efficiency). Relative quantification in qRAT is expressed in two ways:

- **Relative Quantity (RQ):** Used in ΔCq analysis, where samples are normalized to endogenous controls:

$$RQ = 2^{-\Delta Cq} \tag{1}$$

- **Fold Change (FC):** Used in ΔΔCq analysis, where samples are normalized to endogenous controls and expressed relative to a calibrator sample:

$$FC = 2^{-\Delta\Delta Cq} \tag{2}$$

## 11.2 Normalization with Multiple Targets

To increase the robustness of the results, qRAT supports the use of multiple reference genes and calibrator samples:

- **Reference Genes:** If multiple housekeeping genes are selected, the algorithm calculates the **arithmetic mean of their Cq values** for normalization.
- **Calibrator Samples:** If multiple samples are designated as calibrators, the algorithm uses the **mean of these samples** to establish the reference baseline (reference line).

## 11.3 Reference Gene Stability

The accuracy of the relative quantification depends entirely on the stability of the chosen reference genes. While 2–4 reference genes are generally recommended, their stability must be experimentally verified.

qRAT now features an integrated **Reference Finder** tab based on the **geNorm** algorithm. It is highly recommended to use this tool to rank your candidate genes by their $M$-value stability score before proceeding with the final quantification.

# 12 Biological Replicates and Grouping

In many experiments, multiple biological samples receive the same treatment. To analyze these correctly as one experimental group, qRAT provides a specialized **Grouping** tab. This allows users to aggregate individual biological replicates into a single group for statistical comparison and plotting.

**Example Assignment:** Consider an experiment with three control animals and three treated animals. In the input file, each biological sample must have a unique name. In the qRAT Grouping tab, they are assigned as follows:

By defining these groups, qRAT will:

- **Aggregate Data:** Calculate the mean and standard error based on the $n = 3$ biological replicates per group.
- **Statistical Power:** Perform moderated t-statistics based on the variance between the groups rather than between individual technical replicates.
- **Visualisation:** Plots will display the results for "Control" and "Treatment_A" as single entities.

Table 2: Biological Grouping Example

| Sample Name (Input) | Assigned Group (Grouping Tab) | Role |
|---|---|---|
| Sample_1 | Control | Biological Replicate 1 |
| Sample_2 | Control | Biological Replicate 2 |
| Sample_3 | Control | Biological Replicate 3 |
| Sample_4 | Treatment_A | Biological Replicate 1 |
| Sample_5 | Treatment_A | Biological Replicate 2 |
| Sample_6 | Treatment_A | Biological Replicate 3 |

# 13 Inter-Plate Calibration

Inter-plate variation is a common but often underestimated source of error in qPCR experiments spanning multiple runs. qRAT provides a dedicated **Inter-Plate Calibration (IPC)** procedure to detect and remove these technical offsets.

## 13.1 Experimental Strategies

To minimize inter-plate variation, two main strategies are generally recognized:

- **Sample Maximization:** This is the preferred strategy. All samples for a specific gene are analyzed within the same run/plate. Since all comparisons for that gene occur under identical conditions, no inter-plate calibration is required for the samples. In this setup, reference genes do not necessarily need to be on the same plate as the target gene, as they serve to quantify relative concentrations between samples.
- **Inter-Plate Calibrators (IPC):** If sample maximization is not feasible (e.g., due to large sample sizes), a small set of identical samples—the IPCs—must be repeated across all plates. These calibrators allow qRAT to calculate a correction factor to harmonize the data between different runs.

## 13.2 Implementation in qRAT

To activate the correction, navigate to the **Inter-Plate Calibration** tab in the main panel and select *Use Inter-Plate Calibration*.

qRAT identifies IPCs automatically by matching identical **Sample** and **Target** names across different files. The algorithm then calculates the mean difference between the IPCs of each plate to align the Cq levels.

The Inter-Plate Calibration function requires that the IPC samples have **exactly the same name** across all plates. It is only valid if the identical physical material (same cDNA/DNA source) is used as a calibrator on every plate being compared.

# 14 Statistics

qRAT employs a sophisticated statistical framework to evaluate the significance of differences in gene expression between samples or experimental groups. All calculations are powered by the **limma package**, which is specifically designed for high-throughput genomic data.

## 14.1 The Moderated T-Statistic

The core of the analysis is the **moderated t-statistic**. While it is interpreted similarly to a standard Student's t-test, it is much more robust for experiments with small sample sizes. Using a **Bayesian model**, the standard errors are "moderated" across all genes—essentially "squeezing" them towards a common global value. This approach reduces the impact of chance outliers and provides more reliable results when the number of replicates is limited.

## 14.2 Multiple Testing and P-Value Adjustment

When comparing multiple genes or groups simultaneously, the probability of encountering false positives increases. To maintain statistical integrity, qRAT provides several methods to adjust the p-values:

- **Benjamini & Hochberg (FDR):** The default method. It controls the False Discovery Rate, balancing the power to find true differences with the risk of false positives.
- **Bonferroni:** A more conservative approach that strictly controls the family-wise error rate by dividing the alpha level by the number of tests.
- **Holm:** A step-down procedure that is generally more powerful than the Bonferroni method while maintaining similar strictness.

## 14.3 Group Comparisons

Users can choose between comparing exactly **two groups** (e.g., Control vs. Treatment) or performing comparisons across **multiple groups**. The results in the main panel include:

- **p.Value:** The raw, unadjusted p-value.
- **adj.P.Value:** The p-value after applying the selected adjustment method.
- **Significance:** Asterisks indicating the level of statistical significance based on the adjusted p-value.

For valid statistical results, ensure that biological replicates have been correctly assigned in the **Grouping** tab, as this ensures the variance is calculated based on biological rather than technical replicates.

# 15 R Packages and Third-Party Software

qRAT is built using the R statistical programming language and the Shiny web framework. All core qPCR calculations and visualizations rely on high-quality, peer-reviewed Bioconductor and CRAN packages.

Table 3: Software and R Packages

| Category | Software / Package |
|---|---|
| **Core Environment** | R, RStudio |
| **Framework & UI** | Shiny, bslib, shinyjs, waiter, DT, bsicons |
| **qPCR Analysis** | ddCt, HTqPCR, limma |
| **Data Processing** | dplyr, data.table, rlang, tidyr |
| **Visualization** | ggplot2, plotly, ggpubr, scales, RColorBrewer |
| **Reference Finder** | ctrlGene (geNorm algorithm) |

Please note that these packages may have additional dependencies. For detailed documentation on a specific tool, please refer to its respective CRAN or Bioconductor project page.

Icons used in the application are provided by *Font Awesome* and *Freepik* (via www.flaticon.com).

# 16 Release Notes

- **qRAT 0.6.0 (Current)**
  - **Added: Reference Finder** module using the geNorm algorithm ($M$-value stability ranking).
  - **Added:** Manual Filtering via interactive well selection.
  - **Added:** Biological Grouping functionality for replicate aggregation.
  - **Improved:** Full UI migration to Bootstrap 5 using the *bslib* library.
  - **Improved:** Optimized reactivity for data nalysis.
  - **Improved:** Enhanced plot customization (adjustable font sizes, axis titles, and label rotation).
- **qRAT 0.1.0 – 0.5.0**
  - Initial releases: basic ΔΔCq calculation, CSV import, and fundamental plotting.