qPCR Guru

User Guide

Complete reference for the qPCR Guru analysis platform

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ΔΔCq & Pfaffl relative quantification · 5PL Fit Cq (SDM) & Curve Shape QC
MIQE 2.0 compliance with prediction-interval LOD/LOQ · Multi-plate uploads with IPC drift check
geNorm, NormFinder & Global Mean reference normalization · Standard curve diagnostics
Levene-aware ANOVA (Games-Howell when variances unequal) · Publication-ready figure export

Table of Contents

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1. Getting Started

1.1 Supported File Formats

qPCR Guru accepts data exported from virtually any qPCR instrument. Simply export your results to one of the following formats and upload:

Format	Extension	Notes
Microsoft Excel	.xlsx, .xls	Most common; exported from Bio-Rad CFX, Applied Biosystems QuantStudio, Roche LightCycler, etc.
Comma-Separated Values	.csv	Universal text format; works with all instruments
Tab-Separated Values	.tsv	Alternative text format
RDML	.rdml	Standardized qPCR data exchange format (ZIP-wrapped XML)

1.2 Uploading Your Data

From the upload screen, you can drag and drop your file onto the upload area, or click **Browse Files** to select from your computer. qPCR Guru will automatically detect the file format, identify the data sheet, and parse sample names, target names, and Cq values. You can also drop or select multiple files at once for multi-plate experiments — see 1.3.

Tip: If your file contains multiple sheets, qPCR Guru looks for sheets named 'Results', 'Quantification', 'End Point Results', or similar standard names. If no matching sheet is found, it tries each sheet until it finds valid qPCR data.

1.3 Multi-Plate Uploads

When an experiment spans more than one plate, drag-drop or select all the plate files together in a single upload action. qPCR Guru merges them into one analysis automatically: every well retains a reference to its source file, and samples sharing the same name across plates are combined into one replicate group for downstream ΔΔCq, expression, and statistical analyses.

Behind the scenes, the merger uses a **compound key** (source file + well position) when storing amplification and melt curve data. Without this, every plate's "A1" would collide with every other plate's "A1" and the curves on plates 2..N would be silently overwritten. The compound key makes per-well lookups (curve QC, Fit Cq, melt peaks) remain plate-aware, while sample-level data is grouped by sample name as expected.

Mixed file types within a single upload are supported — Excel, CSV, TSV, RDML, and TXT can be combined in one batch. Each file is parsed with the appropriate reader and then merged into the unified well list. The upload-screen status banner reports the number of files loaded once parsing

completes.

Inter-Run Calibrator (IPC) Check. When you upload two or more plates, qPCR Guru automatically scans for samples that appear on multiple plates and treats them as **inter-run calibrators** (IPCs). These samples are evidence of plate-to-plate agreement: if a sample's Cq is stable across plates, the plates can be compared on the same scale; if the Cq drifts, you may need to normalize or repeat a run.

The **Inter-Run Calibrator (IPC) Check** panel appears in the ΔΔCq Analysis view when shared samples are detected. It lists each shared sample × target with: per-plate mean Cq, overall mean Cq, the maximum plate Δ (largest deviation of any one plate's mean from the overall mean), and a status chip:

Max Plate Δ	Status	Interpretation
< 0.5 cycles	Consistent (green)	Excellent plate agreement — plates comparable on the same scale.
0.5 – 1.0 cycles	Minor drift (amber)	Acceptable agreement; consider whether IPC correction is warranted.
> 1.0 cycles	Drift detected (red)	Substantial plate-to-plate offset — investigate cause; repeating the affected run is often safer than correcting after the fact.

Note: No-template controls and other auto-detected negative controls are excluded from IPC detection. Using a sample expected to show no amplification as a plate-drift calibrator is contradictory — negative controls validate contamination status, not Cq stability.

1.4 Understanding the Interface

After uploading, the interface has two main regions:

- **Left panel:** Analysis controls including reference gene selection, calibrator sample, chart options, statistical tests, and export tools.
- **Main area:** Tabbed views for QC Dashboard, Amplification Curves, Melt Curves, and the Analysis tab (charts, tables, visualizations).

Navigation tabs across the top let you switch between views. Each tab becomes active once the relevant data is available.

1.5 Guided Tour

First-time visitors are greeted with a short interactive tour that walks through uploading data, reviewing QC, running ΔΔCq analysis, and exporting results. The tour highlights the relevant UI element at each step and you can skip or finish it at any time.

Tip: You can replay the tour at any time by clicking the **Tour** button in the upload screen header or the application tab bar. This is useful when you've added a new lab member or want a refresher after a feature update.

2. Logging In and Account Management

2.1 Why You Need an Account

qPCR Guru requires a free registered account to access the analysis features. Accounts allow us to:

- apply fair-use rate limits to keep the Service responsive for everyone
- run computationally expensive analyses on our backend without abuse
- provide you with a stable identity for support requests

Registration is free and takes less than a minute. We will never sell your information or use it for advertising profiling. See our Privacy Policy for full details.

2.2 Registering and Logging In

On the login page, click **Register** and provide:

- a valid email address (used as your login identifier)
- a password of at least 8 characters
- first and last name
- job title (e.g., Graduate Student, Principal Investigator)
- organization type (academic, industry, hospital, etc.)
- optional: organization name, research area, country

Once registered, you will be logged in automatically. Your session persists across browser tabs and remains active until you log out or your token expires.

2.3 Email Verification

After registering, qPCR Guru sends a verification link to your email address. Until you click that link, your account exists but the backend-compute features (ΔΔCq analysis, statistical comparisons, Guru Insights, exports, and reports) are gated. You can still log in and use the QC dashboard and amplification curve viewer while unverified.

If you don't see the verification email within a minute or two, check your spam folder. A yellow banner at the top of the application shows your email address and includes a **Resend verification email** button that sends a fresh link. Verification links expire after 24 hours; you can request a new one any time from the banner.

> **Note:** Some corporate and university email systems automatically scan inbound links for malware. To prevent these scanners from accidentally consuming your verification, the email link opens a page with a **Verify Email Address** button — you'll need to click it to complete verification.

2.4 Logging Out

Click your name (top-right corner of the application) and select **Log Out**. This clears your authentication token and any locally cached preferences. Logging out is recommended on shared or public computers.

2.5 Password Reset

If you forget your password, click **Forgot password?** on the login page. Enter your email address and we'll send a password reset link that's valid for 1 hour. Click the link, choose a new password (at least 8 characters), and confirm.

Resetting your password also logs you out of every other active session — useful if you suspect your account has been accessed from a device you no longer control. After resetting, you'll need to log in again with your new password.

> **Note:** The password reset confirmation page always shows the same "check your inbox" message regardless of whether the email is registered. This prevents someone from using the reset form to discover whether a given email has an account.

2.6 Submitting Feedback

qPCR Guru includes an in-app feedback form at **qpcrguru.com/feedback**. Use it to report bugs, request features, flag data issues, or share general feedback. The form captures your name, email, category, and description, and includes browser and page-context information automatically to help us triage and reply.

If you're logged in when you open the feedback page, your name and email are pre-filled from your account. We read every submission personally and reply by email if a response is needed.

2.7 Privacy

Your password is stored as a bcrypt hash and is never visible to us in plain text. Uploaded qPCR data is processed ephemerally and is not retained on our servers after the analysis session ends. For full details, see our Privacy Policy at qpcrguru.com/privacy.

3. QC Dashboard

The QC Dashboard provides an at-a-glance quality assessment of every sample-target combination in your dataset. Each row shows the sample, target, number of valid replicates, mean Cq, standard deviation, coefficient of variation (CV%), and a pass/fail flag.

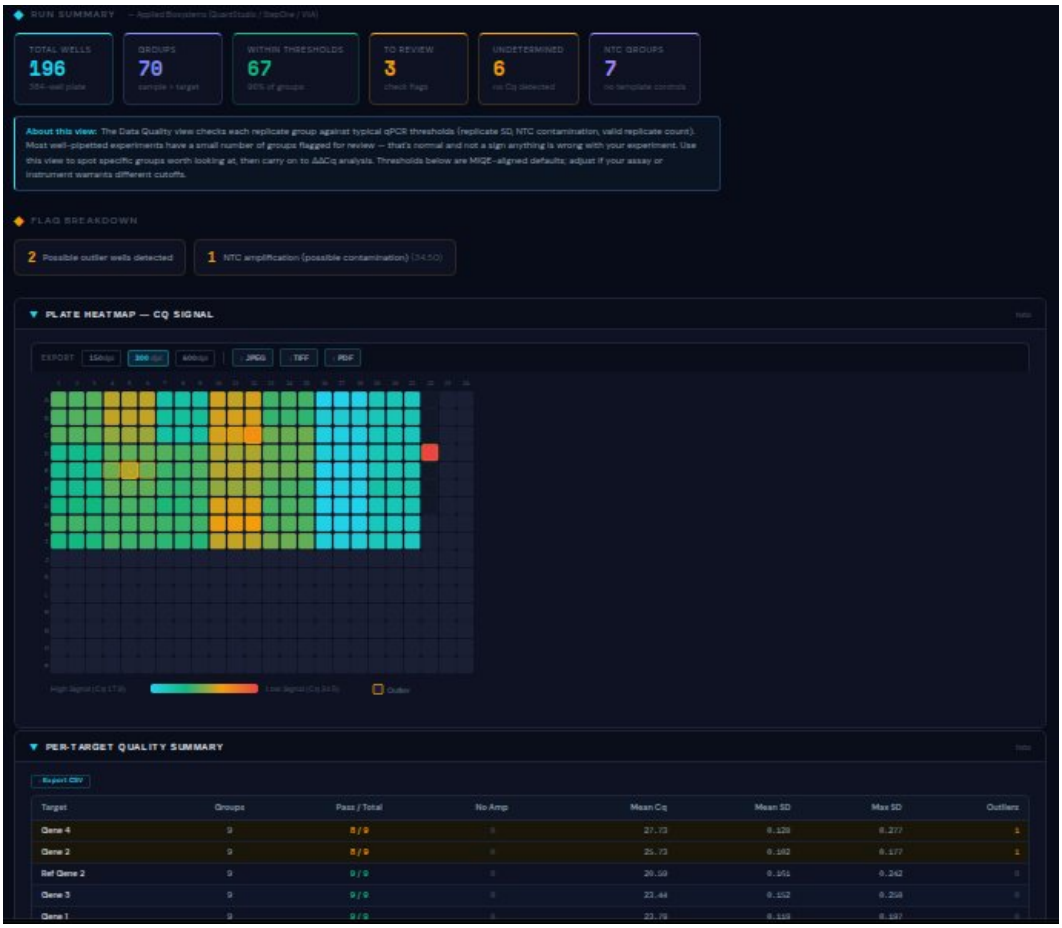

Figure 3.1. QC Dashboard view. The Run Summary tiles at the top track total wells, groups, within-threshold counts, items to review, undetermined wells, and NTC groups at a glance. A plate heatmap visualizes Cq signal across the layout (brighter = earlier amplification), with flagged outlier wells outlined. The per-target Quality Summary table below tracks pass/total replicate counts and mean Cq statistics by gene.

3.1 QC Metrics Explained

Metric	Description	Pass Criteria
Mean Cq	Average quantification cycle across replicates	Must be a valid number
SD	Standard deviation of Cq values within replicates	≤ SD threshold (default 0.5, configurable)

Metric	Description	Pass Criteria
CV%	Coefficient of variation: (SD / Mean) × 100	Lower is better; flags high variability
N Valid	Number of replicates with valid Cq values	≥ 2 replicates recommended

Both the Cq cutoff and the replicate SD threshold are adjustable from the **Quality thresholds** panel inside the QC tab. Defaults follow MIQE 2.0 recommendations (Cq cutoff 35, SD ≤ 0.5), and most users won't need to change them — but assays with intentionally late-cycling targets or instrumentation with wider technical variance can dial these up without editing data or code.

3.2 NTC Validation

No-Template Controls (NTCs) should show no amplification or very late amplification. qPCR Guru flags NTC wells that amplify before the **Cq cutoff** (default cycle 35, same configurable value as used elsewhere in the QC checks). Any NTC issues are highlighted with a warning at the top of the QC Dashboard, since contamination invalidates downstream quantification.

3.3 Replicate Consistency

Replicate groups (sample × target cells) with a standard deviation exceeding the SD threshold are flagged in the dashboard. Flagged groups remain visible by default — qPCR Guru never drops your data silently. If you want flagged groups removed from downstream ΔΔCq, expression, and statistical results, toggle **Exclude flagged groups** in the **QC Options** panel alongside the analysis. When the toggle is on, the panel shows a running count of groups currently being excluded so you know what's changed before reading the results.

Note: Excluding flagged groups affects only the active analysis view — the QC Dashboard, raw data table, and amplification plots continue to show all groups so the exclusion remains transparent and reversible.

3.4 Outlier Detection

When 3+ replicates exist in a sample × target group, qPCR Guru flags individual outliers via the **Median Absolute Deviation (MAD)** test with a **0.5 Cq absolute-deviation floor**. A replicate is flagged only when it is both statistically extreme (> 2.5 × MAD from the group median) and more than 0.5 Cq away in absolute terms.

The absolute floor matters: with tightly clustered triplicates the raw MAD can be tiny, and a pure MAD test would flag replicates whose deviations are well within normal qPCR repeatability. The 0.5 Cq floor — roughly a 1.4-fold expression difference — ensures a flagged replicate is statistically and practically different enough to warrant investigation.

Flagged outliers are never auto-removed — you choose whether to keep, exclude, or investigate. Exclusions feed the recalculated mean Cq, SD, and downstream analyses while the raw well data is preserved.

Note: A **Grubbs test** at the biological-replicate level inside Guru Insights identifies whole-sample ΔΔCq outliers — a distinct QC layer from the per-well MAD check above.

4. MIQE 2.0 Compliance

4.1 What Is MIQE?

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines provide standards for qPCR experimental design, analysis, and reporting (Bustin et al., 2009; updated as MIQE 2.0 in Bustin et al., 2025). They cover sample handling, RNA quality, primer design, reaction efficiency, normalization, replication, and analytical performance metrics (LOD, LOQ, dynamic range). qPCR Guru evaluates your data against the MIQE 2.0 data-analysis recommendations and displays a real-time compliance score in the **Publication Readiness** panel.

4.2 Compliance Checklist

The Publication Readiness panel auto-checks the following five MIQE 2.0 **Essential** criteria from your data and current analysis settings:

- **NTC Controls Included:** at least one no-template-control group is present and detected.
- **Technical Replicates ≥ 2:** every sample × target group has at least two valid replicate wells.
- **Replicate Cq SD ≤ 0.5:** all non-NTC groups fall within the configured replicate-SD threshold (default 0.5 Cq; see 3.1).
- **Multiple Reference Genes:** two or more validated reference genes are used (or Global Mean normalization is active).
- **PCR Efficiency Correction:** Pfaffl efficiency correction is enabled, with measured E values from uploaded standard curves or manual entry (default E = 2.0 fails this check).

Each item shows a green check or red cross with a one-line diagnostic. Reference gene stability values (geNorm M, NormFinder ρ) are reported separately in Step 1 of the analysis workflow, as MIQE 2.0 treats stability as a precondition for selection rather than a binary compliance check.

Figure 4.1. Publication Readiness (MIQE 2.0) panel. Each of the five Essential criteria is shown with a green check or red cross and a one-line diagnostic. The score in the header (here, 4/5) reflects how many criteria pass under the current settings — in this example, PCR Efficiency Correction is failing because efficiency is at the default E = 2.0 rather than measured from a standard curve.

4.3 Analytical Sensitivity (LOD and LOQ)

MIQE 2.0 requires that quantitative qPCR assays report **analytical sensitivity** — the Limit of Detection (LOD) and Limit of Quantification (LOQ) of each target. When you upload a standard curve, qPCR Guru computes both values directly from the regression using a **prediction-interval** construction: LOD from the regression's k = 3·SE upper prediction bound (≈ 99.7% one-sided), and LOQ from the k = 3.3·SE prediction bound (the FDA's "LOQ from LOD" rule, LOQ ≈ 10/3·LOD). Full methodology and references are in 11.4.

LOD and LOQ values are **suppressed** (shown as "—") when the underlying regression does not meet MIQE 2.0 acceptance criteria ($R^2 \geq 0.98$ and efficiency in 90–110%). This is intentional: an LOD claim derived from a poor regression is not defensible for publication, and surfacing it would invite misuse. When values are clamped to the lowest tested standard, the table flags this so you know the prediction bound extrapolated below your dynamic range.

> **Note:** The prediction-interval LOQ is an approximation from regression statistics. For publication-grade LOQ, run replicates at low standard concentrations and report the lowest level at which CV ≤ 25% (Forootan et al., 2017; Klein, 2002). qPCR Guru's value is a useful internal reference but does not replace this empirical validation when claims are central to your paper.

4.4 Scoring

Each criterion in the checklist scores as **Pass** or **Fail**. The panel header color reflects the overall result — green when all items pass, amber when three or four pass, red when fewer. The score is included in generated PDF reports for publication transparency.

Note: MIQE compliance scoring reflects data-analysis checks only. Experimental-design criteria (RNA integrity, primer specificity, contamination controls in wet lab, etc.) must be evaluated and reported separately.

5. Setting Up Your Analysis

5.1 Choosing Reference Genes

Select your primary reference (housekeeping) gene from the dropdown in the left panel. This gene is used for ΔCq normalization. qPCR Guru auto-detects common reference genes (GAPDH, ACTB, 18S, etc.) and pre-selects one, but you should verify this matches your experimental design.

> **Tip:** Using multiple reference genes improves normalization accuracy. Add additional reference genes in the **Reference Gene Validation** section. The geometric mean of all selected reference genes is used for normalization.

5.2 Calibrator Sample

The calibrator (control) sample is the baseline for ΔΔCq calculation. Typically this is your untreated control, time zero, or wild-type sample. The RQ of the calibrator is set to 1.0, and all other samples are expressed relative to it.

5.3 Sample Groups

For experiments with biological replicates, use **Sample Groups** to merge individual samples into groups. For example, group 'Control_1', 'Control_2', 'Control_3' into a single 'Control' group. This pools replicate data for statistical comparisons and simplifies chart presentation.

> **Note:** Sample groups should represent biological replicates, not technical replicates. Technical replicates (same sample, same plate) are already averaged in the QC step.

5.4 Pfaffl Efficiency Correction

The Pfaffl method uses per-gene PCR efficiencies rather than assuming 100% efficiency, producing more accurate RQ values when efficiencies differ between genes. There are three ways to enter efficiency values:

- **Upload a standard curve** (Section 11) — qPCR Guru calculates efficiency, LOD, and LOQ automatically. Click **Apply** or **Apply All** to populate values into the Pfaffl panel.
- **Enter values manually** in the Pfaffl method panel for each target. Use this if you have efficiency values from prior characterization.
- **Leave at default 2.0** (100% efficiency) for genes you have not characterized. Pfaffl mode falls back to 2-^ΔΔCq behavior in this case.

5.5 Standard Curve Workflow

If you ran a dilution series for primer efficiency, upload it from the **Standard Curves** panel in Step 2. The file is parsed locally in your browser, and the data points ($\log_{10}$ quantity, Cq) are sent to the backend for regression analysis. Results include slope, intercept, R^2, efficiency E, efficiency percentage, residual SD, LOD, and LOQ.

Click **Apply** on a single gene to apply its efficiency to matching well targets, or **Apply All** to apply all gene efficiencies at once. See Section 11 for full details.

5.6 Multiple Reference Genes

Add additional reference genes in the **Reference Gene Validation** section. When multiple reference genes are selected, normalization uses the geometric mean of their Cq values (Vandesompele et al., 2002), which is more robust than using a single gene.

For experiments where stable reference genes are difficult to identify — a common situation in miRNA profiling and exploratory expression studies — qPCR Guru also supports **Global Mean normalization**: the mean Cq across all expressed targets is used as the normalization factor, as proposed by Mestdagh et al. (2009). Global Mean works best when many targets are profiled and the majority are expected to be stable, since the mean averages out target-specific variation.

6. Understanding Your Results

6.1 The Data Table

The results table shows one row per sample-target combination with the following columns:

Column	Description
Sample	Sample or group name
Target	Target gene name
Mean Cq	Average Cq value across replicates
SD Cq	Standard deviation of Cq values within the group
ΔCq	Cq(target) − Cq(reference): normalization to reference gene(s)
ΔΔCq	ΔCq(sample) − ΔCq(calibrator): relative to calibrator
SD ΔΔCq	Propagated standard deviation
RQ	Relative Quantification: 2^(−ΔΔCq) or Pfaffl ratio
RQ Min / Max	Asymmetric error range based on propagated SD
p-value	Per-row one-sample t-test of ΔΔCq vs zero (when enabled)
t-stat / df	Per-row t-statistic and degrees of freedom
p adj.	BH-FDR-corrected p-value (when FDR is enabled)

6.2 Data Display Modes

Use the **Y-Axis Data** selector in the Chart Controls to switch between:

- **RQ (Relative Quantification):** Fold change relative to calibrator. Default view.
- **ΔCq:** Normalized Cq difference. Useful for comparing raw expression levels.
- **Mean Cq:** Raw mean Cq values. Useful for checking raw data distribution.
- **Copy #:** Absolute quantification from standard curves. Available only when standard curve data has been uploaded.

6.3 Interpreting Fold Change

An RQ of 1.0 means expression is the same as the calibrator. RQ > 1 indicates upregulation; RQ < 1 indicates downregulation. Common interpretation thresholds:

RQ Value	Interpretation
> 2.0	Upregulated (≥ 2-fold increase)

RQ Value	Interpretation
1.5 – 2.0	Mildly upregulated
0.67 – 1.5	No meaningful change
0.5 – 0.67	Mildly downregulated
< 0.5	Downregulated (≥ 2-fold decrease)

7. Charts & Visualization

7.1 Bar Charts

The main visualization is a bar chart showing RQ (or other data type) per sample-target combination. Bars are drawn from the baseline (0 for linear, 1 for log scale), with error bars representing propagated uncertainty.

7.2 Grouping

Charts can be grouped **by sample** (targets as bars within each sample group) or **by target** (samples as bars within each target group). Toggle this in **Chart Options** to find the view that best tells your story.

7.3 Error Bars

Three error bar modes are available:

- **Asymmetric (default):** Shows RQ Min and RQ Max directly. Reflects the true asymmetric distribution of 2^(−ΔΔCq).
- **Symmetric:** Uses the average of upper and lower bounds. Simpler visually, but less precise.
- **None:** Hides error bars entirely.

7.4 Overlay Options

Overlay individual data points on bars to show the underlying distribution:

- **Individual Points:** Jittered dots for each biological replicate.
- **Violin Plot:** Kernel density estimation showing data distribution shape.
- **Bean Plot:** Combination of individual points with density outline.
- **Box & Whisker:** Standard box plot showing median, quartiles, and whiskers.

7.5 Faceted Panels

Use the **Facet** option to split your chart into one panel per target or per sample. This is especially useful for experiments with many targets or samples. You can choose independent or shared Y-axes across panels.

7.6 Customization

Extensive chart customization is available:

- **Fonts:** Choose font family and size for all chart text.
- **Colors:** Custom colors per sample or target using hex color pickers.
- **Labels:** Custom X and Y axis labels, with bold/italic toggles.

- **Log scale:** Toggle $\log_2$ scale for fold-change visualization.
- **Label angle:** Rotate X-axis labels from 0° to 90° for long names.

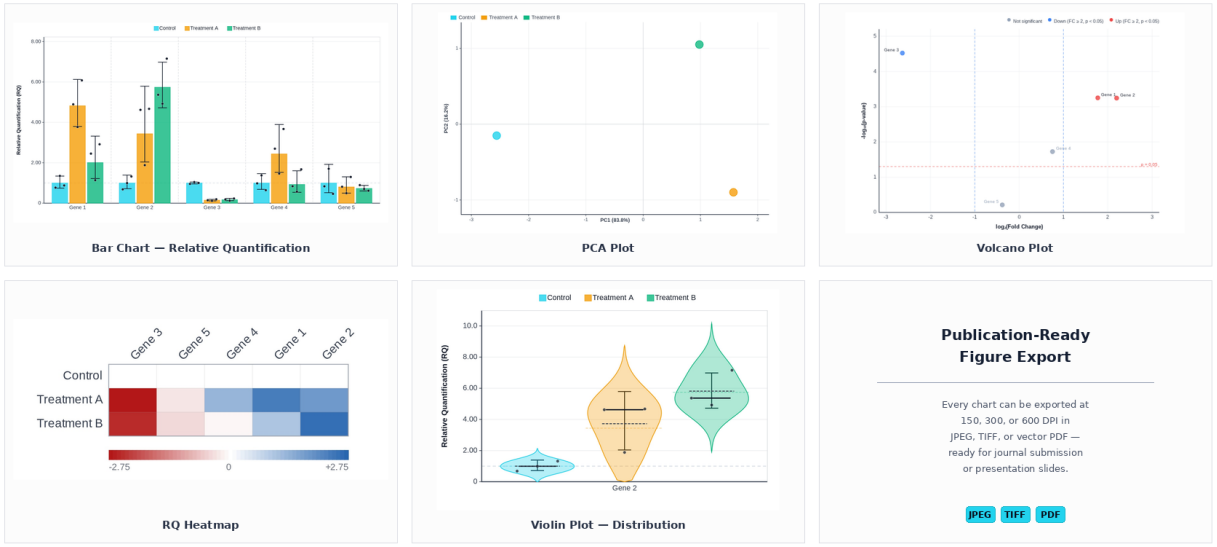

Figure 7.1. A gallery of qPCR Guru's main chart types. The relative quantification (ΔΔCq) bar chart is the canonical output of the analysis workflow; PCA, volcano plots, RQ heatmaps, and per-gene distribution (violin) plots are described in Section 8. Each chart panel is independently customizable and exportable at publication resolution (Section 14.2).

8. Advanced Visualizations

8.1 PCA Plot

Principal Component Analysis (PCA) reduces your multi-gene expression data to 2–3 principal components, revealing clustering patterns among samples. Each point represents a sample, positioned by its overall expression profile. Samples that cluster together have similar gene expression patterns.

Use the axis selectors to view different PC combinations (PC1 vs PC2, PC1 vs PC3, etc.). The variance explained percentage indicates how much of the total variation each component captures.

8.2 Volcano Plot

The volcano plot shows statistical significance ($-\log_{10}$ p-value) vs. magnitude of change ($\log_2$ fold change) for each target gene. Genes in the upper corners are both significantly changed and biologically meaningful. Red dots indicate upregulated genes (FC ≥ 2, $p < 0.05$), blue dots indicate downregulated genes, and gray dots are not significant.

8.3 Correlation Scatter

The correlation plot shows the relationship between two selected samples or two selected targets. Each point represents a gene (sample mode) or sample (target mode). The R^2 value and linear regression line indicate the strength of correlation.

8.4 RQ Heatmap

The heatmap displays all RQ values as a color-coded matrix with samples on one axis and targets on the other. Optional hierarchical clustering (UPGMA algorithm with Euclidean distance) reorders rows and/or columns to group similar expression profiles together. Dendrograms show the clustering hierarchy.

Color scales available: diverging (blue-white-red centered on RQ=1) or linear (white to dark).

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9. Statistical Analysis

qPCR Guru runs two distinct types of statistical tests, each with a different purpose.

9.1 Per-Row One-Sample t-test

Shown in the data table. For each sample-target combination, this test asks whether the per-well ΔΔCq values differ significantly from zero (i.e., from the calibrator). The test requires at least 2 replicate Cq values per group. Results appear in the **p-value**, **t-stat**, and **df** columns. When BH-FDR correction is enabled, the corrected value appears in the **p adj.** column.

9.2 Group Comparison Tests

Shown in the **Group Comparison Statistics** section below the chart. For each target, these tests ask whether expression differs among groups. Available test methods:

- **Auto-select (default):** Welch t-test for 2 groups, ANOVA + Tukey HSD for 3+ groups.
- **Welch t-test:** Two-sample parametric test that does not assume equal variances. Used for 2 groups.
- **One-way ANOVA + Tukey HSD:** Parametric omnibus test (ANOVA F) followed by post-hoc pairwise comparisons (Tukey HSD). Used for 3+ groups. Assumes normality and equal variances across groups.
- **ANOVA + Games-Howell:** Same omnibus ANOVA, but the post-hoc pairwise test uses Welch-style standard errors with Welch-Satterthwaite degrees of freedom and the studentized range distribution. Robust to unequal variances. Recommended when Levene's test rejects equal variances (see 9.3).
- **Kruskal-Wallis:** Non-parametric alternative to one-way ANOVA. Pairwise comparisons use Mann-Whitney with Bonferroni correction.
- **Mann-Whitney U:** Non-parametric two-sample test. With 2 groups, runs as a standard Mann-Whitney U test. With 3+ groups, the omnibus p-value uses Kruskal-Wallis and pairwise comparisons use Mann-Whitney with Bonferroni correction.

Note: Mann-Whitney is inherently a 2-group test. With 3+ groups, qPCR Guru follows standard practice (Sokal & Rohlf; Zar) of using Kruskal-Wallis as the omnibus test and pairwise Mann-Whitney + Bonferroni as the post-hoc method. The test label and inline notes in the UI explain this when it applies.

9.3 Variance Equality and Choice of Post-Hoc Test

Tukey HSD is the standard post-hoc test for one-way ANOVA, but it assumes equal variances across groups (homoscedasticity). When that assumption is violated, Tukey's p-values become biased — typically too small for the high-variance group's comparisons and too large for the low-variance group's comparisons. The consequence is real: false positives where they shouldn't be, missed effects where they should.

qPCR Guru runs **Levene's test (Brown-Forsythe variant)** on every group comparison and surfaces the result inline. The Brown-Forsythe variant uses the median (not mean) of each group as the center, which is more robust to non-normality than the original Levene's. The result appears as Levene p = N.NNNN in the per-target omnibus row:

- **p ≥ 0.10** (green) — group variances consistent; Tukey HSD's assumption holds.
- **0.05 ≤ p < 0.10** (slate) — borderline; interpret pairwise Tukey results with mild caution.
- **p < 0.05** (amber) — variance assumption violated; Tukey HSD's p-values are biased.

When the variance test fails AND the current method is Tukey HSD or Welch, qPCR Guru also shows a warning banner above the chart with a one-click [ANOVA + Games-Howell ↗] button that switches the Statistical Tests dropdown to the heteroscedasticity-robust method.

Why this matters in qPCR: dilution series and treatment groups can produce groups with very different within-group variability. Highly diluted samples near the detection limit amplify with high Cq variability between replicates, while high-concentration samples typically cluster tightly. Tukey HSD pools variance across all groups, so a single high-variance group can inflate the standard error for every comparison — including pairs of clean groups that should be easy to distinguish. Games-Howell uses per-pair Welch-style standard errors, so each comparison is judged against its own variance estimate.

> **Tip:** Tukey HSD remains the qPCR community default and is appropriate for most clean datasets. Games-Howell is the right choice when Levene's flags unequal variances — particularly common with dilution series, LOD studies, and treatment groups where one condition shows much more variability than the others. The warning banner is your prompt to switch; in clean datasets it never appears.

9.4 Significance Stars & Brackets

Enable **Show significance** in Chart Options to display stars above bars:

Symbol	Meaning
***	p < 0.001
**	p < 0.01
*	p < 0.05
ns	Not significant (p ≥ 0.05)

You can also add custom **brackets** between specific groups to highlight pairwise comparisons of interest.

9.5 FDR Correction

When performing multiple comparisons across many targets, you can optionally enable **BH-FDR** in the Statistical Tests section. This applies the Benjamini-Hochberg procedure to control the false discovery rate. Both raw and adjusted p-values are reported (raw in **p**, adjusted in **p adj.**). FDR correction is applied across targets, not across pairwise comparisons within a target.

10. Reference Gene Validation

Accurate normalization requires stable reference genes. qPCR Guru provides two complementary algorithms for assessing reference gene stability.

10.1 geNorm M Value

The geNorm algorithm (Vandesompele et al., 2002) calculates pairwise stability by comparing expression ratios between all candidate reference genes across all samples. The M value represents the average pairwise variation for each gene.

M Value	Interpretation
< 0.5	Excellent stability
0.5 – 1.0	Acceptable stability
> 1.5	Unstable — consider replacing this reference gene

Note: These are Guru-recommended thresholds. The geNorm algorithm is by Vandesompele et al. (2002).

10.2 NormFinder ρ

NormFinder (Andersen et al., 2004) uses a model-based approach to estimate stability, measuring both intra- and inter-group variation independently. The ρ (rho) stability value represents the estimated systematic error introduced by using that gene for normalization.

Rho Value	Interpretation
< 0.3	Excellent stability
0.3 – 0.5	Acceptable stability
> 0.5	Unstable — consider replacing this reference gene

Note: These are Guru-recommended thresholds. The NormFinder algorithm is by Andersen et al. (2004).

10.3 Interpreting Agreement

When both geNorm and NormFinder agree on the most stable gene, you have high confidence in your reference gene choice. When they disagree, investigate further — this may indicate group-specific effects or co-regulation that one algorithm is more sensitive to.

11. Standard Curves & Absolute Quantification

11.1 Uploading a Standard Curve

In Step 2, locate the **Standard Curves for Primer Efficiency and Absolute Quantification** panel and click **Upload Standard Curve**. qPCR Guru accepts QuantStudio exports or any file with Target, Ct/Cq, and Quantity/Concentration columns. The file is parsed in your browser, then the ($\log_{10}$ quantity, Cq) data points are sent to the backend for regression analysis.

For each gene with at least 3 data points, the results table displays:

- **Slope:** regression slope (negative for valid curves)
- **R² (5PL):** coefficient of determination of the 5PL curve fit (target ≥ 0.98)
- **E:** efficiency = 10^(−1/slope) (target 1.8–2.2 = 90–110%)
- **Eff%:** efficiency as percentage
- **LOD:** Limit of Detection from the regression's upper prediction bound (see 11.4)
- **LOQ:** Limit of Quantification from the same prediction-bound construction (see 11.4)

11.2 Smart Name Matching

When you click **Apply** on a gene (or **Apply All**), qPCR Guru matches standard curve gene names to your experiment's well targets using a 3-tier matching strategy:

- **Exact match** — preferred when names match perfectly
- **Case-insensitive exact match** — handles 'GAPDH' vs 'gapdh'
- **Substring match** — handles a 'GAPDH' standard curve matching 'GAPDH_rep1' and 'GAPDH_rep2' wells, or 'ACTB' matching 'ACTB-FAM' and 'ACTB-VIC' probe variants

A green confirmation banner shows which experiment targets received the efficiency value. If no match is found, an error explains which gene names are available so you can correct the curve file or rename targets.

> **Tip:** If your standard curve uses a generic gene name (e.g., 'GAPDH') and your wells use suffixed or prefixed variants (e.g., 'GAPDH_rep1', 'GAPDH_FAM', 'Run2-GAPDH'), qPCR Guru's substring match will apply the same efficiency to all matching wells.

11.3 Efficiency from Slope

PCR efficiency is calculated from the standard curve slope:

$$E = 10^{(-1/\text{slope})}$$

An efficiency of 2.0 (100%) means perfect doubling per cycle. The acceptable range is 1.8–2.2 (90–110%). The efficiency value can be applied to the Pfaffl method via the Apply buttons.

11.4 Limit of Detection and Quantification

qPCR Guru computes LOD and LOQ from the regression's upper prediction bound, rather than the older copies at 3·SD above the highest Cq standard heuristic. The prediction-bound approach accounts for both regression-line uncertainty and the inherent scatter of the standards, which is the construction MIQE 2.0 (Bustin et al., 2025) recommends.

Both LOD and LOQ use the same construction with different multipliers:

- **LOD** — the cycle at which the regression's **k=3·SE_pred** (99.7%) upper prediction bound still produces a measurable signal, back-calculated to copies via the regression.
- **LOQ** — the same construction with **k=3.3·SE_pred**. This follows the FDA "LOQ from LOD" rule (LOQ ≈ 10/3·LOD) and matches Forootan et al. (2017) and Klein (2002) guidance that LOQ corresponds to CV ≤ 25%.

Two safeguards prevent over-confident reporting:

- **Clamping.** If the prediction bound extrapolates below your dynamic range, the displayed value is clamped to the lowest tested standard and prefixed with "≥". This signals that you have no experimental observations there to support a detection claim at lower concentrations.
- **Suppression.** When the regression doesn't meet MIQE 2.0 criteria ($R^2 \geq 0.98$ and efficiency 90–110%), LOD and LOQ are suppressed entirely and displayed as "—". A poor regression doesn't support a defensible limit claim regardless of how the math is set up.

Note: The prediction-bound LOQ is an approximation derived from regression statistics. For publication-grade LOQ, run replicates at low standard concentrations and report the lowest level where CV ≤ 25% (Forootan et al., 2017; Klein, 2002). qPCR Guru's LOQ value is a useful indicator but is not a substitute for empirical CV-based determination.

11.5 Standard Curve Diagnostics

Each gene row in the results table has a **Diag** toggle (▶) that expands a per-point diagnostic subsection. This shows, for every ($\log_{10}$ q, Cq) point that went into the regression:

- **Predicted Cq** — what the regression line says at this $\log_{10}$ q
- **Residual** — observed minus predicted Cq (cycles)
- **Std. Resid.** — standardized (internally Studentized) residual; approximately t-distributed. **|z| > 2** = investigate (amber), **|z| > 3** = likely outlier (red).
- **Leverage** — how much this point pulls the regression toward itself; points at the extremes of the standard range have higher leverage by construction.
- **Cook's D** — how much the regression coefficients change if this point is dropped. Standard threshold: **D > 4/n** flags an influential observation (Cook & Weisberg, 1982).

Points are sorted by Cook's distance, descending — the most influential points appear at the top of the table so you can scan them quickly.

Each row has an **Excl** checkbox. Toggling it drops the point from the regression and refits in place: slope, R^2, efficiency, LOD, and LOQ in the parent row update to reflect the new fit, with an "(N excl.)" indicator next to the gene name. The diagnostic columns also recompute against the new regression, so you can iteratively exclude points and see how the fit responds.

Excluded points appear in the table with strike-through styling so you know what's dropped. A **Restore all N excluded points** button at the bottom of the panel undoes the exclusion. Exclusions are ephemeral (not saved across uploads) — they're for exploring "what if I drop this point?" interactively.

> **Tip:** A high standardized residual with low leverage usually means a noisy replicate that's not bending the regression line — it's worth investigating but doesn't have to be excluded. A high residual with high leverage and high Cook's D is the combination that warrants removal: the point is both an outlier and influential.

11.6 Copy Number Mode

Once a standard curve is uploaded and applied, switch the chart data type to **Copy #** to display absolute quantities calculated from the standard curve. Each sample's mean Cq is back-calculated through the regression to produce a copy number estimate. Tick labels automatically use scientific notation (e.g., 1.5e8) for very large copy numbers.

12. Amplification & Melt Curves

12.1 Viewing Curves

If your data file includes amplification or melt curve data (common in exports from Bio-Rad CFX Maestro, QuantStudio, Roche LightCycler), qPCR Guru will automatically parse and display them. Use the checkboxes to show/hide individual samples or targets.

When raw amplification data is present, qPCR Guru fits a 5-parameter logistic (5PL) curve to each well in the background. The fit powers two downstream features: **Curve Shape QC** (per-well kinetic classification) and **Fit Cq (SDM)** (an independent Cq derivation from the curve geometry). Both are explained below.

12.2 Curve Shape QC

Each well's amplification curve is fit with a 5-parameter logistic (5PL) sigmoid and screened for atypical shape. The per-well status is shown in the **Curve Shape** column of the table beneath the Amplification Curves plot:

Status	Meaning
Normal	5PL fit converges with a well-formed sigmoid shape and no QC flags raised.
Abnormal	One or more curve-shape QC flags raised — see the Flags column for the specific issue.

Each flag corresponds to a distinct kinetic problem with a specific biological or technical interpretation:

Flag	Trigger	What it usually means
Fit did not converge	5PL solver failed	No sigmoid could be fit to the data. Almost always a flat or pure-noise well.
Poor curve fit	$R^2 < 0.95$	The 5PL model doesn't describe the curve well — typically NTC-like wells with no real amplification structure.
Flat curve	Plateau amplitude < 0.05 ΔRn, or amplitude marginal with poor fit, or fitted inflection outside the observed cycle range	No meaningful amplification within the run. Sample may have failed to amplify, or the well was empty / lost to evaporation.
Biphasic	Two distinct slope peaks ≥ 10 cycles apart in the raw fluorescence	Two amplification events in one well — commonly primer-dimer, non-specific product, or low-template stochastic amplification. Reported Cq may reflect the contaminating signal rather than the target. Inspect the melt curve to confirm specificity.

Flag	Trigger	What it usually means
Early amplification	Curve's main rise occurs by cycle 10, observed maximum > 0.40 ΔRn	Cq < 10 is exceedingly rare for any template concentration. Usually indicates carryover contamination, pre-amplified template, plate setup error, or instrument artifact. Reported Cq is not quantitative.
Atypical kinetics	$R^2 \geq 0.99$ but asymmetry parameter f < 0.02 or > 1000	Curve fits well overall but the shape is highly asymmetric — possible secondary product or unusual reaction kinetics.
Noisy baseline	Residual standard error > 10% of plateau amplitude	High noise in the baseline cycles relative to the amplification signal. May affect quantification precision.
Replicate outlier	Inflection cycle > 2 MADs from the replicate group median (requires ≥ 3 replicates)	Curve shape disagrees with the other replicates in its sample × target group. Likely well-specific (bubble, evaporation, pipetting), not biological.

Protecting late, low-abundance signals. When the raw fluorescence shows a real rise above baseline AND the 5PL fit has at least some structural fidelity ($R^2 > 0.80$), the Poor curve fit and Flat curve flags are suppressed. This protects limit-of-detection curves where most of the run is baseline noise — the 5PL fit's R^2 can be unreliable simply because it has few cycles of real signal to learn from. This aligns with the qpcR reference philosophy (Ritz & Spiess, 2008): a curve with valid sigmoidal structure is a valid amplification regardless of how late or how low. Quantitation reliability concerns for late-Cq wells are surfaced separately via the standard Cq > 35 warning.

The Amplification Curves tab includes a segmented control (**All** / **Only abnormal** / **Only normal**) that filters the plot and per-well table. The **Only abnormal** view is the fastest way to scan for problem wells in a large plate.

Note: Abnormal wells aren't automatically excluded from your analysis — they're flagged for your review. You can choose to keep them, exclude them via the QC Dashboard's **Exclude flagged groups** toggle, or investigate specific wells before deciding.

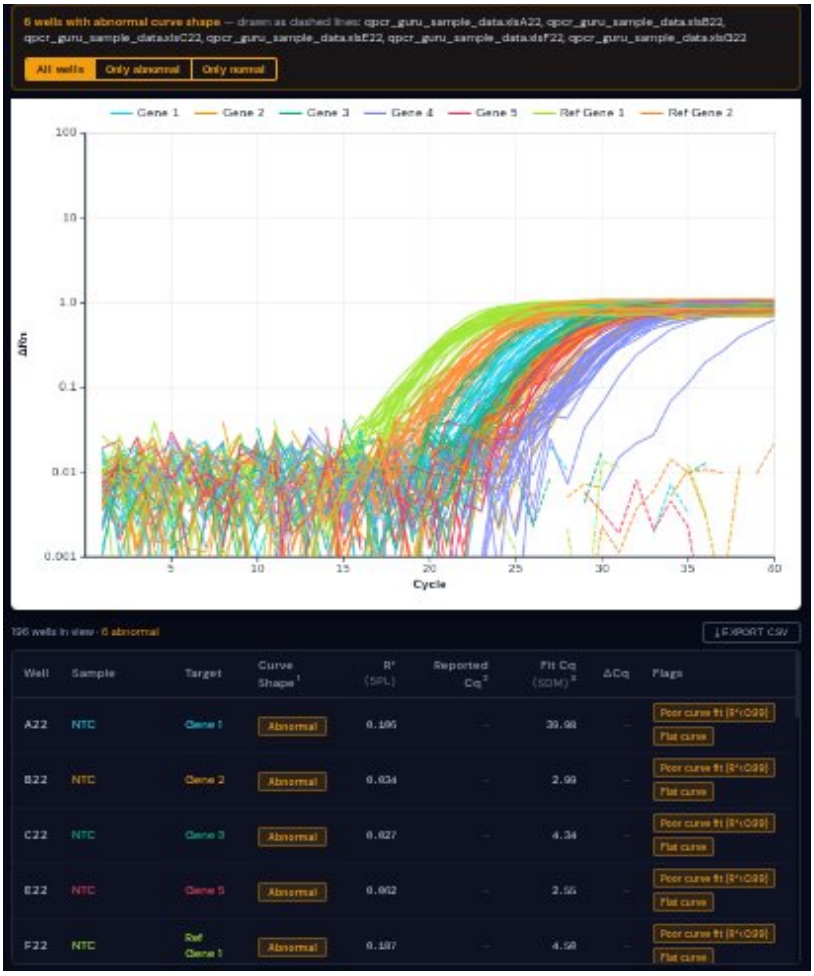

Figure 12.1. Curve Shape QC view. The amplification plot at top shows all wells; those flagged as abnormal are drawn with dashed lines. The per-well table beneath lists each flagged well with its 5PL R², Reported Cq, Fit Cq (SDM), ΔCq, and the specific QC flags raised (e.g. 'Poor curve fit ($R^2 < 0.99$)', 'Flat curve'). The segmented control at the top filters the view to All wells / Only abnormal / Only normal.

12.3 Fit Cq (SDM) and the ΔCq Column

Alongside the **Reported Cq** column (the value present in your imported data file, typically computed by the instrument's threshold-crossing method), qPCR Guru shows a **Fit Cq (SDM)** column. SDM stands for Second-Derivative Maximum — the cycle at which d^2F/dx^2 is largest on the fitted 5PL curve.

Geometrically, this is the cycle where amplification is accelerating fastest — the moment the curve transitions from the lag/baseline phase into the exponential phase. By comparison, the **inflection point** of the sigmoid (cpD1 in qpcR notation) is where amplification is at maximum velocity (steepest part of the curve), which always lies a few cycles later than SDM. Both points are well-defined features of the 5PL fit — SDM is preferred as a Cq surrogate because it lands earlier in the curve, in the part of the kinetics where information about the starting template concentration is least distorted by enzyme saturation effects.

Fit Cq (SDM) is independent of the baseline window and the fluorescence threshold position, both of which can shift between runs and between operators. The methodology is implemented as the qpcR package's cpD2 point (Ritz & Spiess, 2008).

The **ΔCq** column shows Fit Cq − Reported Cq. A small positive offset (~0.3–0.5 cycles, sometimes up to 1.0) is expected for textbook-perfect curves: instrument software typically places the threshold low in the early exponential rise so Cqs are comparable across runs, which puts Reported Cq slightly before SDM on the cycle axis. The signal worth acting on is when |ΔCq| > 1.0 cycles (Guru recommended), which usually points to baseline-subtraction problems, late amplification, or atypical kinetics (Bar et al., 2003).

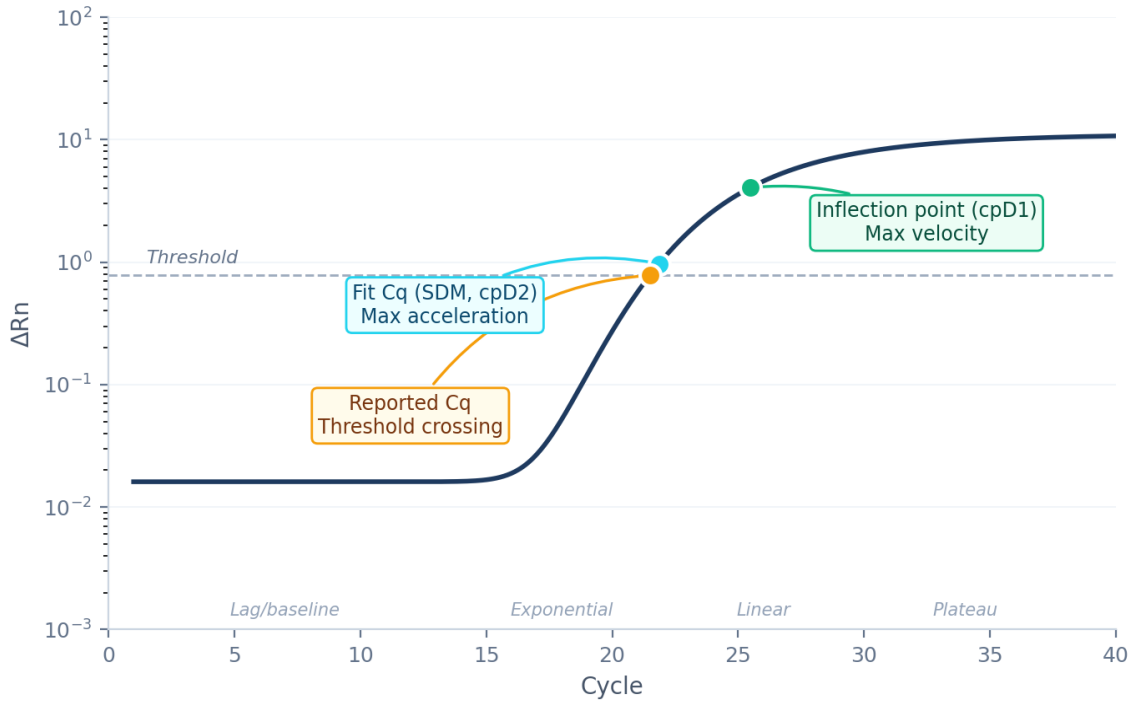

Figure 12.2. A typical qPCR amplification curve with three reference points marked. Reported Cq (threshold crossing) sits where the curve first exceeds the fluorescence threshold. Fit Cq (SDM, cpD2) lands shortly after, at the cycle where the curve is accelerating fastest. The inflection point (cpD1) sits later still, at the steepest part of the curve. For well-behaved curves Fit Cq typically lands 0.3–0.5 cycles after Reported Cq (i.e. ΔCq is positive); larger disagreements warrant investigation.

12.4 When to Use Which Cq

qPCR Guru lets you propagate either Reported Cq or Fit Cq (SDM) through the full analysis pipeline via the **Cq Source** toggle in Step 2. Neither method is universally superior — the right choice depends on your data and your reporting context.

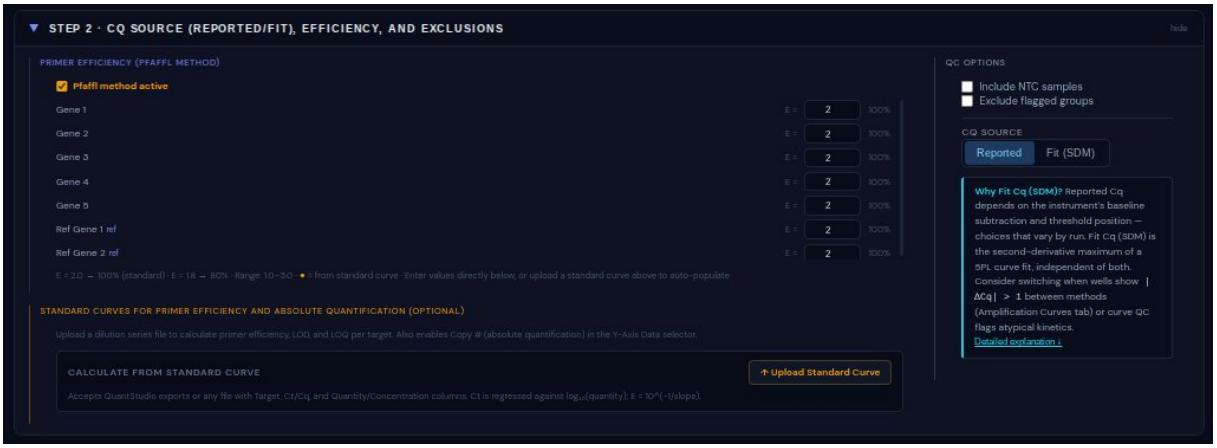

Figure 12.3. Step 2 setup panel. Per-gene Pfaffl efficiency values appear on the left (default E = 2.0 = 100% efficiency; values from a standard curve override the default). The **Cq Source** toggle on the right switches between **Reported** (instrument threshold crossing) and **Fit (SDM)** (second-derivative maximum of the 5PL fit). The 'Why Fit Cq (SDM)?' callout summarizes when to consider switching.

Fit Cq (SDM) tends to be more accurate when:

- Curves have visible baseline drift (autofluorescence build-up, lamp warm-up, dye degradation) that may shift threshold-Cq.
- Amplification is late (low template) and the threshold sits in the still-noisy near-baseline region.
- You're comparing data across different instrument runs or different operators — SDM has lower run-to-run variability because it doesn't depend on threshold placement.
- Curve QC flags the well as Inspect/Abnormal — the disagreement itself is often the diagnostic signal.

Reported Cq remains preferable when:

- Cq values are very early (Cq < ~10) — the 5PL fit becomes unstable when there's no measurable baseline before the curve rises.
- The amplification curve has unusual features the 5PL can't capture (e.g., multi-template wells with two inflection points, or plateaus that crash back down). The threshold method just measures the first crossing and is robust to these.
- You're extending or comparing against prior threshold-Cq results in the same project. Switching mid-study introduces a methodological inconsistency.
- Your downstream audience (reviewers, collaborators, public repositories) expects threshold-Cq. MIQE 2.0 requires the Cq determination method be reported either way, but convention still favors threshold methods in most published qPCR literature.

Tip: The conservative workflow is to run the analysis on Reported Cq (the default), then inspect the ΔCq column for wells with |ΔCq| > 1.0. If only a handful of wells disagree, investigate those specifically. If many disagree, the dataset may have systematic baseline or threshold issues, and switching globally to Fit Cq (SDM) is worth considering. Wells where the 5PL fit was unusable fall back to the reported Cq automatically when Fit Cq mode is on, so the switch never produces missing data.

12.5 Melt Curve Peak Detection

When melt curve data is present, qPCR Guru computes **−dF/dT** for each well, detects peaks, and classifies each well as Normal, Inspect, or Abnormal. The per-well peak table beneath the melt plot shows the number of peaks, their Tm values, and the status.

Classification rules for biological samples:

Status	Criterion
Normal	Exactly 1 peak above the Peak Floor.
Inspect	Multiple peaks, all secondaries below the Inspect → Abnormal threshold. Primers may still be usable, but specificity is worth verifying.
Abnormal	Secondary peak ≥ Inspect → Abnormal threshold (non-specific product), OR 0 peaks above the Peak Floor.

NTCs use inverted rules: 0 peaks = Normal (no template, no melt is the expected outcome); any peak above the floor = Abnormal (contamination signal).

12.6 Adjusting Detection Thresholds

Two sliders in the Melt Curves tab control how peaks are detected and classified:

- **Peak Floor** (default 5%, Guru recommended) — minimum peak height as a percentage of the strongest peak in the currently visible wells. Peaks below this floor are treated as noise.
- **Inspect → Abnormal** (default 50%, Guru recommended) — if a secondary peak is at or above this percentage of the main peak, the well is flagged Abnormal. Below this threshold (but above the Peak Floor) → Inspect.

A **Reset to Guru recommended** button restores the defaults if you want to revert after experimenting. Adjusting either slider re-runs the classification across all wells in place.

12.7 What to Look For

Amplification curves:

- Consistent curve shapes across replicates indicate good reproducibility.
- Curves that plateau at different levels may suggest varying starting quantities or saturation effects.
- Late or absent amplification in NTC wells confirms no contamination.
- Use the **Only abnormal** filter to quickly identify wells flagged by Curve Shape QC.
- Cross-reference the ΔCq column with the curve plot — wells with |ΔCq| > 1 often look subtly off compared to their replicates.

Melt curves:

- A single, sharp peak indicates a specific product.
- Multiple peaks suggest primer dimers, non-specific amplification, or SNPs.

- Shifted peaks between samples may indicate different amplicons or allele variants.
- If many wells are flagged Inspect or Abnormal, try lowering the Peak Floor first — your dataset may have a low-signal baseline where a 5% floor is rejecting real (small) peaks.

13. Guru Insights

Guru Insights provides an automated, expert-level review of your analysis, checking for common issues and highlighting important findings. Access it from the **Guru Insights** tab after running your analysis.

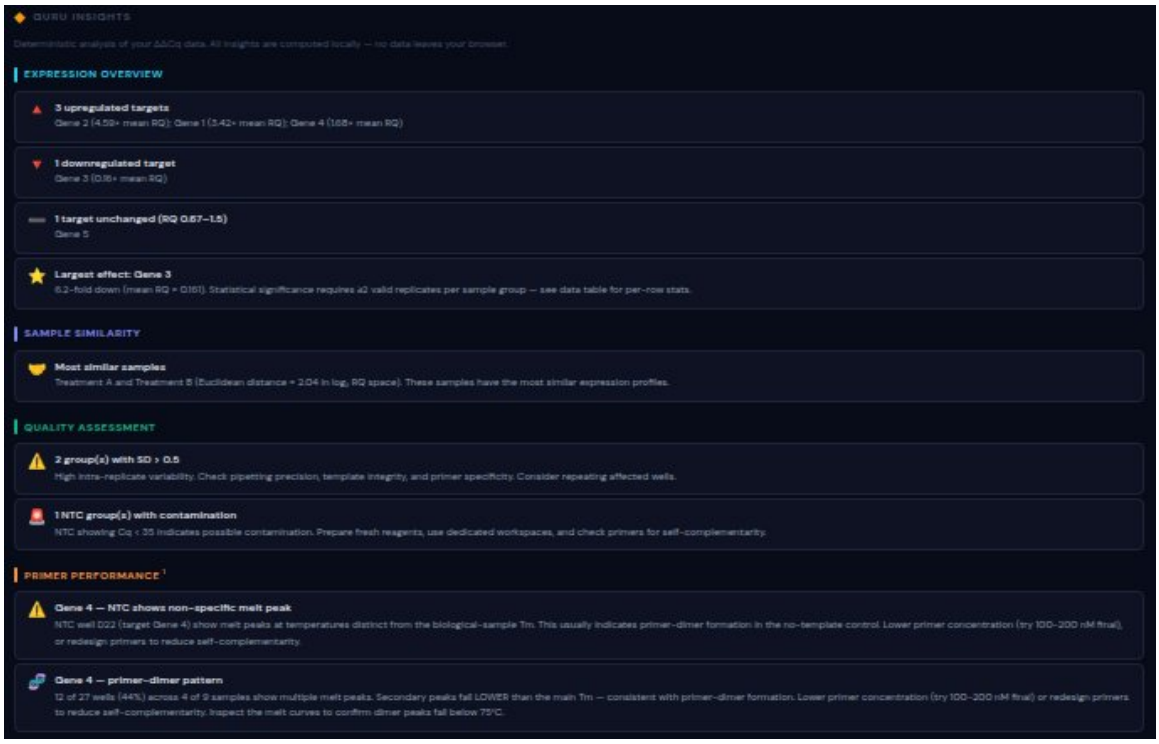

Figure 13.1. Guru Insights output. Findings are grouped into sections — Expression Overview (up/down/unchanged target counts with mean RQ), Sample Similarity (Euclidean distance between groups in log-RQ space), Quality Assessment (groups with elevated SD or NTC contamination), and Primer Performance (melt-peak anomalies suggesting primer-dimer or non-specific amplification). All Insights are computed locally — no data leaves the browser.

13.1 Automated Analysis Review

Guru Insights evaluates:

- Reference gene stability and whether additional reference genes are recommended.
- Significantly up- or downregulated targets.
- Outlier detection and quality issues.
- MIQE compliance status.
- Statistical test results and their interpretation.
- Sample similarity and unexpected clustering.
- Power analysis and follow-up recommendations.

13.2 How to Interpret Recommendations

Insights are categorized as:

- **Findings (blue):** Key observations about your data.
- **Warnings (amber):** Potential issues that may affect data quality.
- **Recommendations (green):** Suggested actions to improve your analysis.

Tip: Guru Insights requires login. Your work is automatically saved before redirecting to the login page if you are not yet authenticated.

14. Exporting Your Work

14.1 CSV Export

Export the full data table as a CSV file for further analysis in Excel, R, Python, or other tools. The CSV includes all columns: sample, target, mean Cq, SD Cq, ΔCq, ΔΔCq, SD ΔΔCq, RQ, RQ Min, RQ Max, p-value, t-stat, df, and (when FDR is enabled) p adj.

14.2 Figure Export

Every chart can be exported as a publication-ready figure:

Format	Best For	DPI Options
JPEG	Presentations, web	150, 300, 600 DPI
TIFF	Journal submissions (lossless)	150, 300, 600 DPI
PDF	Vector-quality documents	150, 300, 600 DPI

The export bar appears above each chart. Select your desired format and DPI, and the figure is generated at full resolution. Section 7 includes a gallery of the chart types available.

14.3 PDF Reports

Click **Generate Report** to produce a comprehensive PDF report including your QC summary, MIQE compliance score, data table, charts, and Guru Insights (if available). This report is designed for lab notebooks, thesis appendices, or publication supplementary materials.

14.4 RDML Import

RDML (Real-time PCR Data Markup Language) is the standardized format for qPCR data exchange, endorsed by the MIQE guidelines. qPCR Guru imports RDML files (v1.1–1.4) including ZIP-compressed variants, so you can bring data into qPCR Guru regardless of which instrument or vendor software produced it.

Note: For sharing your completed analysis with collaborators, use the PDF report or CSV export — these preserve full analysis fidelity across any downstream tool.

Tip: Exports (CSV, figures, reports) are available to all registered users.

15. Saving & Loading Projects

15.1 Local Save

Click the **Save** button in the header to save your current analysis to your browser's local storage. This preserves your wells, format, filename, and instrument data so you can return to your analysis later.

> **Note:** Saved data is stored locally in your browser only. It will not transfer to other devices or browsers. Clearing your browser data will delete saved projects.

15.2 Loading Previous Work

On the upload screen, saved projects appear in the **Project History** section. Click any saved project to reload it instantly, restoring all your data and resuming where you left off. Up to 10 recent projects are retained.

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