USER GUIDE

applied biosystems® by life technologies™

CopyCaller® Software v2.0

for use with:

ViiA7™ Real-Time PCR System
Applied Biosystems 7900HT Fast Real-Time PCR System
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems
Applied Biosystems StepOnePlus™ Real-Time PCR System

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life technologies™

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Part Number 4400042 Rev. C
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Contents

About This Guide

CAUTION! ABBREVIATED SAFETY ALERTS. Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For the complete safety information, see the "Safety" appendix in this document.

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

Purpose

The *CopyCaller*® Software v2.0 User Guide provides reference information for CopyCaller® Software and describes how to analyze the data from TaqMan® Copy Number Assays experiments.

Prerequisites

This guide uses conventions and terminology that assume a working knowledge of the Microsoft® Windows® operating system, the Internet, and Internet-based browsers.

User attention words

Five user attention words may appear in this document. Each word implies a particular level of observation or action as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation or accurate chemistry kit use.

CAUTION! Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

CopyCaller® Software v2.0 User Guide

Overview

About this guide

The guide is intended for novice and experienced users who use CopyCaller® Software v2.0 for copy number variation (CNV) research. It uses conventions and terminology that assume a working knowledge of the Microsoft® Windows® operating system, the Internet, and Internet-based browsers.

Purpose of the software

Applied Biosystems CopyCaller® Software performs relative quantitation analysis of genomic DNA targets using the real-time PCR data from Pre-designed TaqMan® Copy Number Assay, Custom Plus TaqMan® Copy Number Assay, or Custom TaqMan® Copy Number Assay experiments. The software and associated copy number assays can be used to detect and measure copy number variation of specific sequences in human and mouse genomes.

Features

CopyCaller® Software can:

- Open copy number experiment absolute quantitation data collected by the:
  - ViiA 7™ Real-Time PCR System
  - Applied Biosystems 7900HT Fast Real-Time PCR System
  - Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems
  - Applied Biosystems StepOnePlus™ Real-Time PCR System
- Perform copy number analyses with or without a known calibrator sample.
- Report confidence in individual or grouped copy number calls.
- Display the calculated sample copy number data in both table and graphic formats.
- View detailed data analysis information.
- Change analysis parameters and reanalyze the data.
- Simultaneously analyze and view data from multiple experiments.
- Save or print analyzed data for further analysis or for sharing with other laboratories that use CopyCaller® Software.

About the analysis

Applied Biosystems CopyCaller® Software analyzes data generated by TaqMan® Copy Number Assay experiments run on Applied Biosystems real-time PCR systems. In a copy number quantitation reaction, purified genomic DNA is combined with the:

- TaqMan® Copy Number Assay, containing two primers and a FAM™ dye-labeled MGB probe to detect the genomic DNA target sequence.
- TaqMan® Copy Number Reference Assay, containing two primers and a VIC® and TAMRA™ dye-labeled probe to detect the genomic DNA reference sequence.
- TaqMan® Genotyping Master Mix, containing AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure) and dNTPs that are required for the PCR.

  Note: TaqMan® Gene Expression Master Mix, TaqMan® Universal Master Mix (with or without AmpErase uracil-N-glycosylase), or TaqMan® Universal Master Mix II, no UNG, can be used in place of TaqMan® Genotyping Master Mix.

TaqMan® Copy Number Assays are run simultaneously with a TaqMan® Copy Number Reference Assay in a duplex, real-time PCR. Each copy number assay detects a target gene or genomic sequence of interest; the reference assay detects a sequence known to exist in two copies in a diploid genome (for example, the human RNase P H1 RNA gene). After the reaction mix is dispensed on a plate, the reactions are run on an Applied Biosystems real-time PCR system using absolute quantitation settings.

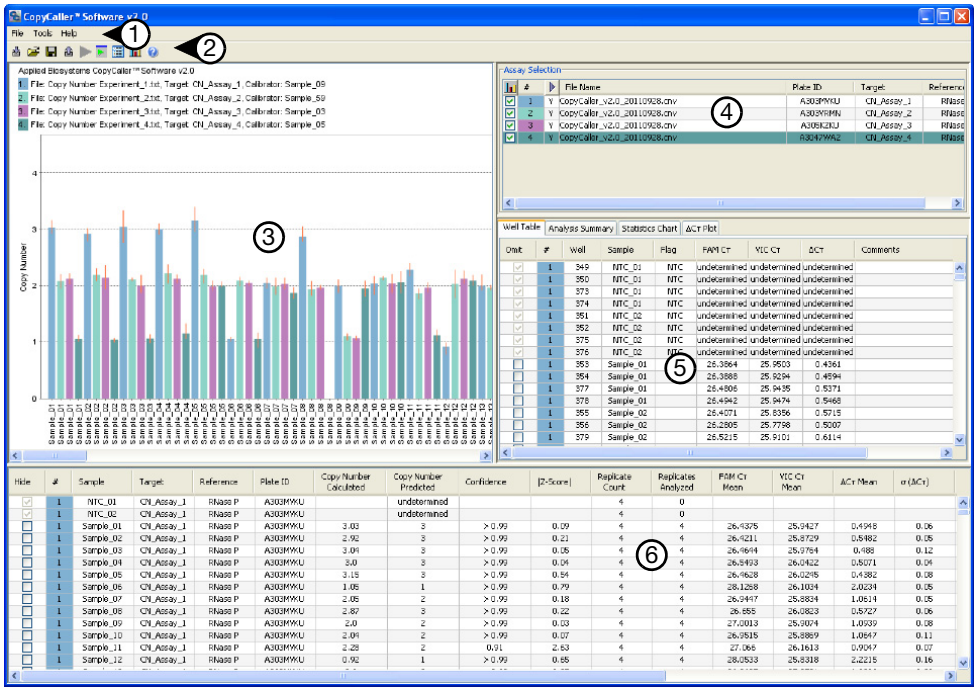
After amplification, the experiment Results Table, containing C_T values for the copy number and reference assay for each well, is exported from the Applied Biosystems real-time PCR system software. The tab-delimited (.txt) file or comma-separated value (.csv) file is then imported into CopyCaller® Software for post-PCR data analysis of the copy number quantitation experiment.

CopyCaller® Software performs a comparative C_T ($\Delta\Delta C_T$) relative quantitation analysis of the real-time data. The analysis determines the number of copies of the target sequence in each test genomic DNA sample. The comparative C_T ($\Delta\Delta C_T$) method first calculates the difference (ΔC_T) between the threshold cycles of the target and reference assay sequences. Then, the method compares the ΔC_T values of the test samples to a calibrator sample that contains a known number of copies of the target sequence. Alternatively, the analysis can be performed without the use of a calibrator sample.

Note: For details on the analysis algorithm that is used by CopyCaller® Software, see Appendix B, "Background Information" on page 55. For more information on TaqMan® Copy Number Assay experiments, refer to the *CopyCaller Software Protocol* (Part no. 4397425).

About the interface

The CopyCaller® Software interface consists of the six major elements shown in the following figure.



Elements of CopyCaller® Software include:

(1) Menu bar

- File menu – Imports real-time PCR quantitation results; opens or saves a CopyCaller® Analysis file; exports copy number analysis results; exits the software.
- Tools menu – Changes the analysis and plot settings.
- Help menu – Opens this document in portable document format (PDF).

(2) Toolbar

- – Adds one or more real-time PCR results export files to the analysis.
- – Opens a CopyCaller® (saved analysis) file.
- – Saves the analysis results in a CopyCaller® (.cnv) file.
- – Exports copy number analysis results.
- – Analyzes the data.
- – Views and edits the analysis settings.
- – Views and edits the Copy Number Plot settings.
- – Opens an enlarged Copy Number Plot.
- – Displays information about the software.

(3) Copy Number Plot – Displays the copy number in each sample for the selected copy number assays. The plot shows samples selected in the Assay Selection Table (see "Review the Copy Number Plot" on page 23 for more information).

(4) Assay Selection Table – Displays all copy number assays imported into the current analysis session (see "Select assays and analyze the data" on page 18 for more information).

(5) Analysis tabs – Displays information and results for the selected copy number assays.

- Well Table tab – Displays the data from the individual wells of the selected assays (see "Review the Well Table" on page 30 for more information).
- Analysis Summary tab – Displays the summary of the analyzed data for each selected copy number assay (see "Review the Analysis Summary" on page 34 for more information).
- Statistics Chart tab – Displays a pie chart or histogram of the analyzed data for each assay that is selected in the Assay Selection Table (see "Review the Statistics Chart" on page 35 for more information).
- ΔC_T – Displays the ΔC_T distribution for the analyzed data for each selected assay (see "Review the ΔC_T Plot" on page 36 for more information).

(6) Results Table – Displays the results of the copy number analysis for assays selected in the Assay Selection Table (see "Review the Results Table" on page 27 for more information).

Analysis workflow

This user guide explains how to prepare files for and perform tasks with CopyCaller® Software according to the following workflow:

Prepare the real-time PCR data

1. Perform TaqMan® Copy Number Assay experiments (page 13)
2. Analyze the experiments using the real-time PCR system (page 15)
3. Export the real-time PCR files (page 16)

Note: To troubleshoot copy number experiments using the real-time PCR system software, see page 41.

Perform the copy number analysis

1. Import the exported real-time PCR files in CopyCaller® Software (page 17).
2. Select assays and analyze the data (page 18).

Note: To troubleshoot CopyCaller® Software error messages, see page 52.

Review the results

1. Review the Copy Number Plot (page 23)
2. Review the Results Table (page 27)
3. (Optional) Edit the analysis settings (page 30)
4. Review the Well Table (page 30)
5. (Optional) Remove outliers and reanalyze (page 32)
6. Review the Analysis Summary (page 34)
7. Review the Statistics Chart (page 35)
8. Review the ΔC_T Plot (page 36)
9. Save, export, and open the analysis (page 39)

Note: To troubleshoot:
- CopyCaller® Software error messages, see page 52.
- Problematic data found during CopyCaller® Software analysis, see page 48.

Note: For information on performing TaqMan® copy number experiments, refer to the TaqMan® Copy Number Assays *Protocol* (see "Related documentation" on page 77).

Perform TaqMan® Copy Number Assay experiments

Perform TaqMan® Copy Number Assay experiments on your real-time PCR system according to the TaqMan® Copy Number Assays *Protocol* (Part no. 4397425) using absolute quantitation settings and the guidelines listed below.

IMPORTANT! CopyCaller® Software can import and analyze real-time PCR data exported from experiments conducted using absolute quantitation settings only.

Experiment setup guidelines

- Use four technical replicates for each gDNA sample.
- Apply to each well of the reaction plate a sample name and a detector/target that includes dye information (reporter and quencher).
- Apply the same sample name to the wells of each technical replicate group. CopyCaller® Software combines data of replicate wells only if they share the same sample name. If the replicate wells are named differently (for example, smpl012a and smpl012b), the software analyzes the wells as different samples.
- Apply unique detector/target names to the wells of plates that contain multiple TaqMan® Copy Number Assays or TaqMan® Copy Number Reference Assays. When a plate contains more than one kind of copy number assay or reference assay, label the wells according to the assay(s) that they contain. CopyCaller® Software can separate and individually analyze the data from multiple assays only if the associated wells are labeled with unique assay names.

  Note: If you use SDS software v1.*X*, you must specify "FAM" and "VIC" as the detector names for the copy number and reference assays respectively. SDS software v1.*X* does not export dye information, so you must specify the reporter dye(s) in the detector name fields.
- Apply the setup data shown in the table below for your real-time PCR system to each TaqMan® Copy Number experiment. CopyCaller® Software requires that exported data files contain both reporter and quencher dye information.

  IMPORTANT! The shaded cells in the table on page 14 indicate that you must enter the specified values exactly as shown.

Required setup information for a TaqMan® Copy Number experiment

Assay	Detector name	Target name	Reporter	Quencher
7900HT Fast System (SDS Software v2.X) – Absolute quantitation plate document‡				
TaqMan® Copy Number Assay	User-defined	N/A	FAM	Nonfluorescent
TaqMan® Copy Number Reference Assay	User-defined	N/A	VIC	TAMRA
7300/7500/7500 Fast System (SDS Software v1.*X*) – Absolute quantitation plate document				
TaqMan® Copy Number Assay	FAM§	N/A	FAM	(none)
TaqMan® Copy Number Reference Assay	VIC§	N/A	VIC	TAMRA
7500/7500 Fast System (7500 Software v2.X) or **Viia7™ Software v1.X** or **StepOnePlus™ System (StepOne Software v2.X) – Quantitation-Standard Curve experiment‡**				
TaqMan® Copy Number Assay	N/A	User-defined	FAM	NFQ-MGB
TaqMan® Copy Number Reference Assay	N/A	User-defined	VIC	TAMRA

‡ If you run more than one TaqMan® Copy Number or Reference Assay on a plate, you can enter the names of the assays in the Detector/Target Name fields so that CopyCaller® Software analyzes the data from each assay separately.

§ If you use SDS software v1.*X*, you must specify "FAM" and "VIC" as the detector names for the copy number and reference assays respectively. SDS software v1.*X* does not export dye information, so you must specify the reporter dye(s) in the detector name.

Note: For complete guidelines on performing experiments using TaqMan® Copy Number Assays, refer to the TaqMan® Copy Number Assays *Protocol* (Part no 4397425).

Analyze the experiments using the real-time PCR system

After running each TaqMan® Copy Number Assay experiment using absolute quantitation settings, perform a preliminary analysis of the real-time data using the software for the real-time PCR system used to run the samples. The analysis generates the threshold cycle (C_T) values for the experiment that CopyCaller® Software uses in the copy number analysis.

Analyze the results

Applied Biosystems recommends the following analysis settings for TaqMan® Copy Number Assay experiments:

- Manual C_T Threshold: **0.2**
- Automatic Baseline: **On**

For each absolute quantitation experiment that you want to include in the analysis:

- Verify that the amplification curves for the reference assay (VIC® signal) in all samples have a distinct, linear amplification phase.
- Verify that the amplification curves for the copy number assay (FAM™ signal) in most wells have a distinct, linear amplification phase.

  Note: Zero-copy samples should amplify minimally, or not at all, for the target of interest.

- Review any displayed quality check (QC) flags, then review the real-time data of the associated samples.

For more information on analyzing your experiment, refer to the user guide or online help for your real-time PCR system.

Export the real-time PCR files

After you use the real-time PCR system software to analyze each TaqMan® Copy Number experiment, export each experiment results or Results Table to one or more real-time PCR files.

IMPORTANT! Do not modify the exported data files. CopyCaller® Software may not be able to import files that have been modified.

Export the results

IMPORTANT! If you run multiple TaqMan® Copy Number or Reference Assays on each plate, make sure to apply distinct assay detector/target names to the document as described in "Experiment setup guidelines" on page 13. User-defined target/detector names allow CopyCaller® Software to arrange and analyze data independently for each assay in a single exported file.

IMPORTANT! If you are using a 7300/7500/7500 Fast system running SDS software v1.*X*, you must specify "FAM" and "VIC" as the detector names for the copy number and reference assays respectively. SDS software v1.*X* does not export reporter dye information, so you must specify the reporter dye(s) as the detector name.

Assays per plate	Did you specify the placement of the assay(s) using separate targets/ detectors?	Action
One	Not applicable	Export the real-time data to a tab-delimited text (.txt) or comma-separated values (.csv) exported file.
More than one	Yes	Export the real-time PCR data to a single exported file (.csv or .txt) that includes all wells of the plate. CopyCaller® Software uses the target/detector names to distinguish the data from the different assays.
	No	Export the real-time PCR data of each assay to a separate exported file. 1. Select the wells of the plate that contain the data from one of the TaqMan® Copy Number assays. 2. Select **File ▸ Export**, then export the data from the selected wells to an data file. 3. Repeat steps 1 and 2 to export the data from the other assays present on the plate. **Note:** To help with organization, name each exported file according to the assay data it contains.

Go to the next section,"Import the exported real-time PCR files in CopyCaller® Software".

Note: For more information on exporting data, refer to the user guide or getting started guide for your real-time PCR system (see "Related documentation" on page 77).

Import the exported real-time PCR files in CopyCaller® Software

Begin the analysis by importing the files exported by the real-time PCR system software.

Guidelines

CopyCaller® Software can import and analyze real-time PCR results files from copy number assay experiments that use absolute quantitation settings.

IMPORTANT! The files exported by the real-time PCR system software must include the setup information described in "Experiment setup guidelines" on page 13.

Import the results files

1. Start CopyCaller® Software.
   - Double-click  (CopyCaller® v2.0) on the desktop.

     or
   - Select **Start ▸ All Programs ▸ Applied Biosystems ▸ CopyCaller Software ▸ CopyCaller v2.0**.
2. In the CopyCaller® Software toolbar, click  (Import real-time PCR results file) or select **File ▸ Import**.
3. In the Import dialog box, select one or more real-time PCR files to analyze.

   To open more than one file:
   - Press the **Ctrl** key while you select noncontiguous files.
   - Press the **Shift** key while you select contiguous files.
4. Click **Open** to import the data from the selected real-time PCR results.
5. Repeat steps 2 to 4 as needed to add additional files to the analysis.

   When you import each results file, CopyCaller® Software displays the assay(s) associated with the data in the Assay Selection Table.
6. Go to "Select assays and analyze the data" on page 18.

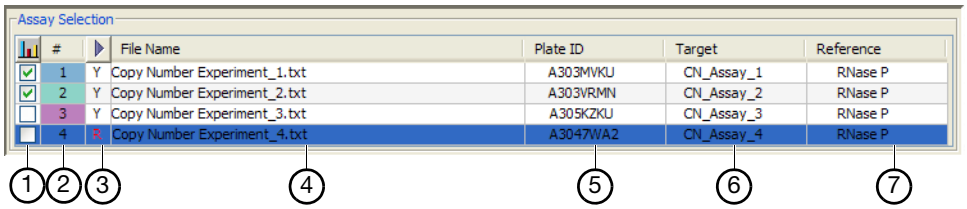
Note: You can open saved CopyCaller® Analysis files for viewing or reanalysis. CopyCaller® Software can open analyzed results files saved by the software (including CopyCaller® Software v1.0 analysis .txt or .csv files and CopyCaller® Software v2.0 analysis .cnv files). For more information, see "Save, export, and open the analysis" on page 39.

Select assays and analyze the data

After you import one or more results files, use the Assay Selection Table to analyze and display the copy number assay data. To begin the analysis, select the desired copy number assay(s), then specify the analysis settings for the assay(s).

About the Assay Selection Table

The Assay Selection Table displays information about the assays that you import into the current session of CopyCaller® Software. Click a table column header once to arrange the data in ascending order (▲), click it again to arrange in descending order (▼), then click it again to reset the column.



Columns of the Assay Selection Table include:

(1) (Display Analysis Results) – Displays the data for the associated assay.

(2) # (Assay ID) – A sequential identifier that is assigned by CopyCaller® Software to each copy number assay that is opened by the software. CopyCaller® Software uses the Assay IDs to identify related data in the Assay Selection Table, Copy Number Plot, Results Table, Well Table tab, Statistics Chart tab, and ΔC_T Plot tab. The column also displays the unique color assigned to the assay by the software. (The software displays a color only if the assay has been added to the analysis.)

Note: CopyCaller® Software assigns new IDs for each analysis session.

(3) (Analysis Status) – Indicates if the associated assay has been analyzed by the software, where Y = analyzed, R = reanalysis required, and (blank) = not analyzed.

(4) File Name – The name of the file that is imported into CopyCaller® Software.

(5) Plate ID (editable) – The plate barcode associated with the assay that was run using a 384-well plate. If a 96-well plate was used, the software displays a blank cell.

(6) Target – The TaqMan® Copy Number Assay used to detect and quantify the genomic region of interest.

(7) Reference – The TaqMan® Copy Number Reference Assay used to normalize the genomic DNA input.

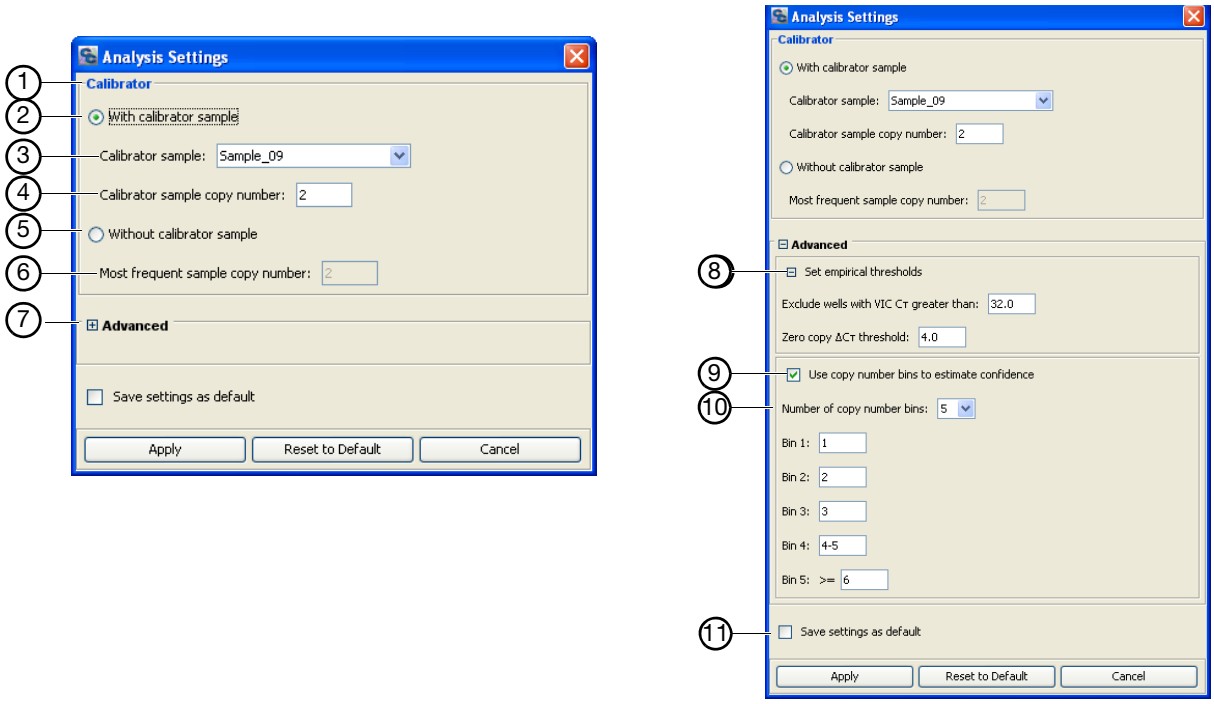
Note: If a file contains data from more than one copy number/reference assay combination (for example, plate wells were given multiple target or reference assay names), CopyCaller® Software splits the file into multiple rows in the Assay Selection Table (one row for each assay combination). See "Export the real-time PCR files" on page 16 for more information.

Select and analyze the copy number assays

CopyCaller® Software can analyze copy number experiment data with or without a calibrator sample. If you select "with calibrator sample" in the Analysis Settings dialog box, the software calculates copy number values using the comparative C_T method of relative quantitation. If you select "Without calibrator sample" in the Analysis Settings dialog box, the software calculates copy number values using a maximum likelihood algorithm. For more information, see Appendix B, "Background Information" on page 55.

About the analysis settings

The Analysis Settings dialog box allows you to specify the analysis settings for the assay(s) selected in the Assay Selection Table.



(1) Calibrator selection – Choose with calibrator sample or without calibrator sample analysis method.

(2) With calibrator sample – Configures CopyCaller® Software to perform the quantitative analysis of the selected assays using a calibrator sample as the basis for comparison. When selected, this option activates the Calibrator Sample Name drop-down list and the Calibrator Sample Copy Number field.

(3) Calibrator sample – The sample to be used as the calibrator sample. Enter the name of the calibrator or select a sample from the drop-down list. If you selected for analysis:

- A single assay, the software adds the "(median ΔC_T)" suffix to the sample(s) with the median ΔC_T for the population of analyzed samples. The median sample may be a candidate for use as the calibrator if most samples have an equivalent copy number. Also available is an option to select the **Median ΔC_T** per assay value as the calibrator. If there are two median ΔC_T samples for an assay, the Median ΔC_T is the average of these values.
- Multiple assays, the drop-down list displays samples common to all selected assays so that you can select a common calibrator sample. Also available is an option to select the **Median ΔC_T** per assay value as the calibrator. If there are two median ΔC_T samples for an assay, the Median ΔC_T is the average of these values.

(4) Calibrator sample copy number – The known copy number of the target in the calibrator sample.

(5) Without calibrator sample – Configures the software to perform an analysis of the selected assays without a calibrator sample. When selected, this option activates the Most Frequent Sample Copy Number field.

(6) Most Frequent Sample Copy Number – The number of copies that the majority of samples in the analysis are expected to contain.

Note: If the most frequent sample copy number is the same for all experiments, you can use the "Without Calibrator Sample" option to analyze multiple assays that do not share a common calibrator sample.

(7) Advanced – (Optional) Click the [+] to expand the advanced settings section to review and/or edit empirical thresholds or create copy number bins for confidence estimates.

[8] Set empirical thresholds:

- **Exclude wells with VIC C_T greater than** – Defines the filtering threshold used by CopyCaller® Software to remove poor quality data before the analysis (default value is 32). If a replicate has a VIC C_T greater than the threshold value, then CopyCaller® Software excludes the well from the analysis.
- **Zero copy ΔC_T threshold** – Defines the filtering threshold used by the software to categorize samples with relatively weak, background amplification as having zero copies of the target (default value is 4.0).

[9] Use copy number bins to estimate confidence – Enables grouping copy numbers into bins for estimation of confidence in the predicted copy number calls. Bins may contain successive single numbers or range of copy numbers; the final bin contains a single number and covers all copy numbers greater than or equal to this value. When this option is used, |z-score| values are not provided as part of the confidence estimates. For more information on quality metrics, see "Review the Results Table" on page 27 and '"About the quality metrics" on page 64.

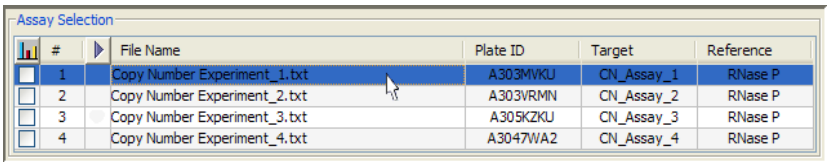
[10] Number of copy bins – Select the number of copy number bins to use for the data analysis (from 2-10). The appropriate number of bin boxes appears. Enter sequential, non-overlapping single numbers or range of numbers in each bin box; the final '>=' box must contain a single number value.

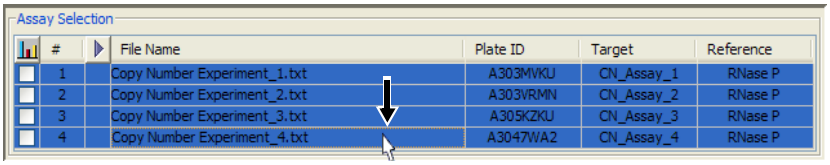
(11) Save Settings As Default – Configures CopyCaller® Software to apply the current analysis settings to all new assays added to the analysis.

Select and analyze the assay data

1. In the Assay Selection Table, select one or more assays to analyze. To select an assay, click anywhere in a row of the Assay Selection Table (the software highlights the selected row in blue).

   To select more than one assay in the Assay Selection Table, press the **Ctrl** or **Shift** key while you select individual assays, or click and drag across the list of assays.





   Note: If you select multiple assays, the selected assays are analyzed using identical analysis settings.

2. In the toolbar, click (Analysis Settings).

   Note: See "About the analysis settings" on page 19 for a complete description of the analysis settings.

3. In the Analysis Settings dialog box, Calibrator selection panel: specify the calibrator sample settings for the selected assay(s) depending on whether or not a calibrator sample of known copy number is available.

Calibrator present?	Action
Yes	1. Select **With Calibrator Sample**. 2. In the Calibrator Sample Name drop-down list, select or enter a sample to use as the calibrator for the analysis. 3. In the Calibrator Sample Copy Number field, enter the number of copies of the target sequence that are in the calibrator sample. The number of copies must be a whole number greater than zero.
No	1. Select **Without Calibrator Sample**. 2. In the Most Frequent Sample Copy Number field, enter the number of copies of the target sequence expected in the majority of samples. The number of copies must be a whole number greater than zero.

4. (*Optional*) Expand the Advanced settings box to review and/or edit empirical thresholds, or to create copy number bins for confidence estimates:
   - **Exclude wells with VIC C_T greater than** —Enter a cycle number to specify the threshold above which CopyCaller® Software excludes samples from the analysis. We recommend a default VIC threshold value of 32.
   - **Zero copy ΔC_T threshold** — Enter a ΔC_T value to specify the threshold above which CopyCaller® Software will classify samples as zero copy samples. We recommend a default ΔC_T threshold value of 4.0.
   - **Use copy number bins to estimate confidence** — To use this option:

     First, select the number of copy number bins to use for the data analysis (from 2-10). The selected number of bin boxes appears.

     Enter sequential single numbers or range of numbers in each bin box; the final '>=' box must contain a single number value.

     When the copy number bins option is used, calculated confidence values for predicted copy numbers will be based on the copy number bins. Note that |z-score| values are not provided as part of the confidence estimates when the copy number bins option is used, because copy number bins are not normal distributions.

     For more information on quality metrics, see "Review the Results Table" on page 27 and "About the quality metrics" on page 64.

5. Click **Apply** to apply the analysis settings and perform copy number analysis using the revised analysis settings.

   After the analysis is complete, CopyCaller® Software displays "Y" in the (Analysis Status) column of the Assay Selection Table for each analyzed assay.

6. Repeat steps 1 through 5 as necessary to analyze any remaining assays.

7. Display the results of the analysis:
   a. In the Assay Selection Table, verify that the assays that you want to add to the analysis show "Y" in the (Analysis Status) column. If not, analyze the unanalyzed assay(s) as explained above.
   b. In the (Display Analysis Results) column, select the check boxes for up to 10 analyzed assays that you want to display.

8. Go to "Review the Copy Number Plot" on page 23.

Remove an assay

Removing a copy number assay from the Assay Selection Table removes the assay results from the current display and the analysis.

1. In the Assay Selection Table, select the row corresponding to the assay that you want to remove.
2. Right-click the selected row, then select **Remove from analysis** (or press **Delete**).
   The selected assay is deleted from the current session of CopyCaller® Software.

Note: Deleting an assay from CopyCaller® Software does not delete the associated exported real-time PCR file from the computer drive.

Multiplate analysis

When you right-click an assay row in the Assay Selection Table, there is also the option **Combine plates for multiplate analysis**. We highly recommend single plate copy number analysis whenever possible because the highest quality copy number data is generated when measurement variation is very low. When data from multiple plates is combined for analysis, plate-to-plate experimental variation may be introduced and may impact the quality of the copy number analysis results. For more information about multiplate analysis, see Appendix C on page 69.

Open CopyCaller® Analysis files

1. Start CopyCaller® Software.
   - Double-click  (CopyCaller® Software) on the desktop.
     or
   - Select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **CopyCaller Software** ▸ **CopyCaller v2.0**.
2. In the CopyCaller® Software toolbar, click  (Open analysis) or select **File** ▸ **Open**.
3. In the Open dialog box, select one or more CopyCaller® Analysis files (.cnv).
   To open more than one file:
   - Press the **Ctrl** key while you select noncontiguous files.
   - Press the **Shift** key while you select contiguous files.
4. Click **Open**.

Note: CopyCaller® Software v1.0 analysis files (.txt or .csv suffix) can be opened in CopyCaller® Software v2.0 by choosing **All Files** in the Open dialog box.

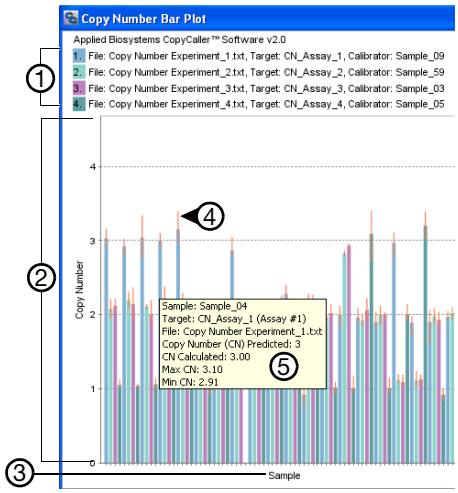
Review the Copy Number Plot

After you select one or more assays for display, perform an initial review of the Copy Number Plot. For each selected copy number assay, the plot displays the calculated or predicted copy number of each sample, and bars that indicate the copy number range for the associated replicate group. You can view the copy number data in the Copy Number Plot (see page 24), edit the plot settings from the Plot Settings dialog box (see page 25), and print or save the plot (see page 26).

About the Copy Number Plot

The Copy Number Plot displays a bar graph of the data in the Copy Number Calculated column of the Results Table. The software displays one bar for each analyzed sample. Within each bar, the software displays a red line (bar) that represents the minimum and maximum copy number calculated for the sample replicate group. To compare the results from several copy number assays, select multiple assays in the Assay Selection Table. See "Select and analyze the copy number assays" on page 18 for more information.

Note: To view a large version of the plot, click (View enlarged plot).



Elements of the Copy Number Plot include:

(1) Legend – The assays that are analyzed by the software and displayed by the plot.

(2) y-axis (Copy Number) – Displays the copy number for each sample associated with the assays selected in the Assay Selection Table.

(3) x-axis (Samples) – Displays the samples associated with the assays selected in the Assay Selection Table.

Note: The Copy Number Plot does not display data for "Undetermined" samples, where the copy numbers for the associated samples cannot be calculated. For example, a sample could be "Undetermined" if it shows no amplication for the reference assay (a VIC C_T greater than the threshold that is specified in the Analysis Settings dialog box). The software displays the names of "Undetermined" samples in red.

(4) Copy Number Range Bars – Indicates the minimum and maximum copy number (CN) calculated for the sample replicate group.

(5) Tooltip – For the bar of interest, displays the sample name, target assay name, and copy number data (predicted, calculated, maximum, and minimum values).

About the assay colors

CopyCaller® Software automatically assigns a unique color to each assay that you open for analysis. After you select an assay to be displayed, the software uses the same color to identify data for the assay in the Well Table, the Results Table, the Copy Number Plot, and the ΔC_T Plot view.

CopyCaller® Software uses only ten colors to display assay data. The software assigns colors to assays as they are selected for display. When an assay is removed from display (by deselecting the associated checkbox in the Assay Selection Table), then the color assigned to the assay becomes available for the software to reassign to another assay.

Note: You can change the colors that the software assigns to assays. For more information, see "View or edit the plot preferences" on page 25.

Guidelines for reviewing the plot

Review the Copy Number Plot for the following:

- Samples should have calculated copy number values close to integers and small range bars.
- Review the plot for intermediate copy numbers. The presence of intermediate, calculated copy number values (such as 1.5) can indicate that the calibrator or copy number was specified incorrectly, or a potential problem exists with the associated test sample or calibrator sample. See "Troubleshooting CopyCaller® Software analysis results" on page 48 for more information.
- Review the copy number range bars for each sample. Large bars may indicate that the technical replicates of the associated sample exhibit a broad range of ΔC_T values, possibly indicating that sample data quality is suboptimal. To examine the individual ΔC_T values for the technical replicates, see "Review the Well Table" on page 30.

  Note: The copy number range of replicates is frequently larger for samples that have high target copy numbers (>3) as a result of their smaller ΔC_T values.

View the plot

Use the following methods to view data in the Copy Number Plot:

Display copy number tooltips	In the Copy Number Plot, put the mouse pointer over a bar or a sample name of interest. A tooltip displays the sample name, target assay name, and copy number data (predicted, calculated, maximum, and minimum values).
Select sample bars in the copy number plot	In the Copy Number Plot, select a bar or bars of interest. CopyCaller® Software automatically: • Displays the bar and the sample name on the x-axis in blue. • Selects the assay(s) in the Assay Selection Table, and the related data in the Results Table, Well Table tab, and ΔC_T Plot tab. To select more than one sample, press the **Ctrl** key while you select individual samples.

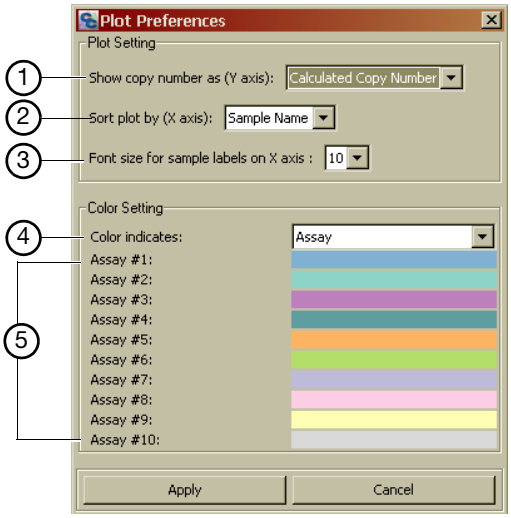
Zoom in on a copy number region of the plot	To zoom in for detailed viewing, click and drag the mouse pointer vertically down the Copy Number Plot to highlight a region of interest. Release the mouse button to expand the plot. To return to the default Copy Number Plot view, click and drag the pointer vertically up the plot, then release the mouse button.
View an enlarged Copy Number Plot in a new window	Double-click the left mouse button on the Copy Number Plot or click on the View enlarged plot icon .

After you review the Copy Number Plot, review the results of the analysis in the Result Table. Go to "Review the Results Table" on page 27.

View or edit the plot preferences

About the Plot Settings

Elements of the Plot Settings dialog box include:



(1) Show Copy Number As (Y axis) – Changes the data displayed by the Copy Number Plot, where:

- **Calculated Copy Number** – Configures the plot to display the data shown in the Copy Number Calculated column of the Results Table (see page 27).
- **Predicted Copy Number** – Configures the plot to display the data shown in the Copy Number Predicted column of the Results Table (see page 27).

(2) Sort Plot By (X axis) – Changes the sequence of the samples in the plot, where:

- **Sample Name** – Sorts the samples in the plot by name.
- **Assay** – Sorts the samples in the plot by assay ID.
- **Copy Number** – Sorts the samples in the plot by target copy number in the sample.

[3] Font size for sample labels on X axis – Changes font size. Choose 10, 12, or 14 size font.

(4) Color indicates – Changes the way the software applies color to the bars, where:

- **Assay** – Configures the plot to display each assay in a different color.
- **Predicted Copy Number** – Configures the Copy Number Plot, Statistics Chart pie chart, and ΔC_T Plot to display each predicted copy number (up to copy number 10) in a different color.

(5) Colors – Change the colors used for the assay color or for the predicted copy number color.

Note: To change a color, click a color bar, use the color picker utility to select a color, then click **OK** to apply the color.

To view or edit the plot settings:

1. In the toolbar, click  (View Plot Preferences).
2. In the Plot Settings dialog box, change the plot and color settings as desired.
3. Click **Apply** to apply the settings and close the dialog box.

Copy, save, or print the plot

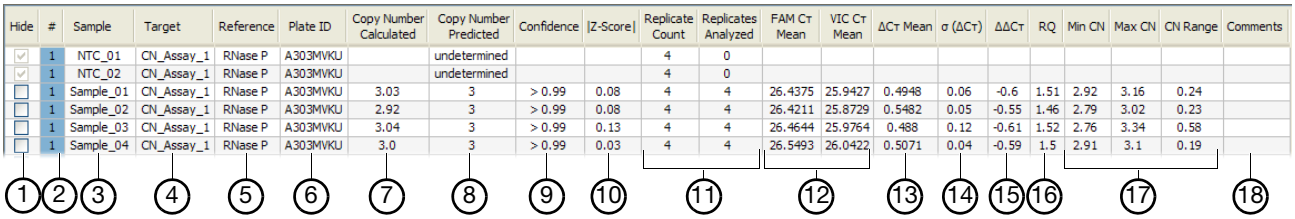
You can copy, save, or print the Copy Number Plot for use in documents or presentations. For instructions, see "Copy, save, or print the Copy Number Plot, Statistics Chart, or ΔC_T Plot" on page 40.

Review the Results Table

Review the Results Table for information about the assay, reaction plate, and real-time PCR for each assay that you selected in the Assay Selection Table.

About the Results Table

The Results Table displays the results of the copy number analysis of individual samples. Each row of the table represents the combined values of the technical replicates for a single sample.



Hide	#	Sample	Target	Reference	Plate ID	Copy Number Calculated	Copy Number Predicted	Confidence	|Z-Score|	Replicate Count	Replicates Analyzed	FAM CT Mean	VIC CT Mean	ΔCT Mean	σ (ΔCT)	ΔΔCT	RQ	Min CN	Max CN	CN Range	Comments
☑	1	NTC_01	CN_Assay_1	RNase P	A303MVKU		undetermined			4	0										
☑	1	NTC_02	CN_Assay_1	RNase P	A303MVKU		undetermined			4	0										
☐	1	Sample_01	CN_Assay_1	RNase P	A303MVKU	3.03	3	> 0.99	0.08	4	4	26.4375	25.9427	0.4948	0.06	-0.6	1.51	2.92	3.16	0.24	
☐	1	Sample_02	CN_Assay_1	RNase P	A303MVKU	2.92	3	> 0.99	0.08	4	4	26.4211	25.8729	0.5482	0.05	-0.55	1.46	2.79	3.02	0.23	
☐	1	Sample_03	CN_Assay_1	RNase P	A303MVKU	3.04	3	> 0.99	0.13	4	4	26.4644	25.9764	0.488	0.12	-0.61	1.52	2.76	3.34	0.58	
☐	1	Sample_04	CN_Assay_1	RNase P	A303MVKU	3.0	3	> 0.99	0.03	4	4	26.5493	26.0422	0.5071	0.04	-0.59	1.5	2.91	3.1	0.19	

Columns of the Results Table include:

(1) Hide – Includes or excludes the data of the associated sample in or from the Copy Number Plot.

(2) # (Assay ID) – A sequential identifier that is assigned by CopyCaller® Software to each copy number assay that is opened by the software. CopyCaller® Software uses the Assay IDs to identify related data in the Assay Selection Table, Copy Number Plot, Results Table, Well Table tab, Statistics Chart tab, and ΔC_T Plot tab.

(3) Sample – The sample whose data are associated with the row.

(4) Target –The TaqMan® Copy Number Assay used to detect and quantify the genomic region of interest.

(5) Reference – The TaqMan® Copy Number Reference Assay used to normalize the genomic DNA input.

(6) Plate ID – The bar code of the plate used to run the assay.

(7) Copy Number Calculated – The copy number for the associated sample calculated by the relative quantitation method as:

$cn_{sample} = RQ_{sample} \times cn_{calibrator}$

where RQ = relative quantity of the associated sample; and *cn* = copy number.

(8) Copy Number Predicted – The predicted copy number of the associated sample.

(9) Confidence – The confidence value of the associated predicted copy number.

(10) |Z-Score| – The absolute z-score value of the associated copy number integer. See "Deviation |z-score| estimate" for more information.

(11) Replicate Count/Replicates Analyzed – For the associated sample, the number of technical replicates for the sample and the number of replicates included in the analysis.

Note: The Replicates Count and Replicates Analyzed values differ when replicate well data are omitted from the analysis.

(12) FAM/VIC C_T Mean – The arithmetic mean of the C_T that is calculated for the technical replicates of the associated sample, where the:

- FAM column displays the mean C_T for the signal associated with the FAM™ dye-labeled probe of the copy number assay.
- VIC column displays the mean C_T for the signal associated with the VIC™ dye-labeled probe of the reference assay.

(13) ΔC_T Mean – The arithmetic mean of the ΔC_T of the technical replicates for the associated sample.

(14) $\sigma(\Delta C_T)$ – The standard deviation of the ΔC_T of the technical replicate population for the associated sample.

(15) $\Delta\Delta C_T$ – The $\Delta\Delta C_T$ value for the associated sample, which is calculated as:

$(\Delta\Delta C_T)_{s,t} = \mu(\Delta C_T)_{s,t} - \mu(\Delta C_T)_{calibrator}$

where: *s* = sample; *t* = target copy number assay.

(16) RQ – The relative quantity for the associated sample, which is calculated as:

$$RQ_{(s,t)} = 2^{-(\Delta\Delta C_T)_{s,t}}$$

where: *s* = sample; *t* = target copy number assay.

(17) Min CN/Max CN/CN Range – The limits and the range for the calculated copy numbers (CN) of the technical replicates for the associated sample.

(18) Comments – Any comments that you want to include with the associated sample.

Guidelines for reviewing the Results Table

Review the Results Table for the following:

- Compare the calculated and predicted copy number values to the related data in the Copy Number Plot, Well Table, and ΔC_T Plot (see "Review the Well Table" on page 30 and "Review the ΔC_T Plot" on page 36 for more information).
- Examine the confidence values and absolute z-score values to assess the reliability of each copy number call.
- Review samples having a predicted copy number of "Undetermined."

  A sample is "Undetermined" if CopyCaller® Software cannot analyze the sample because the:
  - Reference assay did not amplify sufficiently, possibly indicating low sample quality.
  - Replicate data for a sample were conflicting.
- Review samples having a predicted copy number of 0 (zero-copy samples).

  Zero-copy samples produce reference assay amplification (passing VIC C_T values) and weak or nonexistent target amplification (generating the NOFAM or DCTET QC flags respectively). If a sample replicate group does not produce FAM amplification (an undetermined C_T), then the software calculates only the mean VIC C_T for the samples. If a sample replicate group produces measurable but weak FAM amplification, the software calculates a copy number that is approximately zero. (For more information on quality flags, see "Quality flags of the Well Table tab" on page 31.)

  Note: CopyCaller® Software cannot calculate confidence values for zero-copy-number samples. However, samples that produce no FAM signal are, by definition, high-confidence calls because no target DNA was amplified.
- Review samples having a predicted copy number that is greater than or equal to 1.

  CopyCaller® Software calculates confidence and absolute z-score values for each sample set that has a non-zero predicted copy number value and sufficient data for the estimation.

  Note: The software cannot calculate the confidence and absolute z-score values for sample sets that have fewer than seven samples of a single copy number because the algorithm requires a minimum number of data points (see "About the quality metrics" on page 64 for more information).

- Under optimal experimental conditions where samples are of high quality, copy number and reference assays have amplified, and sample replicates have similar C_T and ΔC_T values:
  - Samples that have low copy numbers (1, 2, or 3) commonly have confidence values greater than 95%.
  - As copy numbers increase, confidence progressively decreases due to the decreased separation of ΔC_T subdistribution values of copy numbers (see "Confidence estimate" on page 64 for more information).
  - Review samples that have confidence values greater than 95%.

    Samples that have high confidence values can sometimes deviate significantly from the mean copy number for the copy number subdistribution. For sample copy number calls having confidence values greater than 95%, look at the absolute z-scores, then consider accepting or rejecting the copy number call based on the following:

|Z-Score|	Status
< 1.75	Pass
2.65 > *z* ≥ 1.75	Pass with caution
≥ 2.65	Fail

Note: The thresholds in the table above are based on empirical observations and are provided only as guidelines.

Note: For more information on troubleshooting Results Table data, see "Troubleshooting CopyCaller® Software analysis results" on page 48.

When you finish reviewing the Results Table, adjust the analysis settings as explained below, or go to "Review the Well Table" on page 30.

Note: If you are working with targets that have high copy numbers in some samples (for example, 4 or more), and discrete high copy number values are not required in your analysis, consider using copy number bins to estimate confidence values. This option may provide higher confidence that a sample target copy number is within a given range or above a given value, as opposed to being equal to a discrete copy number. For example, a sample with a low confidence, predicted copy number of 5 may have a high confidence, predicted copy number of 4 or more. Note that confidence, but not absolute z-score values (which are an attribute of normal distributions), are calculated when the copy number bins option is used (see "Select and analyze the assay data" on page 20).

Export and save the Results Table data

You can export and save the Results Table data for use in documents, presentations, or downstream analysis. For specific instructions, see "Save, export, and open the analysis" on page 39.

Note: If you select more than one assay in the Assay Selection Table, the data for the assays are displayed vertically.

(Optional) Edit the analysis settings

After you perform an initial review of the analyzed data in the Copy Number Plot and the Results Table, you can optimize the analysis settings if necessary.

View or edit analysis settings

1. Select one or more assays in the Assay Selection Table, then click  (View Analysis Settings) in the toolbar.
2. Revise the analysis settings as needed.

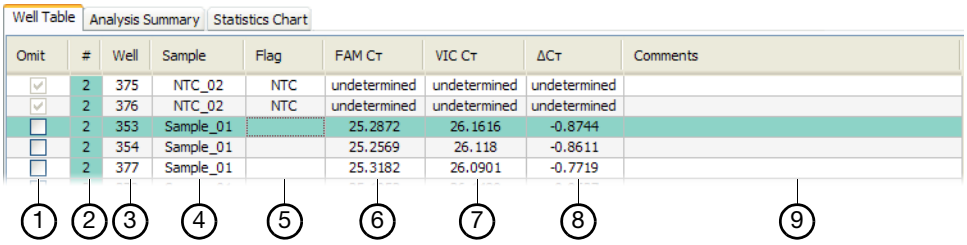
   For example, if the quality of the calibrator data is poor, you can select a different calibrator sample and reanalyze the data.
3. Click **Apply** to perform the copy number analysis using the revised analysis settings.

Review the Well Table

Use the Well Table tab to review and manage the well data of the analyzed results files. After you analyze and display one or more assays, use the Well Table tab to review the sample replicate data (including quality flags that were generated during the analysis; see page 31), and to omit or include well data (see page 32).

About the Well Table tab

The Well Table tab displays the well-level data for the assays selected in the Assay Selection Table. You can arrange data by selecting the column headers of the table. Click a table column header once to arrange the data in ascending order (▲), click it again to arrange in descending order (▼), then click it again to reset the column.



Columns of the Well Table include:

(1) Omit – A check box that includes or excludes the data of the associated well in or from the copy number analysis. When a check box is selected, CopyCaller® Software omits the associated well data from the analysis.

(2) # (Assay ID) – A sequential identifier that is assigned by the CopyCaller® Software to each copy number assay that is opened by the software. The CopyCaller® Software uses the Assay IDs to identify related data in the Assay Selection Table, Copy Number Plot, Results Table, Well Table tab, Statistics Chart tab, and ΔC_T Plot tab.

(3) Well – The position of the well on the associated plate.

Note: For 7900HT Fast system data, the well column displays numbered positions instead of coordinate positions. The wells on the plate are numbered sequentially left to right, top to bottom (for example, the well A1 is "1" and the well H12 is "96").

(4) Sample – The name of the sample that is in the well.

(5) Flag – The quality flags that are generated by the associated well data. See "Quality flags of the Well Table tab" on page 31 for more information.

(6) FAM C_T – The C_T for the TaqMan® Copy Number Assay.

(7) VIC C_T – The C_T for the TaqMan® Copy Number Reference Assay.

(8) ΔC_T – The difference between the FAM C_T and the VIC C_T.

(9) Comments – Any comments that you want to add to the associated well.

Quality flags of the Well Table tab

CopyCaller® Software assigns nine quality flags that indicate the quality status (not error) for a sample replicate (or well).

Category	Flag	Assigned when the associated well...
Undetermined wells/samples	NOVIC	VIC C_T value is undetermined (not measurable by the real-time PCR system).
	VICET	ΔC_T exceeds the "Exclude Wells With VIC C_T Greater Than" threshold defined in the Analysis Settings dialog box (see "About the analysis settings" on page 19).
	UNDET	Is associated with a replicate group where half of the replicates are zero-copy (as determined by NOFAM or DCTET rules) and half of the replicates have nonzero-copy number values (assigned the UNDET flag).

Category	Flag	Assigned when the associated well...
Zero-copy number wells/ samples	NOFAM	FAM C_T value is undetermined (not measurable by the real-time PCR system).
	DCTET	ΔC_T exceeds the "Zero copy ΔC_T threshold" defined in the Analysis Settings dialog box (see "About the analysis settings" on page 19).
Outlier wells	OZERO	Is an outlier in a replicate group where the majority are zero-copy.
	OCONF	Exceeds the outlier confidence determined by the software (see "About the quality metrics" on page 64).
	OUSER	Is manually omitted by the user.
No Template Control	NTC	Is assigned as a no-template control.

Guidelines for reviewing the Well Table

Review the Wells Table for the following:

- Compare the Well Table data to the related data in the Copy Number Plot and the Results Table (see "Review the Results Table" on page 27 for more information).
- For each replicate group, review the Flag column for any quality flags generated during the analysis, and determine the source of the warning.

  Some quality flags indicate potential issues with wells or samples. For example, wells that generate NOVIC or VICET flags did not amplify the reference assay target properly and may contain low-quantity or poor-quality DNA.

Note: For more information on troubleshooting the Wells Table data, see "Troubleshooting CopyCaller® Software analysis results" on page 48.

Omit wells

You can use the Well Table tab to omit wells from the copy number analysis. For information on removing outliers, see "(Optional) Remove outliers and reanalyze" on page 32.

(Optional) Remove outliers and reanalyze

After reviewing the results of the copy number analysis, you can use the Well Table tab to review well quality flags and sample replicate consistency, and to manually omit well data from the analysis, if necessary.

About outliers

Outliers occur when a factor other than initial sample quantity affects the PCR amplification and the measured C_T value. Outliers can result from either random experimental error or a number of laboratory errors, such as contamination, plate seal leaks, pipetting inaccuracies, or instrument issues. In the context of relative quantitation for duplex PCR, the range of the ΔC_T of the technical replicates is used as the basis for outlier removal.

CopyCaller® Software uses an automatic outlier removal algorithm to remove erroneous data values. The algorithm first estimates plate-level variability by calculating the ΔC_T standard deviation. The algorithm then eliminates technical replicates with ΔC_T values that deviate from the mean of the sample replicates by more than four standard deviations.

The outlier removal method used by CopyCaller® Software is conservative; We do **not** recommend a more aggressive approach. Subjective removal of too many data points can change the copy number prediction or confidence for a sample. For analyses that do not use a calibrator sample, outlier removal can affect the copy number of multiple samples. We recommend manually removing outliers only when the rationale to remove a technical replicate well is objective and obvious.

Note: For more information on the outlier removal algorithm, see "Outlier detection" on page 61.

Guidelines for identifying and removing outliers

Use the following guidelines to identify outliers:

Note: CopyCaller® Software

- Review the Well Table for QC flags. If any wells have generated QC flags:
  - Click the Sample header of the Well Table to arrange the wells by sample, then review the C_T values for the associated sample replicates.
  - Use your real-time PCR system software to view and troubleshoot the raw data.
- Review the calculated copy number values. The presence of copy number values between integers and large copy number ranges may (but not necessarily) indicate the presence of one or more replicate outliers.
- Review the standard deviation of ΔC_T displayed in the $\sigma(\Delta C_T)$ column of the Results Table. In general, an acceptable standard deviation of ΔC_T is less than 0.15.

Note: For more information on troubleshooting outliers, see "Troubleshooting CopyCaller® Software analysis results" on page 48.

Remove outliers

1. Select the **Well Table** tab.
2. Locate the well(s) that you want to omit from the copy number analysis.
3. For each outlier well, select its corresponding check box in the Omit column.

   Note: After omitting a well, the analysis status of the associated assay in the Assay Selection Table changes from "Y" to "R," indicating that the assay must be reanalyzed.
4. Click ▶ (Analyze) to perform copy number analysis for the assay without using the data from the outlier well(s).

Review the Analysis Summary

Review the Analysis Summary tab for the summarized results of the copy number analysis. While viewing the summary, you can copy and paste the data into other applications.

About the analysis summary

The Analysis Summary tab displays a summary of the results from the copy number analysis of the assays selected in the Assay Selection Table. If multiple assays are selected, then the Analysis Summary tab displays the additional summaries horizontally.

Elements of the Analysis Summary tab include:

[1] Assay drop-down list – Changes the Assay #, Target name.

(2) Assay # (Assay ID) – A sequential identifier that is assigned by CopyCaller® Software to each copy number assay that is opened by the software. CopyCaller® Software uses the Assay IDs to identify related data in the Assay Selection Table, Copy Number Plot, Results Table, Well Table tab, Statistics Chart tab, and ΔC_T Plot tab.

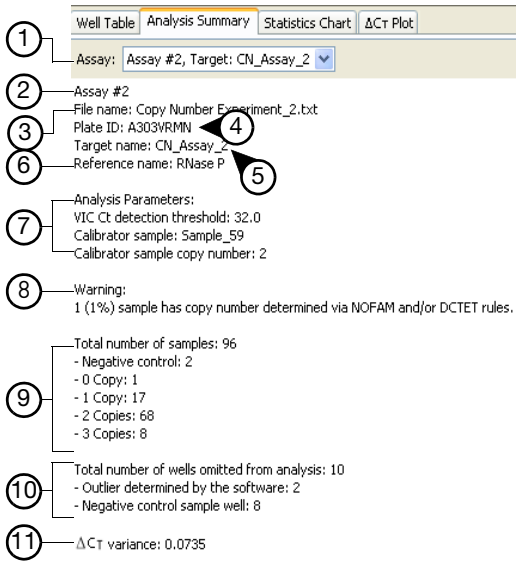
(3) File name – The name of the file opened by CopyCaller® Software.

(4) Plate ID – The bar code of the plate(s) used to run the samples associated with the assay.

(5) Target name – The copy number assay that is used to detect and quantify the genomic region of interest.

(6) Reference name – The reference assay that is used to normalize the genomic DNA input.

(7) Analysis Parameters – A summary of the analysis settings used to analyze the assay.



(8) Warnings Summary – A summary of warnings and quality flags generated during the analysis of the assay.

(9) Copy Number Summary – A summary of the copy number assignments, including the total number of analyzed samples, negative controls (NTC), and samples according to predicted copy number.

(10) Omitted Well Summary – A summary of the wells omitted from the analysis (wells identified as outliers and no-template [negative] controls).

[11] ΔC_T variability – The plate level variability standard deviation value.

Copying and pasting the Analysis Summary

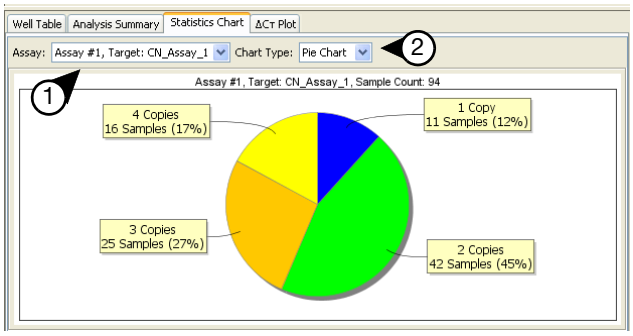
1. In the Analysis Summary tab, select the text that you want to copy.
2. Press **Ctrl+C** to copy the text.
3. Create or open a document in a word processing application (for example, Microsoft® Word), then press **Ctrl+V** to paste the text from the Analysis Summary tab into your document.

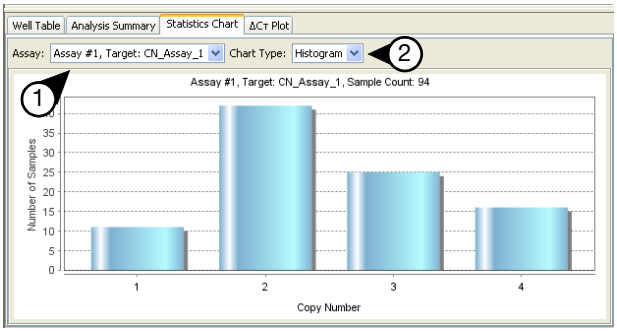
Review the Statistics Chart

Review the Statistics Chart tab for information about the distribution of copy numbers among the samples in a copy number assay. While viewing the Statistics Chart, you can copy, save, or print it.

About the Statistics Chart

The Statistics Chart tab displays the range of predicted copy numbers (in integers), the number of samples with a particular copy number, and the percentage of samples for each copy number. You can view the copy number statistics in a pie chart or in a histogram.





Elements of the Statistics Chart tab include the:

[1] Assay drop-down list – Changes the Assay #, Target name.

[2] Chart type drop-down list – Changes the plot type displayed.

[3] Pie chart – Displayed using the Predicted Copy Number color settings in the Plot Preferences. See "View or edit the plot preferences" on page 25.

[4] Histogram - Displayed using the assay color assigned by CopyCaller® when an assay is added to the analysis. See "View or edit the plot preferences" on page 25.

Copy, save, or print the pie chart or histogram

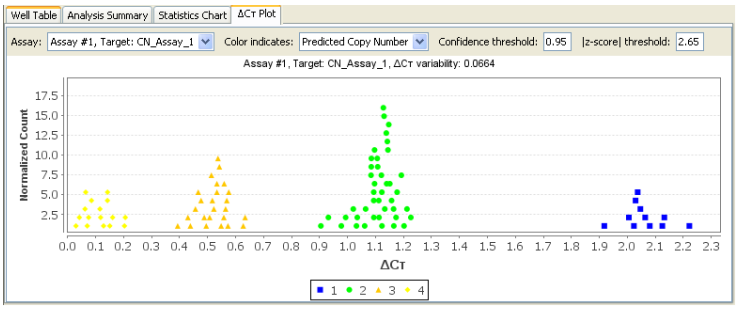
You can copy, save, or print the Statistics Chart for use in documents or presentations. For specific instructions, see "Copy, save, or print the Copy Number Plot, Statistics Chart, or ΔC_T Plot" on page 40.

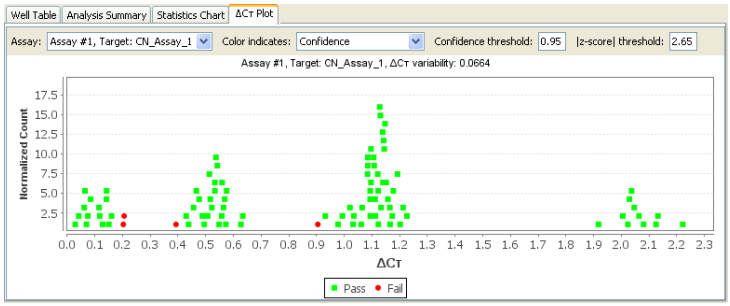
Review the ΔC_T Plot

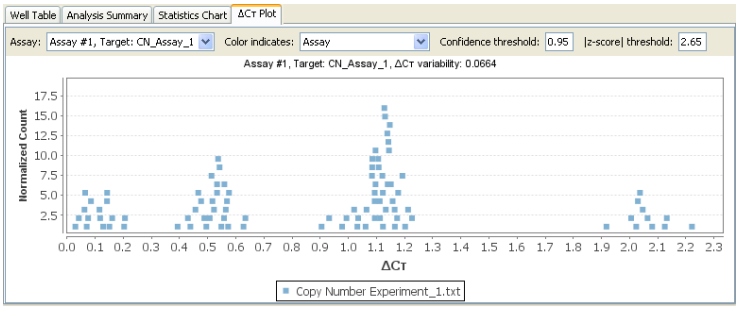
Review the ΔC_T Plot tab for information about the distribution of sample ΔC_T values in a copy number assay experiment. While viewing the ΔC_T Plot, you can copy, print or save it.

About the ΔC_T Plot

The ΔC_T Plot tab displays the distribution of sample ΔC_T values for a selected assay in a pseudohistogram. The X-axis corresponds to ΔC_T values and the Y-axis corresponds to a normalized count (in percentage) of the number of samples within a particular ΔC_T bin. The ΔC_T values are plotted in a fine grid of ΔC_T bins (0.04 C_Ts in size) at a height (y value) that corresponds to the order of appearance of a data point in the bin and at the exact ΔC_T value (x value). The ΔC_T Plot can be viewed with color coding that shows the predicted copy numbers or confidence pass/fail status for each sample, or the assigned plate assay color. The ΔC_T Plot views enable you to visually examine the copy number group ΔC_T normal distributions, to discern the data quality with respect to subdistribution clustering and separation, and to help evaluate why a particular copy number call and confidence estimate was made for a given sample.







Elements of the ΔC_T Plot include:

- **Assay** drop-down list — Changes the Assay #, Target name.
- **Color indicates** drop-down-list — Changes the plot view to show:

Predicted Copy Number	You can edit the color settings in Plot Preferences (**Tools** ▸ **Preferences**). See "View or edit the plot preferences" on page 25.
Confidence	Displays the pass or fail status of a sample based on the selected confidence and |z-score| thresholds.
Assay	The assay color assigned by CopyCaller® when an assay is added to the analysis See "View or edit the plot preferences" on page 25.

- Confidence threshold — Changes the confidence threshold used for the confidence value color coded plot view. Samples with confidence values greater than or equal to the chosen value (95% is the default value) will be designated as passing in the plot view unless they fail the |z-score| threshold.
- |z-score| – Changes the absolute z-score threshold used for the confidence value color coded plot view. Samples with absolute z-score values less than the chosen value (2.65 is the default value) will be designated as passing in the plot view unless they fail the confidence threshold. If copy number bins were used to estimate confidence, the |z-score| threshold option will be unavailable because |z-score| values were not calculated (see "About the analysis settings" on page 19).

  Note: For more information on confidence and absolute z-score values, see "Guidelines for reviewing the Results Table" on page 28 and "About the quality metrics" on page 64.

- Plot title— Includes the Assay #, Target name, and the ΔC_T variability.
- ΔC_T variability – The plate level ΔC_T variability is a standard deviation value that is an estimate of the variability in the ΔC_T measurements for an assay across all samples in the analysis.

Guidelines for reviewing the ΔC_T Plot

Review the ΔC_T Plot for the following values:

- Compare the Predicted Copy Number plot sample data to the related data in the Copy Number Plot and the Results Table. When sufficient data points are available and the data is of high quality (for example, samples of the same copy number have similar ΔC_T values), well defined normal distributions of ΔC_T values representing low copy number groups (1, 2, or 3) are typically present. Note that as copy number increases, the separation between ΔC_T subdistributions (one per copy number) decreases and becomes more difficult to resolve.
- Examine the Confidence plot sample data to evaluate each copy number call. The confidence and absolute z-score thresholds can be changed to be more or less stringent. In high quality data sets wherein ΔC_T subdistributions are clearly delineated, confidence values for low copy number samples (1, 2, or 3) commonly

have confidence values greater than 95%. As copy numbers increase, confidence progressively decreases due to the decreased separation of ΔC_T subdistribution values of copy numbers. (for more information, see "Confidence estimate" on page 64).

Note: CopyCaller® Software cannot calculate confidence values and |z-score| values for zero copy-number samples or for data sets that contain fewer than seven samples of a single copy number. Such samples will have undetermined confidence in the ΔC_T plot (for more information, see the "Review the Results Table" on page 27 and "About the quality metrics" on page 64).

- Review the ΔC_T variability value to evaluate the data quality. This value represents the plate level standard deviation for the observed copy number normal distributions; the lower the value, the higher the quality of the data (for more information, see "About the quality metrics" on page 64).

View the ΔC_T Plot

To view data in the ΔC_T Plot:

Display copy number tooltips	In the ΔC_T Plot, place your mouse over a sample of interest. A tooltip displays the sample name, target assay name, file name, and copy number data (predicted, calculated, maximum, and minimum values).
Select samples in the ΔC_T Plot	In the ΔC_T Plot, left-click to select a sample of interest. CopyCaller® Software automatically: • Displays the sample in blue. • Selects the assay(s) in the Assay Selection Table, and the related data in the Copy Number Plot, Well Table tab, and the Results Table. To select more than one sample, press the **Ctrl** key while you select individual samples.
Expand a region of the ΔC_T plot for detailed viewing of sample data	Click the mouse button and drag the cursor down and right across the ΔC_T Plot to highlight a region of interest. To return to the default ΔC_T Plot view, click and drag the pointer up or left across the plot, then release the mouse button.
View an enlarged ΔC_T Plot in a new window	Double-click the left mouse button on the ΔC_T Plot.

Copy, save, or print the ΔC_T Plot views

You can copy, save, or print the Statistics Chart for use in documents or presentations. For specific instructions, see "Copy, save, or print the Copy Number Plot, Statistics Chart, or ΔC_T Plot" on page 40.

Save, export, and open the analysis

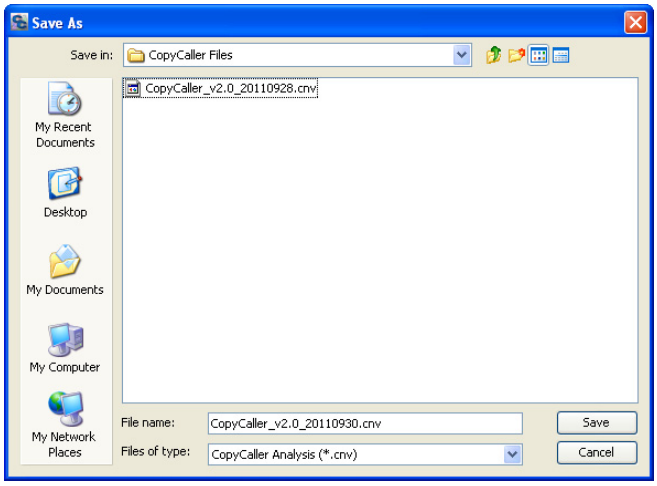
After completing the copy number analysis, you can save the analysis for later use by CopyCaller® Software. You can also export the results for use in documents, presentations, or downstream analysis.

Save the results to a CopyCaller® analysis file

CopyCaller® Software can save the analyzed data for all assays selected in the Assay Selection table.

To save the analysis:

1. Click (Save analysis) or select **File** ▸ **Save As**.
2. If you selected Save As, select the folder to save files in, and optionally edit the **CopyCaller® Analysis** file name (keep the .cnv extension).
3. Click **Save**.



Export the results to a CopyCaller® file

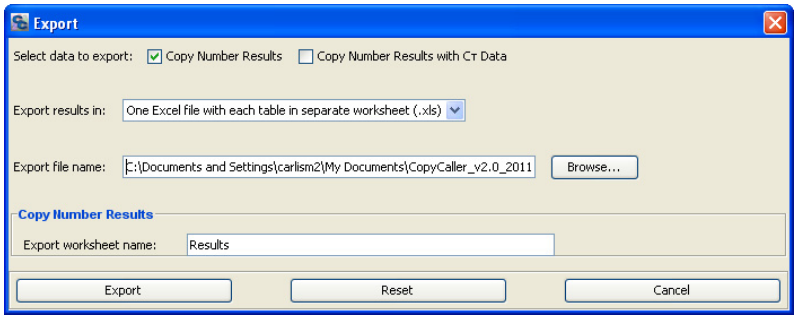
CopyCaller® Software can export the analyzed results table for all assays selected in the Assay Selection table to Microsoft® Excel® or text files.

To export the analysis results:

1. Click (Export) or select **File** ▸ **Export**.
2. Select the data to export:

Copy Number Results	Saves only the processed data in the Results Table.
Copy Number Results with C_T Data	Saves the processed data in the Results Table plus the C_T Data from the Well Table tab.

3. In the **Export results in** drop-down list, select the file type: a Microsoft Excel file (.xls), comma-separated values file (.csv), or tab-delimited text file (.txt).
4. In the **Export file name** field, edit the file name and browse for a location to save the results.
5. In the **Copy Number Results** section, edit the worksheet name or file name.
6. Click **Export**.



Open a saved analysis with CopyCaller® Software

After saving an analysis as a CopyCaller Analysis file, you can resume the saved analysis by opening the (.cnv) file: click (Open) or click **File** ▸ **Open**, select the file in the Open dialog box, then click **Open**.

Note: Analyzed data files saved in CopyCaller® Software v1.0 have the extension .txt or .csv and can be opened by selecting **All Files** in the Open dialog box.

Note: If you open a CopyCaller® file that contains the analyzed results of multiple assays, CopyCaller® Software displays the individual analyzed assays in the Assay Selection Table.

Copy, save, or print the Copy Number Plot, Statistics Chart, or ΔC_T Plot

You can save the Copy Number Plot, the Statistics Chart, or the ΔC_T Plot as a Portable Net Graphic file (PNG) or a Joint Photographic Experts Group file (JPG) for use in documents or presentations. You can also print the Copy Number Plot or the statistics chart.

To copy the plot or chart:

1. Right-click the plot or chart, then select **Copy**.
2. Paste the copied plot or chart into an open application of your choice.

To save the plot or chart:

1. Right-click the plot or chart, then select **Save as**.
2. Navigate to the folder where you want to save the file.
3. In the Files of type drop-down list, select the file format of the exported file.
4. Enter a file name, then click **Save**.

To print the plot or chart:

1. Right-click the plot or chart, then select **Print**.
2. In the Page Setup dialog box, select page setup options, then click **OK**.
3. In the Print dialog box, click **OK**.

A Troubleshooting

This appendix covers:

Troubleshooting real-time PCR data

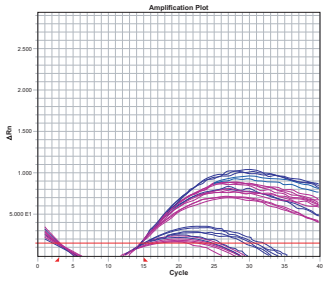
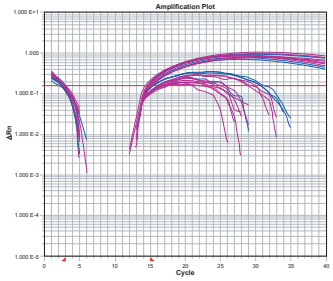
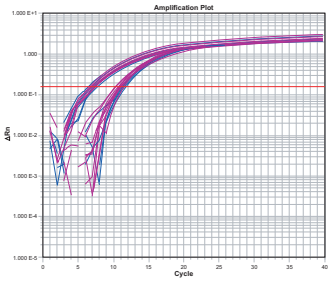
This section contains information for troubleshooting TaqMan® Copy Number Assay experiments using the Applied Biosystems real-time PCR system software (such as the SDS software or the StepOne™ software).

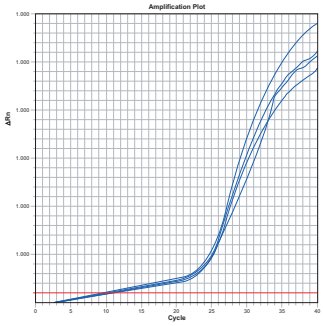
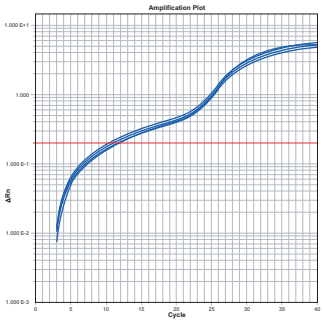
Overview

The table below summarizes the symptoms that are covered in this section, and indicates the relevant troubleshooting pages. The table organizes the information by the interface elements (plots) in which the symptoms are observed.

Plot	Observation	See page
Amplification Plot: R_n vs. Cycle	R_n value in the R_n versus Cycle plot is very high.	47
	R_n value shifts during the early cycles of the PCR (cycles 0 to 5).	47
	ΔR_n is small.	47
Amplification Plot: ΔR_n vs. Cycle	Amplification curve shows abnormal plot and/or low ΔR_n values.	42
	Amplification curve shows a rising baseline.	43
	Amplification curve shows weak amplification.	43
	Amplification curve shows samples within the same assay that have differently shaped curves.	44
	Amplification curve shows no amplification of the sample (C_T is undetermined).	45
	No template control (NTC) shows amplification.	46
	High level of variation exists between replicates (inconsistent data, C_T value varies).	46
	Signal above the threshold is noisy.	47
Multicomponent Plot	ROX™ dye fluorescence (passive reference dye) is low.	46
	Fluorescence from both the passive reference (ROX™) dye and the reporter dye(s) increase simultaneously.	46
	Multicomponent signal for ROX™ dye is not linear.	47

Plot	Observation	See page
Other	Reference assay (VIC® dye) C_T values vary.	46

Observation	Possible cause	Recommended action
Amplification curve shows abnormal plot and/or low ΔR_n values. Linear view:  Log view:  	Baseline set incorrectly (some samples have C_T values lower than the baseline stop value)	Enable automatic baselining, or manually move the baseline stop value to a lower C_T (2 cycles before the point at which the amplification curve for the sample crosses the threshold). **Note:** Refer to your real-time PCR system user guide for procedures on setting the baseline. Corrected log view: 
	Amplification detected in the early cycles of the PCR (prevents the software from setting the baseline automatically)	• Verify that the concentration of the sample is within the recommended range. • Dilute the sample to increase the C_T value.

Observation	Possible cause	Recommended action
Amplification curve shows a rising baseline. Linear view:  Log view:  	• Primer and probe interaction • Bubble in a well	• Adjust the threshold and/or baseline manually. • Select another assay from the same genomic region. Before thermocycling, verify that the reaction plate does not contain bubbles.
Amplification curve shows weak amplification.	Sequence mismatch between target region and the copy number assay	• Perform bioinformatics. For example, verify the presence of SNPs in the target region. • Select another assay from the same genomic region.
	Degraded reagents and/or assays	• Verify that the reagents have not expired. • Follow the correct handling and storage conditions. • Avoid excessive freeze-thaw cycles. Consider diluting the 60X TaqMan® Copy Number Assay to a 20X working stock.
	Degraded or contaminated template	• Improve sample integrity (extraction methods). • Verify each template preparation by agarose gel electrophoresis or bioanalyzer to determine the: – Purity – Level of degradation • Use DNase-free, sterile, filtered water.
	Inhibitors present in the reaction	Verify the presence of an inhibitor: 1. Create a serial dilution of your sample. 2. Run the serial dilution with an assay known to detect a target in the sample (for example, the reference assay). If a PCR inhibitor is present, dilute DNA samples yield higher-than-expected C_T values in comparison to higher-concentration samples. 3. Rerun the assay with repurified template.

Observation	Possible cause	Recommended action
Amplification curve shows samples within the same assay that have differently shaped curves.	Poor sample quality	1. Verify the quality of the sample. 2. If necessary, reextract the sample.
	Imprecise pipetting (different concentrations)	Follow accurate pipetting practices.
	Contamination	Verify that your workspace and equipment are cleaned correctly.
	Precipitation in the TaqMan® buffer	Perform the copy number experiments using TaqMan® Genotyping Master Mix. Be sure to mix the solution thoroughly to produce a homogenous solution. **Note:** TaqMan® Gene Expression Master Mix or TaqMan® Universal Master Mixes are acceptable alternatives.
	Incorrect baseline and/or threshold setting	Refer to your real-time PCR system user guide for procedures on setting the baseline: • Change the method used to set the baseline or threshold. If you set them: – Manually, enable automatic baselining and/or thresholding. – Automatically, set the baseline and/or threshold manually. • Increase the upper or lower value of the baseline range.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_T is undetermined).	Copy number sequence not present in the test sample(s) (for example, the copy number variation region is deleted)	• Verify that the reference assay (VIC® dye) C_T values are normal in samples lacking the copy number assay (FAM® dye) signal. • Verify the result by: – Rerunning the sample using the same assay. – Running the sample using an alternative assay in the same genomic region. • Verify the known copy number variation of the target genomic region.
	Assay/target sequence mismatch	• For custom assays, perform bioinformatics. For example, verify that the sequence submitted to assay design contains the correct target sequence. • Select an alternative target region for assay design or select another assay from the same genomic region.
	Missing PCR component(s)	Verify that gDNA, TaqMan® Copy Number Assay, TaqMan® Copy Number Reference Assay, and TaqMan® Master Mix were added to the reaction plate. **Note:** If the master mix was not added to the reaction, the passive reference also fails.
	Dye components specified incorrectly	Verify the dye components settings (reporter, quencher, and passive reference dyes).
	Annealing temperature too high for the primers and/or probes	Verify that the thermal cycler is set to the correct annealing and extension temperatures. Ensure that the thermal cycler is calibrated and maintained regularly.
	Degraded template	• Determine the quality of the template. • Rerun the assay with fresh template. • Use DNase-free, sterile, filtered water.
	Inhibitors present	Verify the presence of an inhibitor: 1. Create a serial dilution of your sample. 2. Run the serial dilution with an assay known to detect a target in the sample (for example, the reference assay). If a PCR inhibitor is present, dilute DNA samples yield higher-than-expected C_T values in comparison to higher-concentration samples. 3. Rerun the assay with purified template.
	Incorrect baseline and/or threshold setting	• Change the method used to set the baseline or threshold. If you set them: – Manually, enable automatic baselining and/or thresholding. – Automatically, set the baseline and/or threshold manually. • Lower the threshold value to within the appropriate range. **Note:** Refer to your real-time PCR system user documentation for information on setting the baseline and threshold.

Observation	Possible cause	Recommended action
No template control (NTC) shows amplification.	Reagent contamination (gDNA, amplicon, or plasmid clones)	• Rerun the assay using new reagents. • Be sure your workspace and equipment are cleaned correctly. • Use AmpErase® UNG (in TaqMan® Gene Expression or Universal Master Mixes) and adjust the protocol to include the UNG activation step (50°C/10 min HOLD before the standard PCR protocol steps).
	Template or amplicon contamination	Follow good laboratory practices for preventing PCR contamination.
Reference assay (VIC® dye) C_T values vary.	Reference sequence not present or contains polymorphisms in test sample(s)	Use an alternate TaqMan® Copy Number Reference Assay.
	High variation in sample concentrations	Quantitate and normalize samples before running them. **Note:** In general, the calculation of sample-level ΔC_T accounts for variability in sample concentration.
	Pipetting inaccuracy	Verify that your pipettors and/or liquid transfer robot(s) are calibrated and working correctly.
High level of variation exists between replicates (inconsistent data, C_T value varies).	Insufficient mixing	• Increase the time spent mixing the reagents. • Validate your mixing process by running a replicate plate.
	Pipetting inaccuracy	Verify that your pipettors and/or liquid transfer robot(s) are calibrated and working correctly.
	Incorrect threshold setting	Using your real-time PCR system software, set the threshold above the noise and within the region where the amplification curves for the technical replicates are clustered tightly. **Note:** Refer to your real-time PCR system user documentation for information on setting the threshold.
	Low concentration of one or more reaction components	Verify that correct amounts of copy number assay, reference assay, and master mix were added to the reaction plate.
	Low target concentration	Rerun the reaction using more sample.
	High sample concentration	• Reduce the amount of sample. • Quantitate and normalize the sample.
	Template or amplicon contamination	Follow good laboratory practices for preventing PCR contamination.
ROX™ dye fluorescence (passive reference dye) is low.	Degraded TaqMan® buffers	Verify that the assays have not expired and that they have been stored according to the instructions on the packaging.
	Pipetting inaccuracy (insufficient master mix)	Follow accurate pipetting practices.
Fluorescence from both the passive reference (ROX™) dye and the reporter dye(s) increase simultaneously.	Evaporation	Verify that the seal of the optical adhesive cover is intact (no leaks).

Observation	Possible cause	Recommended action
Multicomponent signal for ROX™ dye is not linear.	Incorrect pure dye components spectra	Perform a pure dye calibration, then reanalyze the plate document/experiment using the new dye spectra.
	Dye components specified incorrectly	Select the correct dyes for the plate document/experiment.
R_n value in the R_n versus Cycle plot is very high.	Incorrect passive reference setting	Select the ROX™ dye as the passive reference for the plate document/experiment.
R_n value shifts during the early cycles of the PCR (cycles 0 to 5). **Note:** This condition does not affect PCR or the results.	Fluorescence did not stabilize to the buffer conditions of the reaction mix	• Manually set the lower value of the baseline range. *or* • Enable automatic baselining.
ΔR_n is small.	Poor PCR efficiency	Verify that the concentrations of reagents and assay are correct.
	Low copy number of target	Increase the quantity of the sample.
Signal above the threshold is noisy.	Evaporation	Verify the seal of the optical adhesive cover (no leaks).
	Empty well (pipetting inaccuracy)	Verify that your pipettors and/or liquid transfer robot(s) are calibrated and working correctly.
	Detector/target incorrectly applied to an empty well (The empty well is labeled with a detector in the plate document/experiment.)	1. Verify that the detector/target settings for the well in the plate document/experiment are correct. 2. Exclude the well, then reanalyze the data.

Troubleshooting CopyCaller® Software analysis results

This section contains information for troubleshooting TaqMan® Copy Number Assay experiments using CopyCaller® Software.

Overview

The table below summarizes the symptoms covered in this section and indicates the relevant troubleshooting page. The table organizes the information by the interface elements in which the symptoms are observed.

Interface element	Observation	See page
Copy Number Plot	Large copy number range bars in the Copy Number Plot (technical replicates exhibit a broad range of ΔC_T values).	49
Results Table	Calculated copy numbers are between integers (for example, 1.5 copies).	49
	Calculated copy numbers are between integers (for example, 1.5 copies).	49
	Copy number confidence values are high (>95%), but the absolute z-scores are also high (>2.65).	50
	Confidence value for a sample changes after one or more outliers are manually omitted from the analysis.	51
	Copy number confidence calls are very low.	50
	Predicted copy number call for a sample changes after one or more outliers are manually omitted from the analysis.	51
	No confidence values are assigned.	51
Wells Table	ΔC_T of a technical replicate deviates significantly from the replicate group, but is not identified as an outlier.	50

Observation	Possible cause	Recommended action
Large copy number range bars in the Copy Number Plot (technical replicates exhibit a broad range of ΔC_T values).	Inefficient mixing of reagents	• Increase the time that you mix the reagents. • Validate the mixing process by running a replicate plate or by repeating the experiment.
	Inaccurate pipetting	Verify that your pipettors and/or liquid transfer robot(s) are calibrated and working correctly.
	Threshold set incorrectly	Using your real-time PCR system software, set the threshold above the noise and within the region where the amplification curves for the technical replicates are clustered tightly. **Note:** Refer to your real-time PCR system user documentation for procedures on setting the threshold.
	Concentration of one or more reaction components is low	Verify that the correct amounts of copy number assay, reference assay, and master mix were added to the reaction plate.
	• Target concentration is low • C_T values are high (>32.0)	• Quantitate the sample DNA. • Rerun the assay using more template.
	Template or amplicon contamination	Follow good laboratory practices for preventing PCR contamination.
Calculated copy numbers are between integers (for example, 1.5 copies).	Calibrator sample ΔC_T mean does not accurately represent other samples having the same target copy number	• Select a different calibrator sample, then reanalyze the data. • Reanalyze the data without a calibrator sample. **Note:** For more information on selecting a calibrator sample, see "Select assays and analyze the data" on page 18.
	When many samples are affected, an incorrect calibrator sample or copy number was selected	• Select a different copy number, then reanalyze the data. • Select a different calibrator sample, then reanalyze the data. • Reanalyze the data without a calibrator sample.
	Sequence mismatch between the copy number target region and the assay sequences in the sample.	• Verify the result by rerunning the sample using: – The same assay. – An alternative assay in the same genomic region. • Perform bioinformatics. For example, verify the presence of SNPs in the target region.
	Reference gene contains polymorphisms in the sample	Use an alternate TaqMan® Copy Number Reference Assay.
	Sample quality is poor	• Verify the sample quality. • If necessary, reextract the sample.

Observation	Possible cause	Recommended action
Copy number confidence calls are very low.	ΔC_T variability across the plate is too high (such as, large copy number range bars and high standard deviations are observed for all samples)	Repeat the experiment using good laboratory practices to reduce the sample replicate variability.
	Number of sample replicates are too low for high confidence calls	Repeat the experiment using at least four replicates per sample.
	Sample copy number is high (>3)	The confidence values are often lower for high-copy-number samples even under optimal experimental conditions due to the compression of the ΔC_T subdistributions at higher copy numbers. Consider using copy number bins (that is, group higher copy numbers) to estimate confidence (see "About the analysis settings" on page 19). Follow best laboratory practices to minimize the experimental variability.
	Target assay is not robust	Select another target for assay design or another assay from the same genomic region.
Copy number confidence values are high (>95%), but the absolute z-scores are also high (>2.65).	ΔC_T variability across the plate is too high (such as, large copy number range bars and high standard deviations are observed for all samples)	• Repeat the experiment using good laboratory practices to reduce the sample replicate variability. • Verify that the sample preparation is of high quality, then rerun the sample.
	Poor sample quality	1. Verify the quality of the sample. 2. If necessary, reextract the sample.
	Assay/target mismatches	1. Select the correct calibrator sample. 2. Verify the sequence information for copy number assay. 3. If the experiment fails again, reorder the assay.
ΔC_T of a technical replicate deviates significantly from the replicate group, but is not identified as an outlier.	ΔC_T of the well is not significantly different from the wells of the plate	In the Well Table of the software, manually omit the outlier, then reanalyze the data.
	Symptom may be normal and within the expected mathematical distribution for sample replicates	Do not remove outliers unless you are sure that the replicate data are of poor quality. **Note:** Subjective removal of data points can erroneously change the copy number of a sample or decrease the confidence of a call. The copy number of many samples may be affected if you use the "Without Calibrator Sample" analysis method.
	Replicate well data are of low quality when compared to other replicate wells	Determine the quality of the wells by reviewing the data for the entire sample replicate group using both the real time PCR system software and CopyCaller® Software. (An acceptable sample ΔC_T standard deviation for a replicate group is generally less than 0.15.) If the well data are poor, omit the outlier well and reanalyze the assay data.

Observation	Possible cause	Recommended action
Predicted copy number call for a sample changes after one or more outliers are manually omitted from the analysis.	Omitting the outlier changed the calculated ΔC_T value of the sample	Determine the quality of the well data and whether or not the data point should have been removed by reviewing the data for the entire sample replicate group. Review the data using both the real-time PCR system software and CopyCaller® Software. (An acceptable sample ΔC_T standard deviation for a replicate group is generally less than 0.15.) Verify the sample copy number by rerunning the copy number experiment and/or by selecting another assay from the same genomic region.
	Can occur for multiple samples when the "Without calibrator sample" analysis method is used	• Rerun the copy number experiment. • Remove the outliers cautiously. Omit only those wells that contain poor quality C_T values for experimental reasons. Reinstate outliers that have been removed subjectively and reanalyze. • Verify the copy number results by repeating the experiment using recommended practices to reduce sample variability. • Verify the copy number results by selecting another assay from the same genomic region.
Confidence value for a sample changes after one or more outliers are manually omitted from the analysis.	Omitting the outlier changed the ΔC_T value for the replicate group	
No confidence values are assigned.	Samples are zero-copy for the target of interest	No action is required. CopyCaller® Software cannot assign confidence values to zero-copy samples because ΔC_T values are required for the calculations. However, samples that have strong reference assay amplification and no or very weak copy number assay amplification are assigned as zero-copy number samples with highest confidence.
	Number of samples tested is too low (The confidence algorithm needs at least 7 samples of the same copy number to calculate confidence values.)	Increase the number of samples used in the experiment.

Troubleshooting CopyCaller® Software error messages

This section contains information for troubleshooting error messages produced by CopyCaller® Software.

Overview

The table below summarizes the error messages covered in this section and indicates the relevant troubleshooting pages.

Error Message	See page
<file> contains data from an unknown detector dye and cannot be added to the analysis. Verify that the detector dye setting used in the experiment are spelled and set correctly.	53
<file> contains data from an unknown reporter dye and cannot be added to the analysis. Verify that the reporter dye setting used in the experiment are spelled and set correctly.	
<file> does not contain sample raw C_T data.	
<file> is missing column data and cannot be added to the analysis.	
<file> is not is not a compatible file and cannot be added to the analysis. Verify that the file format is compatible with the software.	
<sample> cannot be used as the calibrator sample because it is predicted to have a zero-copy number.	
All the samples have replicates with undetermined FAM C_T and/or have exceeded the zero copy ΔC_T threshold in <assay>.	
Insufficient data points to estimate confidence and z-score values for <assay>.	54
No calibrator sample has been set.	
The calibrator sample <sample> is not present in <assay>. Verify that the calibrator sample is present and that the sample name is spelled correctly.	
The most frequent sample copy number must be greater than 0.	
Unable to load the export file to CopyCaller™ Software.	

Error Message	Possible cause	Recommended action
<*file*> contains data from an unknown detector dye and cannot be added to the analysis. Verify that the detector dye setting used in the experiment are spelled and set correctly.	The incorrect reporter dye was applied to the detector/assay using the real-time PCR system software	Verify that the results file contains the correct dye assignments (FAM and VIC). If necessary, use the real-time PCR system software to correct the dye assignments, then export the file again using the correct settings.
<*file*> contains data from an unknown reporter dye and cannot be added to the analysis. Verify that the reporter dye setting used in the experiment are spelled and set correctly.		
<*file*> is missing column data and cannot be added to the analysis.	Results file is missing one or more columns of data	Recreate the CopyCaller file and raw data by exporting the data again using your real-time PCR system software.
<*file*> does not contain sample raw C_T data.	Results file is missing the raw C_T data	
<file> is not is not a compatible file and cannot be added to the analysis. Verify that the file format is compatible with the software.	Exported .csv file is not a result or Results Table	Verify that the file contains only exported results created by the real-time PCR system software.
	Exported .xls file was exported using the StepOne™ software	Export the results as a .txt file.
<*sample*> cannot be used as the calibrator sample because it is predicted to have a zero-copy number.	The sample used as the calibrator to analyze the assay has: • A copy number of 0 *or* • An undetermined copy number	For the sample selected as the calibrator, verify that: • The copy number is greater than 0. • No more than two technical replicates have undermined FAM C_T values or ΔC_T values greater than 4. • The sample has more than one replicate included in the analysis.
All the samples have replicates with undetermined FAM C_T and/or have exceeded the zero copy ΔC_T threshold in <*assay*>.	• FAM C_T values are absent or insignificant for all samples associated with the assay • FAM C_T values for all the test samples are significantly lower than the VIC C_T values	Add additional test samples to the analysis.
		Verify that the exported results table contains the information required by CopyCaller® Software.
		For custom assays, perform bioinformatics. For example, verify that the sequence submitted to target design is correct.
	Copy number assay is designed for a sequence on the Y chromosome	Avoid testing all female samples with copy number assays designed from the Y chromosome.

Error Message	Possible cause	Recommended action
Insufficient data points to estimate confidence and z-score values for <*assay*>.	• The assay was evaluated using less than 7 samples having the same copy number • All samples generated a copy number of 0 • All samples generated an undetermined copy number	Add additional test samples to the analysis.
		Verify that the exported results table contains the information required by CopyCaller® Software.
		For custom assays, perform bioinformatics. For example, verify that the sequence submitted to target design is correct.
		Select an alternate target region for assay design or select another assay from the same genomic region.
No calibrator sample has been set.	A sample has not been assigned in the Analysis Settings dialog box	Select the correct calibrator sample as explained in "Select assays and analyze the data" on page 18.
The calibrator sample <*sample*> is not present in <*assay*>. Verify that the calibrator sample is present and that the sample name is spelled correctly.	If analyzing a single assay, the sample selected as the calibrator is not present among the imported data	In the Analysis Settings dialog box, select the correct calibrator sample. See "Select assays and analyze the data" on page 18 for more information.
	If analyzing multiple assays, the sample selected as the calibrator is not present in at least one assay	In the Analysis Settings dialog box, select a calibrator sample common to all assays, or analyze the assays without the use of a calibrator. See "Select assays and analyze the data" on page 18 for more information.
The most frequent sample copy number must be greater than 0.	In the Analysis Settings dialog box, the Most Frequent Sample Copy Number field contains a value of "0"	Enter a value greater than 0 in the Most Frequent Sample Copy Number field as explained in "Select assays and analyze the data" on page 18.
Unable to load the export file to CopyCaller™ Software.	• Results file was exported using the incorrect file format • Results file contains the incorrect data tables	Verify that the file is exported by the real-time system software contains the Results Table and that the file is exported in the correct format. If necessary, export the file again using the correct settings.

B Background Information

This appendix covers:

About the TaqMan® Copy Number Assays

CopyCaller® Software analyzes data generated by TaqMan® Copy Number Assay experiments that are run on Applied Biosystems real-time PCR systems. This section describes the mechanics of the TaqMan® Copy Number Assay and how real-time PCR data are generated from the reactions.

About duplex reactions

In a copy-number quantitation experiment, TaqMan® Copy Number Assays are run together with a TaqMan® Copy Number Reference assay in a duplex real-time polymerase chain reaction (PCR). The Copy Number assay detects the target gene or genomic sequence of interest, and the Reference assay detects a sequence that is known to exist in two copies in a diploid genome. This method of relative quantitation is used to determine the relative copy number of the target of interest in a gDNA sample, normalized to the known copy number of the reference sequence.

About the reaction components

In a copy-number quantitation experiment, there are four reaction components:

- TaqMan® Copy Number Assay (see below)
- TaqMan® Copy Number Reference Assay (see below)
- Genotyping Master Mix, containing AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), and dNTPs required for the PCR reactions (can substitute TaqMan® Gene Expression Master Mix, TaqMan® Universal Master Mix, or TaqMan® Universal Master Mix II, no UNG)
- Purified genomic DNA (gDNA) sample

Components of TaqMan® Copy Number Assays

Each Copy Number Assay contains:

- Two unlabeled primers for amplifying the target sequence of interest.
- One TaqMan® MGB probe for detecting the target sequence of interest. The probe includes:
  - FAM™ reporter dye, attached to the 5′ end.
  - A nonfluorescent quencher (NFQ) and a minor groove binder (MGB), attached to the 3′ end.

    MGBs increase the melting temperature (T_m) without increasing probe length. They allow for the design of shorter probes.

Components of TaqMan® Copy Number Reference Assays

Each Copy Number Reference Assay contains:

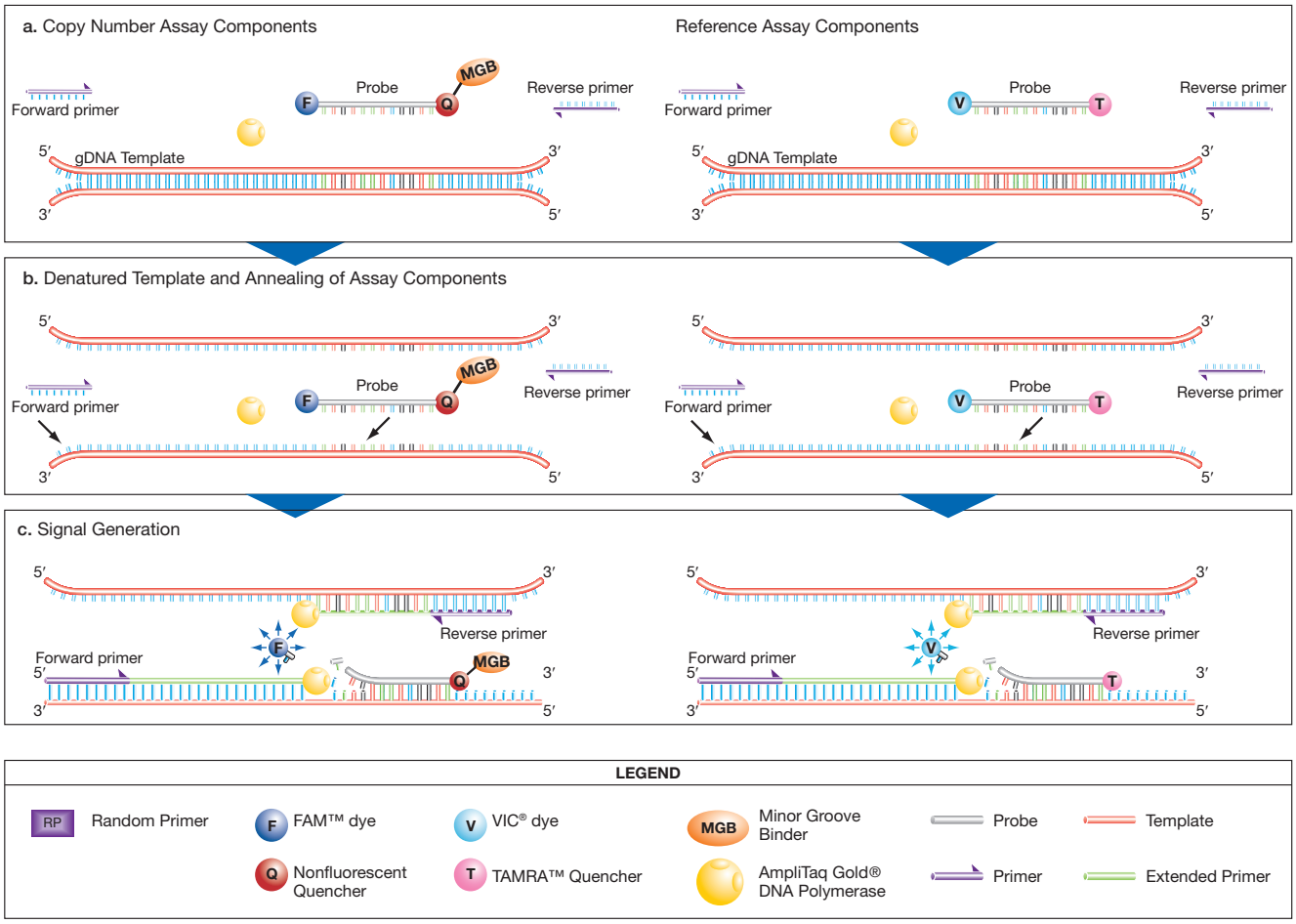
- Two unlabeled primers for amplifying the reference sequence.
- One TaqMan TAMRA™ probe for detecting the reference sequence. The probe includes:
  - VIC® reporter dye, attached to the 5′ end
  - TAMRA™ quencher, attached to the 3′ end

About the 5′ nuclease assay

The figure below shows the steps in a duplex PCR reaction containing copy-number target and reference assays, both of which are 5′ nuclease assays.

During PCR:

- A TaqMan® Copy Number Assay, a TaqMan® Copy Number Reference Assay, Genotyping Master Mix, and a gDNA sample are mixed together in a single well or tube.
- The gDNA template is denatured and each set of assay primers anneals to its specific target sequences. Each TaqMan® probe anneals specifically to its complementary sequence between forward and reverse primer binding sites.
- When each oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye causes the reporter dye signal to be quenched.
- During each round of PCR, the target and reference sequences are simultaneously amplified by AmpliTaq Gold® DNA Polymerase. This enzyme has a 5′ nuclease activity that cleaves the probes that are hybridized to each amplicon sequence.
- When an oligonucleotide probe is cleaved by the AmpliTaq Gold® DNA Polymerase 5′ nuclease activity, the quencher is separated from the reporter dye, increasing the fluorescence of the reporter. Accumulation of PCR products can be detected in real time by monitoring the increase in fluorescence of each reporter dye at each PCR cycle.



For more information

For more information about copy number experiments or real-time PCR using TaqMan® probes, see "Related documentation" on page 77.

Overview of the analysis algorithm

The algorithms used by CopyCaller® Software assume that:

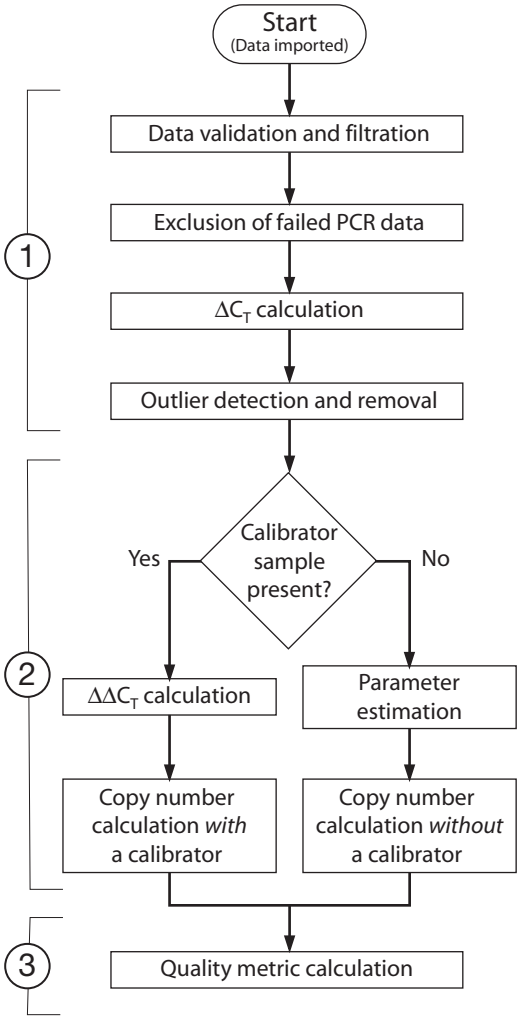
- Instrument readings are proportional to the underlying PCR reactions of the target genetic material.

  and

- multiple sources of variability combine to result in measurements being normally distributed about nominal values.

Analysis workflow

CopyCaller® Software calculates copy number assignments from the data of copy number experiments using the following series of steps:



(1) Preprocessing. The software detects invalid data, removes outlier(s), and calculates values for independent variables (such as ΔC_T values).

(2) Sample copy number assignment. The software calculates copy numbers using one of two algorithms. If a calibrator sample is present, the software applies a relative quantitation algorithm similar to the algorithm that is used by the software of your real-time PCR system. If a calibrator sample is not present, the software applies an algorithm that calculates copy number assignments that maximize the likelihood of the observed data with respect to a theoretical model (see "About the theoretical model" on page 59).

(3) Quality metric assignment. For each sample, the software calculates two quality metrics:

- **Confidence metric** – An estimate of the confidence that the assigned copy number is the true copy number relative to any of the other possible assigned copy numbers for the group of analyzed samples. For example, a confidence value of 90% indicates a 10% chance that the actual copy number differs from the called copy number.
- **Absolute z-score metric** – An estimate of how much the ΔC_T value of a sample deviates from the mean ΔC_T value of the assigned copy number category. For example, a copy number of 2 and an absolute z-score of 1 indicate that the ΔC_T for the sample is ±1 standard deviation from the mean ΔC_T for copy number 2.

For more information

For more information about the copy number analysis or relative quantitation using the Comparative C_T, see "Related documentation" on page 77.

About the theoretical model

CopyCaller® Software determines copy numbers from real-time PCR data and related confidence measures using a theoretical model that is taken to adequately describe the data.

Biochemical basis

The data generated by the copy number assay experiment is a ΔC_T value. The value is the difference in cycle threshold (C_T) values between a genomic target sequence of unknown copy number and an endogenous reference sequence of known and invariant copy number. A C_T value is associated with the PCR cycle at which the concentration of free emitter dye of the TaqMan® probe is high enough such that its fluorescence can be detected by the real-time PCR instrument. Samples with more copies of the target sequence achieve C_T earlier (at lower cycle numbers) because the PCR has more genetic material. The endogenous reference gene is presumably a gene that has been observed to have little, if any, variation in copy number. Hence, if an unknown target C_T value is the same as the endogenous reference C_T, presumably, the unknown target has the same copy number. Because the PCR process doubles the amount of genetic material at each cycle, if the unknown C_T is one cycle higher, the unknown must have half the number of copies relative to the endogenous reference. If the unknown C_T is one cycle lower, it must have double the number of the endogenous reference.

In practice, the relationship between C_T values and target copy number that is described above can be modified because the PCR process does not exactly double the concentration of genetic material at each cycle. The unknown and endogenous reference genes are monitored by different dyes that can differ in levels of delectability by the real-time PCR instrument. In general, the sequence context of the unknown and endogenous reference genes is not the same, and therefore may differentially influence the PCR process. Furthermore, mutations in the unknown and endogenous reference genes may also influence the PCR process. The net result is that the actual relationship between C_T values and gene copy number deviates from the ideal described above. A statistical model that can account for this deviation is described in the next section.

About the statistical model

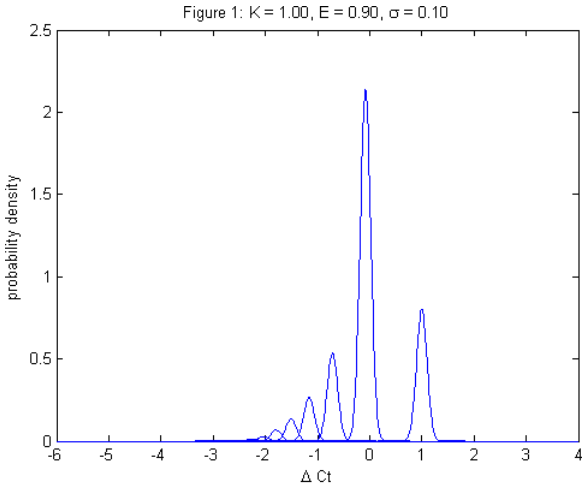
The exponential nature of the PCR process determines the main feature of the model: as the number of gene copies increases, the difference between successive ΔC_T values decreases. The following equation expresses this behavior:

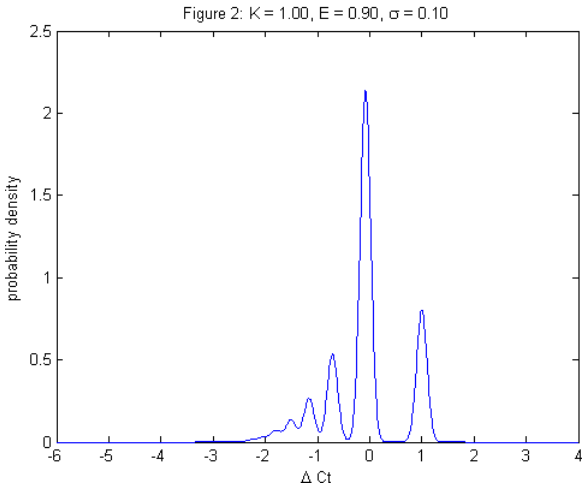
$$\Delta C_T = K - \log_{1+E} cn$$

where K is a constant, E is the PCR efficiency of the assay of interest, and *cn* is copy number with the range $[1, \infty)$. If the biochemical processes and instrumentation behaved perfectly as described in the previous section, then $K = 0$ and $E = 1$. In practice, however, $K \neq 0$ and $E < 1$. The previous section cited several possible causes for this deviation from the ideal. There are many other causes, such as thermal fluctuations, the binding behaviors of PCR primers and TaqMan® probes, and the starting concentration of a sample. The use of the endogenous reference presumably handles the main effect of starting concentration, but this probably does not nullify all its contributions to variation. The net effect is that, even if all samples have the same copy number, the ΔC_T values are not necessarily the same. To model this variation, it is assumed that ΔC_T values for samples of a given copy number are normally distributed around a mean that is calculated by the equation above. An additional simplifying assumption is that the variability of the normal distribution, σ^2, for a given copy number is the same as that for all other copy numbers. Different copy numbers have

different probabilities of occurring within the sampled population, and these probabilities are determined by the characteristics of the genes in question. Consequently, the total probability covered by the normal distribution for a given copy number is modulated by the probability of that copy number within the sampled population. This completes the statistical model relating ΔC_T values to *cn*.

The upper plot (below) shows an example model in which 20% of the samples have a *cn* = 1, 54% have a *cn* = 2, 13% have a *cn* = 3, and 13% are distributed among *cn* > 3; *K* is set at 1; *E* is set at 0.9; and σ is set at 0.1. The subdistributions for each copy number are shown separately to make it easier to see the component distributions. The lower plot shows the same model where the sub-distributions have been combined to show the actual probability density function.





Data preprocessing

Data validation

Before analyzing the data to assign copy number calls, CopyCaller® Software excludes wells in which the PCR reaction has failed. The software omits data from wells with VIC C_T values greater than the threshold defined in the Analysis Settings dialog box (see "About the analysis settings" on page 19). If all replicates of a sample have VIC C_T values greater than the threshold, CopyCaller® Software does not generate a copy number call for the associated sample.

Note: In general, wells with a VIC C_T greater than 32 (default) are defined as having failed amplification, possibly due to an insufficient quantity of gDNA.

CopyCaller® Software analyzes zero-copy samples differently from nonzero-copy samples. Wells in which sufficient gDNA is present (where the well has a VIC $C_T \leq 32$) but in which no FAM target is detected (FAM C_T = undetermined) may indicate either a failed copy number assays reaction or a zero-copy sample. If all replicates of a particular sample have VIC ≤ 32 and undetermined FAM C_T, the sample is assumed to be a zero-copy sample. Additionally, samples having extremely weak FAM signal relative to the VIC signal ($\Delta C_T > 4.0$) are also predicted to be zero-copy for the target sequence. The weak signal is assumed to be nonspecific.

ΔC_T calculations

After filtering the well data, CopyCaller® Software:

1. Calculates $(\Delta C_T)_w$, the well-level ΔC_T, for each target/reference combination in each well.

   The software begins by normalizing the C_T of each target assay in each well with the C_T of the corresponding reference assay in that well:

   $$(\Delta C_T)_w = (C_T)_{t,w} - (C_T)_{r,w}$$

   where: t = target assay; w = well; r = reference assay.

   Note: This step helps negate variations in volume and pipetting inconsistencies in the experiment.

2. Calculates $\mu(\Delta C_T)$, the sample-level ΔC_T arithmetic mean of the replicates, for each target/reference combination in the analysis.

   This gives an estimate of the actual ΔC_T values and can help reduce experimental error.

   For each replicate group of n wells representing a specific target/reference combination, a $\mu(\Delta C_T)$ value is calculated as:

   $$\mu(\Delta C_T) = \frac{\Sigma_{w=1}^{n} (\Delta C_T)_w}{n}$$

Outlier detection

After calculating ΔC_T values, CopyCaller® Software removes from the analysis data outlier wells that produce ΔC_T values that differ significantly from the mean of all applicable wells (such as pre-filtered wells). The differences in the ΔC_T values of outlier wells is commonly attributed to samples that amplify insufficiently or not at all due to experimental error (such as pipetting inaccuracy or contamination).

To identify outliers among the replicate groups, the software:

1. Estimates the plate level standard deviation.
2. Excludes data points greater than four standard deviations from the mean ΔC_T of the replicate group.

To estimate the standard deviation, the software excludes replicates that deviate from the replicate median by more than the distance between the one- and two-copy distributions, using a conservative value of 0.8 for PCR efficiency.

Note: We do **not** recommend a more aggressive approach to outlier removal. The validity of the statistical analyses for copy number determination and sample quality metrics assume that the data are distributed as described in "About the theoretical model" on page 59.

Copy number assignment

The way that CopyCaller® Software calculates copy number varies depending on the availability of a calibrator sample that has a known copy number for the target of interest.

Copy number assignment with a calibrator sample

When a calibrator sample that has a known copy number is available, CopyCaller® Software:

1. Calculates $\Delta\Delta C_T$ for each sample/target combination in the analysis.

   The software calculates $\Delta\Delta C_T$ values for each target assay and each sample by subtracting the $\mu(\Delta C_T)$ of the calibrator sample from the corresponding $\mu(\Delta C_T)$ of each test sample (including the calibrator sample) for each target assay.

   $$(\Delta\Delta C_T)_{s,t} = \mu(\Delta C_T)_{s,t} - \mu(\Delta C_T)_{c,t}$$

   where: s = sample; t = target assay; c = calibrator.

2. Calculates copy number for each sample/target combination in the analysis.

   When a calibrator sample with a known copy number is available, CopyCaller® Software calculates copy number by rounding the product of the following equation to the nearest integer:

   $$\text{Copy number} = cn_c 2^{-\Delta\Delta Ct}$$

   where cn_c is the copy number of the target sequence in the calibrator sample, and $\Delta\Delta C_T$ is the difference between ΔC_T for the unknown sample and the calibrator sample.

   Note: The calibrator sample is specified in the analysis settings (see "Select assays and analyze the data" on page 18).

Copy number assignment without a calibrator sample

When a calibrator sample is not specified for analysis, CopyCaller® Software finds the parameter values using the model described in "About the theoretical model" on page 59. The software uses the model to estimate *K*, which is an estimate of the mean ΔC_T for copy number 1. Using the calculated estimate, the software then employs the same copy number assignment described in "Copy number assignment with a calibrator sample" on page 62.

The software calculates the parameter values for the model as follows:

- *Efficiency* – Assumed to be 100%.
- *K* – Determined by maximizing the likelihood of the observed ΔC_T replicate means as a function of this parameter. The likelihood of the observations is taken to be the sum of probability density across all samples for a given model.

For each candidate value for *K*, the following initial model is assumed, then refined:

- Probability of a copy number – Calculated as the range of copy numbers with non-zero probability, where the limits are from 1 copy to the copy number that could be assigned to the sample with the lowest value for the replicate mean ΔC_T, given the candidate value for *K*. Initially, the software assumes that all copy numbers with non-zero probability have the same probability.
- Standard deviation of the sub-distributions – The software assigns an initial value.

Note: When using the "Without calibrator sample" likelihood analysis method, the asymmetry of the statistical model around the mean ΔC_T value for each copy number may cause the predicted copy number value for a sample to be higher (or lower) than expected if the calculated copy number was rounded up (for example, a calculated value of 2.4 may correspond to a predicted value of 3).

CopyCaller® Software uses the initial model to calculate the proximity of the samples to the means of each copy number sub-distribution. The proximity measure for a given sample is the probability density at the replicate mean ΔC_T value divided by the total probability density for the sample. Using this proximity measure, the software updates the model as follows:

- Probability of a copy number – The software calculates the sum of the proximity measures over samples as an estimate of the number of samples associated with a given copy number. The software then divides the sum by the total number of samples to calculate a revised estimate of the copy number probability.
- Standard deviation of the sub-distributions – Using the proximity measures as the weightings, the software calculates a weighted sum of squared deviations between the replicate means and the means of the copy number sub-distributions. The software calculates the square root of the average value of the weighted sum over the samples as a revised estimate of the standard deviation of the sub-distributions.

The software applies the revised model to calculate likelihood for a given value of *K*.

About the quality metrics

CopyCaller® Software generates two quality metrics for each analyzed sample: a confidence estimate and a deviation estimate. Additionally, the software generates a plate level ΔC_T variability estimate.

Confidence estimate

The confidence estimate for a given sample is the probability that the calculated copy number is the correct assignment compared to other copy numbers that have non-zero probability of occurring. CopyCaller® Software uses the copy number and observed ΔC_T values of a sample to calculate the confidence estimate for the sample relative to the theoretical model described in "About the theoretical model" on page 59. The confidence estimate model is identical to that used for estimating copy number except that amplification efficiency is not assumed to be 100%, and the parameter values are estimated differently.

The software calculates the model parameters as follows:

- *K* and *E* – Using the two most frequently assigned copy numbers and the equation describing means of the copy number sub-distributions as a function of copy number (see "About the theoretical model" on page 59), the software forms two linearly independent equations that can be solved for *K* and *E*. The maximum likelihood estimate for the means of the copy number sub-distributions completes the estimate for the parameters. When all samples are assigned the same copy number, *E* is assumed to be 1.
- Standard deviation of the sub-distributions – Using *K*, *E*, and copy numbers to estimate means of the copy number sub-distributions, the software calculates the maximum likelihood estimate for this parameter for all samples with non-zero assigned copy numbers. The calculation reduces the degrees of freedom by two because *K* and *E* are estimated from the data.
- Probability of a copy number – For each nonzero-copy number, the software estimates the probability by dividing the number of samples assigned the copy number by the total number of samples.The software modifies the estimate slightly by assigning a small amount of probability to copy numbers for which there are no samples, and then normalizing the total probability to 1.

The estimate of confidence as a function of assigned copy number and replicate mean is calculated as:

$$Confidence(\mu_r, cn_{assigned}) = \left[1 + \sum_{cn \neq cn_a} \frac{\Pi_{cn}}{\Pi_{cn_a}} e^{-\Omega}\right]^{-1}$$

where *a* (subscripted) = assigned, μ_r = replicate mean for the sample, $cn_{assigned}$ = copy number assigned to the sample, Π_{cn} = probability of copy number *cn*, Ω is calculated as:

$$\Omega = \frac{1}{\sigma^2 \log(1+E)} \log\left(\frac{cn}{cn_a}\right)\left((\hat{\mu}_r - K) + \frac{\log(cn_a cn)}{2\log(1+E)}\right)$$

where σ = standard deviation of the sub-distributions, *E* = PCR efficiency of the target assay, *K* = constant in the function relating the sub-distribution mean (μ_{cn}) to copy number (*cn*) calculated as:

$$\mu_{cn} = K - \frac{1}{\log(1+E)}\log(cn)$$

Using bootstrap techniques, the software calculates an estimate of a 5% lower bound for confidence. For example, the software estimates the value for which there is only a 5% chance that the actual value for confidence is lower than that reported. A consequence of this approach is that there will be some variation in the values reported.

Deviation |z-score| estimate

The deviation estimate is the absolute z-score—the absolute value of the number of standard deviations separating the replicate mean $\Delta C_{T,\mu r}$ of a sample from the mean subdistribution, μ_{enr}, of the assigned copy number.

The deviation estimate uses the model estimated for the confidence metric and is calculated as:

$$absoluteZscore(\mu_r, cn_{assigned}) = \left|\frac{\mu_r - \mu_{cn_a}}{\sigma}\right|$$

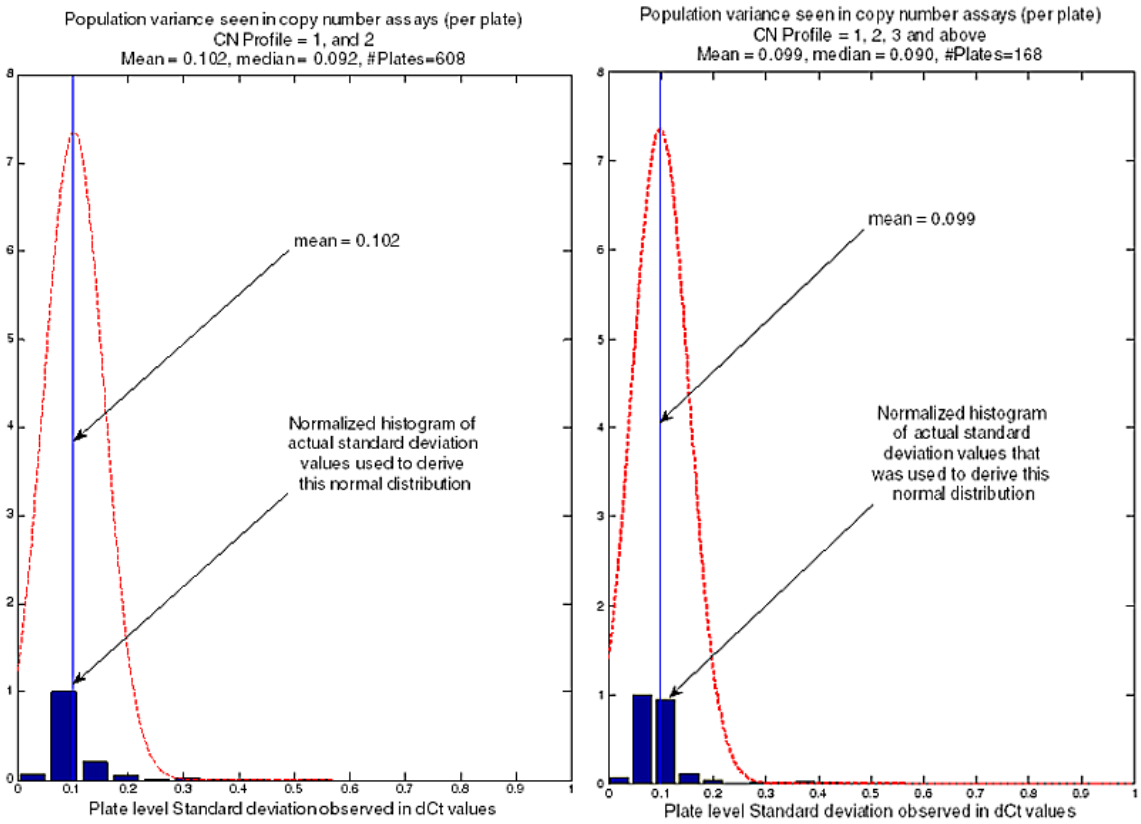
Using bootstrap techniques, the software calculates an estimate of a 5% lower bound for the deviation. A consequence of this approach is that there will be some variation in the values reported.

Note: If copy number bins are used to estimate confidence values, |z-score| estimates cannot be calculated because copy number bins are not normal distributions.

ΔC_T variability estimate

The plate level ΔC_T variability is a standard deviation value that is an estimate of the variability in the ΔC_T measurements for an assay across all samples in an analysis. It is derived by fitting the sample ΔC_T values to a model which is essentially a superimposition of the copy number group normal distributions with their centers at the average ΔC_T of each copy number group. The variability is assumed to be the same in all copy number groups (that is, the set of normal distributions has the same standard deviation). This standard deviation value estimated for the model is reported as the estimate for the plate level variability.

The two figures on the next page show the distribution of plate level variability seen in copy number assay analyses on sample populations having (A) copy numbers 1 and 2 only, and (B) assays with copy numbers 1, 2, 3 and above. The line denotes the mean standard deviation for these normal distributions (0.10). In general, ΔC_T variability values below this limit may be considered to have low plate ΔC_T measurement variability.



For multiplate analysis, plate ΔC_T variability values can be used to aid evaluation of whether or not plate data should be combined for copy number analysis. If the plate ΔC_T variability is higher than the empirically derived threshold limit of 0.10 for an assay, then combining data from different plates may not be able to improve the sample copy number call rate because the additional plate-to-plate variation may lead to spreading of the data points and loss and/or overlap of the copy number group subdistributions.

Miscellaneous information

Recommended minimum data requirements

The table below illustrates the relationship between the number of data points used in an analysis and the accuracy of the analysis. The table shows the accuracy of estimates of the mean and standard deviation of a normal random variate as a function of the number of points used to make the estimate. For estimates of the mean and standard deviation, the table shows "half point" percent deviations from actual values as a function of the number of points used to make the estimates.

Note: The term "half point" describes the deviation at which half the estimates would have lower deviations, and half would have higher deviations from actual values. For the mean, the percentage is relative to the standard deviation of the underlying distribution.

The values in the table show that, for the number of replicates or samples typically used in copy number studies, estimates of mean and variability deviate considerably from actual values. Note that the gains in accuracy (estimate of the mean and standard deviation) decrease rapidly as the number of data points increases.

Number of points used in estimation	Percent deviation from the actual for the estimate of the...	
	Mean‡ (%)	Standard deviation§ (%)
2	57.74	36.42
3	44.16	28.98
4	37.03	24.78
5	32.5	21.99
6	29.29	19.97
7	26.88	18.42
8	24.97	17.19
9	23.42	16.17
10	22.13	15.31
11	21.03	14.58
12	20.08	13.94
23	14.29	10.01
24	13.98	9.80
46	10.02	7.06
47	9.92	6.98
91	7.1	5.01
92	7.06	4.98
183	5.00	3.53

‡ Percent deviation from the actual mean (relative to the standard deviation of the distribution) exceeds the listed value for 50% of the estimates.

§ Percent deviation from the actual standard deviation exceeds the listed value for 50% of the estimates.

Selecting the number of replicates

When selecting the number of replicates to use in a copy number study, one possible method is to select the number at which the benefit of adding one more replicate falls below a target percentage. For example, if you choose 5% as the minimum percentage of improvement, the optimum number of replicates is:

- Four data points if estimating standard deviation. (The difference in the estimate of standard deviation between 4 and 5 replicates is 37.03 – 32.5 = 4.53.)

  or

- Three data points if estimating the mean. (The difference in the estimate of the mean between 3 and 4 replicates is 28.98 – 24.78 = 4.2.)

In the example above, because the copy-number problem involves estimating many types of parameters, using four replicates may be preferable.

For the copy-number problem, the mean for each sample (replicate mean) must be estimated. Also, four more parameters must be estimated from the data when there are two unique copy numbers among the samples. One parameter must be added for each additional unique copy number beyond the first two. Given the example above, where four replicates are used per parameter, four replicates would be needed to estimate the replicate means, and $4 \times (2 + N_{cn})$ samples are needed to estimate the other parameters, where N_{cn} is the number of unique copy numbers among the samples.

Interaction of copy number and the confidence metric

The tables below show that for the theoretical models described in page 59, the ability to distinguish between adjacent copy numbers decreases as copy number increases. Consequently, the confidence metric is generally lower at high copy numbers. The interaction of several parameters in the model influences the severity of the effect. For this reason, several models, selected by examining the variation in data over a database of 282 assays, are used to show the effect.

The table below lists models used to show the relationship between copy number and confidence metric. For this table, standard deviations of ΔC_T values were obtained from an analysis of 282 assays, each applied to 91 samples.

Model‡	Copy number subdistribution standard deviation
Optimal	0.05
Typical	0.09
Poor	0.2

‡ Rated by ability to resolve copy numbers.

The table below shows the confidence metric value at which 95% of the probability for a given copy number is captured. Consequently, 95% of the samples with the given copy number should have a confidence value of that shown in the table or greater.

Model	Copy number (CN)									
	1	2	3	4	5	6	7	8	9	10
Optimal	1.0000	1.0000	1.0000	1.0000	0.9998	0.9903	0.8976	0.6619	0.4324	0.2934
Typical	1.0000	1.0000	0.9916	0.7468	0.2833	0.1938	0.1477	0.1299	0.1196	0.0856
Poor	0.9570	0.6251	0.1092	0.0897	0.0769	0.0760	0.0747	0.0682	0.0527	0.0306

C

Multiplate Analysis

This appendix covers:

Guidelines for multiplate analysis

IMPORTANT! Life Technologies Corporation has not validated the multiplate method of analysis. We highly recommend single plate data analysis as the best practice method. Use multiplate analysis at your own risk.

Multiple plates containing samples run with the same assay may be combined for copy number analysis. We highly recommend single plate copy number analysis be done whenever possible because the highest quality copy number data is generated when measurement variation is very low. Experimental variation can be introduced when data from multiple plates is combined for analysis, and variation can impact the quality of the copy number analysis.

Copy number quantitation requires high quality real-time PCR data for the best results. Measurement variation can be controlled by use of high quality samples, avoiding process errors, and accurately following the recommended *TaqMan® Copy Number Assay Protocol* to achieve the highest confidence results. Generally, we highly recommend that all samples to be analyzed together are run on the same plate to avoid experiment variation introduced by plate-to-plate variation. However, in specific instances, such as high throughput analysis of many samples or assays, it may be desirable to combine data from multiple plates for copy number analysis. In some situations, combining data may improve the call rate for samples falling into copy number groups that are otherwise under-represented on single plates.

The following sections provide basic guidelines for multiplate analysis. First, the data for each individual plate in your study should be analyzed and the data quality evaluated. Plates with high quality assay data can then be selected in CopyCaller® Software and analyzed together in the same way that single plate data is analyzed.

Experimental setup and single plate assay analysis

Each plate in a study should contain enough data points for copy number analysis and data quality evaluation, because only best quality plate data is suitable for multiplate analysis. To set up an experiment and perform single plate analysis:

- Minimize plate-to-plate ΔC_T variation differences by running each copy number reaction plate on the same real-time PCR instrument using the same experimental process. For example, use high quality gDNA samples prepared by the same method, the same amount of gDNA, the same master mix lot, and optimally the same copy number and reference assay lots.
- Run enough samples on each plate to enable copy number analysis for each assay used. All sample replicates *must* be run on the *same* plate; replicates run on different plates cannot be combined in multiplate analysis. Seven samples of the same copy number group must be present for confidence and absolute z-score values, and ΔC_T variability, to be calculated. If fewer samples are run, we recommend that a well-characterized calibrator sample for copy number analysis be included on each plate.
- Analyze each assay on a per-plate basis and review the copy number analysis results. Review the ΔC_T plot to assess the quality of the copy number data. In general, the copy number group ΔC_T subdistributions should be distinct, with clear boundaries between distributions. Confidence values should be high for most samples, and the ΔC_T variability should generally be ≤ 0.10 for each plate to be combined in multiplate analysis. If data from plates with high subdistribution and ΔC_T variability are combined, the copy number group ΔC_T distribution boundaries may be ill-defined and overlap between groups, and the quality of the copy number analysis results may be poor.

IMPORTANT! High quality samples and a highly reproducible experimental process are required to generate the best quality data with low ΔC_T subdistribution and ΔC_T variability for each plate to be combined for multiplate analysis. If those requirements cannot be met, use the copy number analysis results from single plate analysis.

Note: All plates to be combined in multiplate analysis must have the same target name (copy number assay) applied to each sample well for the plate data to be analyzed together.

Select assays for multiplate analysis

The procedure for selecting assays for multiple analysis depends on the qualifications for multiplate analysis listed in the previous section, "Experimental setup and single plate assay analysis". For each plate that meets or exceeds the qualifications, do the following procedure:

1. In the Assay Selection Table, select the rows corresponding to the assays that you want to combine.
2. Right-click on a selected row, then select **Combine plates for multiplate analysis**.
3. A new assay row appears and the assay name for the multiplate follows this convention: `Multiplate_target-name_reference-name_#`

   You can select the multiplate assay and analyze the data as described in "Select assays and analyze the data" on page 18.
4. Review the analyzed multiplate data as described in the following sections:
   - "Review the Copy Number Plot" on page 23
   - "Review the Results Table" on page 27
   - "Review the Well Table" on page 30
   - "Review the Analysis Summary" on page 34
   - "Review the ΔC_T Plot" on page 36
5. If necessary, repeat the multiplate analysis using a subset of the single plates to improve the data analysis quality.

Note: Samples having the same sample name on different plates are analyzed on a per-plate basis; that is, the ΔC_T values for wells having the same sample name will not be combined between plates in multiplate analysis. If the same calibrator sample is run on multiple plates in the analysis and the calibrator sample name is the same on each plate, then the ΔC_T value of the calibrator from the first selected assay plate will be used as the calibrator for the multiplate analysis.

D

Software Installation

System requirements

Computer	Monitor	Operating system
• Pentium 4 or compatible processor, 1.0 GHz • 512 MB of RAM • 700 MB disk space • UL listed • CD drive (if installing the software from a CD) • Internet browser with a network connection (if installing from the internet)	• 1280 ∞ 1024 resolution for full-screen display • 16-inch or larger • 32-bit color • UL listed	Microsoft Windows® XP, Service Pack 2 or later

Install CopyCaller® Software

IMPORTANT! To install the software, you must have administrative privileges to the Microsoft® Windows® operating system.

IMPORTANT! The CopyCaller® Software Installer also installs the MATLAB® software, a critical component of the CopyCaller™ product. Uninstalling the MATLAB® software makes CopyCaller® Software inoperable.

1. Download CopyCaller® Software:
   a. Go to **www.appliedbiosystems.com**
   b. In the Home page of the Applied Biosystems web site, click **Support**.
   c. In the Support page, select CopyCaller® Software in the Software Downloads, Patches & Updates drop-down list, then click **Continue**.
   d. In the CopyCaller® Software page, click **Download Free Software**.
   e. Complete the registration as directed by the web site, then follow the instructions to download and install CopyCaller® Software.
2. Close all open applications.

3. Start the CopyCaller® Software Installer:
   - Double-click  (CopyCaller® Software Installer).

     or
   - Load the Applied Biosystems CopyCaller® Software CD into the computer.
4. Install the software as instructed by the software.
5. When the installation is complete, click **Finish**.

Bibliography

Livak, K., Schmittgen, T., Analysis of Relative Gene Expression Data Using Real-Time Quantitation PCR and the $2^{-}\Delta\Delta^{Ct}$ Method, *Methods* 25: 402-408 (2001).

Application Note: *Analysis of Copy Number Variation: Design Pipeline and Validation of TaqMan® Copy Number Assays*. Publication 135AP03-0

National Institute of Standards and Technology: **www.nist.gov**

Documentation and Support

Related documentation

This section provides references that can provide more information on copy number experiments and analysis.

Portable document format (PDF) versions of this guide and the documents listed below are also available at **www.appliedbiosystems.com**.

Note: To open the documentation available from the Applied Biosystems web site, use the Adobe® Acrobat® Reader® software available from **www.adobe.com**.

Note: For additional documentation, see "Obtaining support" on page 78.

Copy number analysis

For more information on CopyCaller Software experiments, refer to the *Applied Biosystems TaqMan® Copy Number Assays Protocol* (Part no. 4397425).

To view and order Pre-designed TaqMan® Copy Number Assays, Custom Plus TaqMan® Copy Number Assays, Custom TaqMan® Copy Number Assays, and TaqMan® Copy Number Reference Assays, go to **www.appliedbiosystems.com.**

For general information about relative quantitation using the Comparative C_T method, refer to:

- *Applied Biosystems Real-Time PCR Systems User Bulletin: Relative Quantitation (RQ) Algorithms in Applied Biosystems Real-Time PCR Systems Software* (Part no. 4378622).
- Applied Biosystems Real-Time PCR Systems Getting Started Guides listed in the following section"Instrument operation".

Instrument operation

For more information, refer to the documents that are shipped with your Applied Biosystems Real-Time PCR System:

- **User guide** – Procedures for using and maintaining the instrument, including performing instrument calibrations.
- **Getting started guides for quantitation experiments** – Background information on real-time PCR, experiment examples, and guidelines for experiment design, setup, run, and analysis.

Real-time system	Software	For more information, refer to the...
7900HT Fast system	SDS Software	*Applied Biosystems 7900/7900HT Fast Real-Time PCR Systems Getting Started Guide for Absolute Quantitation*
7300/7500/7500 Fast system	SDS Software	*Applied Biosystems 7300, 7500, 7500 Fast Real-Time PCR Systems Getting Started Guide for Absolute Quantitation*
7500/7500 Fast system	7500 Software	*Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*
StepOnePlus™ system	StepOne™ Software	*Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*
ViiA 7™ System	ViiA 7™ System Software	*Applied Biosystems ViiA™ 7 Real-Time PCR System Getting Started Guide for Standard Curve Experiments*

- **Chemistry guide or reagent guide** – Information on real-time PCR experiments, PCR options, and reagents that are supported on your real-time PCR system

Obtaining support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Glossary

Δ	Delta. A symbol indicating a normalized value.
$\Delta\Delta C_T$ method	Also known as the Comparative C_T method. A quantitative PCR method used to determine the quantity of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. Relative quantity units are abbreviated as RQ.
ΔR_n	Abbreviation for baseline-corrected normalized reporter.
μ	See arithmetic mean (μ).
σ_{SD}	See standard deviation (σ_{SD}).
amplicon	A segment of DNA amplified during PCR.
amplification	Part of the instrument run during which PCR amplifies the target. For quantitation experiments, fluorescence data collected during amplification are displayed in an amplification plot and used to calculate results.
amplification plot	Display of data collected during the cycling stage of PCR amplification. Can be viewed as: • Baseline-corrected normalized reporter (ΔR_n) vs. cycle • Normalized reporter (R_n) vs. cycle • Threshold cycle (C_T) vs. well
arithmetic mean (μ)	A measure of the central tendency of a sample. Calculated as the sum of the individual values in the sample divided by the number of elements in the sample.
assay	A PCR reagent that contains primers to amplify a target, and a probe to detect amplified target.
assay ID	Value assigned by CopyCaller® Software to TaqMan® Copy Number Assays.
automatic baseline	Analysis setting in which the software calculates the baseline start and end values for the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_T).
automatic C_T	Analysis setting in which the software calculates the threshold and baseline in the amplification plot automatically. The software uses the threshold and baseline to calculate the threshold cycle (C_T).

baseline	The initial cycles of PCR in which little change in fluorescence occurs. In the amplification plot, a line fit to the fluorescence levels for a defined range of cycles. If you use the manual baseline analysis setting, we recommend that you select early cycles of PCR to determine the baseline.
baseline-corrected normalized reporter (ΔR_n)	The magnitude of normalized fluorescence generated by the reporter during PCR amplification, calculated as: $\Delta R_n = R_n$ (endpoint) – R_n (baseline), where R_n = normalized reporter.
bootstrapping	A statistical resampling method used to estimate the precision of sample statistics by drawing randomly, with replacement, from a set of data points. Bootstrapping is often used to derive robust estimates of standard errors and confidence intervals of a population parameter like a mean, median, proportion, odds ratio, correlation coefficient, or regression coefficient.
calibrator	A sample with a known copy number for the target of interest used in the $\Delta\Delta C_T$ method to compare the quantity of a nucleic acid sequence in all samples in the same study. Also known as the *calibrator sample*. **Note:** CopyCaller® Software can perform a quantitative analysis of experiments that do not contain a calibrator sample.
comparative C_T ($\Delta\Delta C_T$) method	Quantitation method for quantitation experiments. With the comparative C_T ($\Delta\Delta C_T$) method, results from a calibrator sample and a reference genomic sequence are used to determine relative quantities of a target in samples.
C_T	Abbreviation for threshold cycle. The fractional cycle number at which the fluorescence exceeds the threshold.
cycle threshold	See *threshold cycle (C_T)*.
cycling stage	Stage in a thermal profile that is repeated. If the cycling stage is used to perform PCR, it is called an amplification stage.
detector	A virtual representation of a nucleic acid primer and probe combination used in assays. The user specifies which detector to use for each target sequence.
EFF%	See *amplification efficiency (EFF%)*.
experiment	The entire process of performing a run using the real-time PCR system, including setup, run, and analysis.
experiment name	Entered during experiment setup, the name that is used to identify the experiment. Experiment names cannot exceed 100 characters and cannot include any of the following characters: forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), or semicolon (;).
flag	In the real-time system or CopyCaller™ Software, a indicator that the data from a well did not pass a specific quality control parameter.

forward primer	Oligonucleotide that flanks the 5′ end of the target. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
holding stage	Stage in the thermal profile that includes one or more steps. For example, you can add a holding stage to the thermal profile to activate enzymes, to inactivate enzymes, or to incubate a reaction.
manual baseline	Analysis setting in which you enter the baseline start and baseline end values for the amplification plot. The software uses the baseline and the threshold to calculate C_T values.
manual C_T	Analysis setting in which you enter the threshold value and select whether to use automatic baseline or manual baseline calculations. The software uses the threshold value that you enter and the baseline to calculate the threshold cycle (C_T).
maximum likelihood estimation (MLE)	A statistical method used to fit a mathematical model to real world data. For a fixed set of data paired with a probability model, maximum likelihood estimates for parameters of the model are those estimates that result in a statistical model for which the observed data are maximally probable under that model compared to other configurations of that model.
mean difference (μ_D)	The difference between two calculated mean values. Note that $\Delta\Delta C_T$ is a mean difference.
multicomponent plot	Display of data collected during the cycling stage of real-time PCR. The multicomponent plot shows fluorescence for all cycles in the run.
negative control (NC)	The task assigned to targets assays in wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells. Negative control wells show background fluorescence and allow for the detection of contamination.
no template control (NTC)	See *negative control (NC)*.
nonfluorescent quencher-minor groove binder (NFQ-MGB)	Molecules that are attached to the 3′ end of TaqMan® probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence. Because the NFQ does not fluoresce, it produces lower background, resulting in better precision in quantitation. The minor groove binder (MGB) increases the melting temperature (T_m) without increasing probe length. It also allows the design of shorter probes.
normalized reporter (R_n)	Fluorescence from the reporter dye normalized to the fluorescence signal of the passive reference.
omit well	[1] Action performed after an analysis to exclude one or more wells from a reanalysis. [2] Action that the software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well.

outlier	For a set of data, a datapoint that is significantly smaller or larger than the others. For PCR, a well that amplifies insufficiently or not at all due to experimental error. Outliers typically produce C_T values that differ significantly from the average for the associated replicate wells. These values may not be included in the calculations for a $\Delta\Delta C_T$ study.
passive reference	Dye that produces fluorescence. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for fluorescence fluctuations caused by minor well-to-well differences in concentrations or volume. Normalization to the passive reference signal allows for high data precision.
PCR	Polymerase chain reaction. A method to amplify specific genetic sequences.
probability density function	A function that describes the distribution of probability over the possible values of a variable.
pure dye	Reagent that contains the fluorescent dye. Pure dyes are used to perform a pure dye calibration on the real-time system. Also see *system dye*.
quantitation method	With quantitation experiments, the method used to determine the quantity of target in the samples. For quantitation experiments, there are three types of quantitation methods are available: standard curve, comparative C_T ($\Delta\Delta C_T$), and relative standard curve.
quantitative PCR	A PCR method used to measure the quantity of a nucleic acid sequence within samples.
quantity	In quantitation experiments, the amount of target in the samples. Absolute quantity can refer to copy number, mass, molarity, or viral load. Relative quantity refers to the fold-difference between normalized quantity of target in the sample and normalized quantity of target in the calibrator sample.
quencher	Molecule attached to the 3' end of TaqMan® probes to prevent the reporter from emitting fluorescence while the probe is intact. With TaqMan® reagents, nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher.
raw data plot	Display of fluorescence amplitude for the selected wells for all filters. Displays fluorescence amplitude from all data collection points in the run.
real-time PCR	A polymerase chain reaction (PCR) technology in which the progress of the PCR is monitored as the amplification occurs. Real-time PCR data are used to calculate results for quantitation experiments.
reference sample	See the calibrator sample.
reference sequence	Target is present in two copies in all samples, used in comparative C_T ($\Delta\Delta C_T$) experiments to normalize fluorescence signals for the copy number target you are quantifying.

relative quantitation	A type of quantitation used to analyze the quantity of a nucleic acid sequence (target) in a given sample relative to another calibrator sample. Relative quantity units are often abbreviated as RQ.
replicates	A set of identical reactions containing identical components and identical volumes.
reporter	Fluorescent dye used to detect amplification. In reference to TaqMan® probes, the reporter dye is attached to the 5′ end.
reverse primer	Oligonucleotide that flanks the 3′ end of the target. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.
R_n	Abbreviation for normalized reporter.
samples	gDNA samples in which the copy number of the target is unknown. See also unknown.
SDS	Sequence Detection System.
standard curve method	Quantitation method for quantitation experiments. With the standard curve method, results from standards are used to determine absolute quantities of a target in samples.
standard deviation (σ_{SD})	A measure of statistical variability representing the deviation of measured values from the arithmetic mean of the measured values. Calculated as the square root of the mean of squared deviations from the sample mean. All standard deviations described in this document are sample estimates and, therefore, use unbiased (or N – 1 type) calculations.
statistical variability (σ_{SV})	A statistic (calculated value) used to estimate the variability associated with measured or calculated values. In relative quantitative (RQ) analysis, statistical variability depends both on the chosen control method (internal or external) and on the analysis method (advanced or basic). Consequently, in this document, statistical variability is the generic term used for calculating RQ Min and Max intervals.
subdistributions of ΔC_T	A distribution of ΔC_T values for a specific copy number value in isolation. By contrast, the total distribution of ΔC_T values is a weighted sum of the subdistributions.
TaqMan® reagents	PCR components that consist of primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.
target	The nucleic acid sequence that you want to amplify and detect.
task	Specifies how the software uses the data collected from a well during analysis. Available tasks in copy number experiments are: • Unknown • Negative Control • Standard (standard curve and relative standard curve experiments)

template	Type of nucleic acid to add to the PCR reaction. Genomic DNA templates are used in copy number analysis experiments.
thermal profile	Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the instrument run.
threshold	A level of ΔR_n (automatically determined by the software or manually set) used for C_T determination in real-time PCR. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification curve defines the C_T. The threshold can be determined automatically (see *automatic C_T*) or can be set manually (see *manual C_T*).
threshold cycle (C_T)	The PCR cycle number at which the ΔR_n meets the threshold in the amplification plot.
unknown	The task for the target in wells that contain sample template with unknown target quantities.
variance	The square of the standard deviation, where the standard deviation is a measure of the spread of a quantity of interest (for example, the ΔC_T value for a specific copy number) about the mean of that quantity.
z-score	A statistical measure that indicates the number of standard deviations an observation is above or below the (absolute) mean of a normally distributed population.

Index

P

Q

R

S

T

U

V

W

Z

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