USER GUIDE

applied biosystems®
by life technologies™

Mutation Detector™ Software

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life technologies™

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How to Use this Guide

Purpose

The *Mutation Detector™ Software User Guide* describes how to use the Mutation Detector™ Software to analyze data from TaqMan® Mutation Detection Assay experiments.

Prerequisites

This guide is intended for novice and experienced users.

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

Understanding user attention words

Two user attention words 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 or software operation or accurate chemistry kit use.

Obtaining documents

To obtain...	See...
Documents that are referenced in this guide	"Documentation and Support" on page 65

1 Product Information

Purpose of the software

Life Technologies Mutation Detector™ Software is an automated data analysis tool that performs analysis of real-time PCR quantitation data from TaqMan® Mutation Detection Assay research experiments.

Use the Mutation Detector™ Software to:

- Import instrument results files (*.csv or *.txt files) from TaqMan® Mutation Detection Assay experiments that were run and analyzed on supported Applied Biosystems® real-time PCR systems
- Perform mutation detection analyses to determine the:
  - Mutant allele assay detection ΔC_T cutoff values for your sample type
  - Presence or absence of a mutation
  - Percent mutation in samples when a calibration ΔC_T value is available
- Use predetermined or real-time calculated calibration ΔC_T values and detection ΔC_T cutoff values in the data analysis
- Combine data from technical replicates
- View detailed data analysis information
- View and analyze data for multiple experiments simultaneously
- Save or print analyzed data for further analysis or for sharing with other laboratories that use the Mutation Detector™ Software

About the data analysis

The Mutation Detector™ Software analyzes real-time PCR results from TaqMan® Mutation Detection Assay experiments that were performed on an Applied Biosystems® real-time PCR system.

The TaqMan® Mutation Detection Assays detect and measure somatic mutations in genes that are associated with cancer. The assays are powered by castPCR™ technology, which refers to Competitive Allele-Specific TaqMan® PCR.

For more information

Refer to the *TaqMan® Mutation Detection Assays Protocol* for detailed assay information and the real-time PCR experiment procedures; see "Product documentation" on page 65.

About the mutation detection experiment

In a mutation detection experiment, a sample of unknown mutation status is run in individual real-time PCRs with one or more assays that target mutant alleles within a gene and the corresponding gene reference assay. In each PCR, a purified gDNA sample is combined with:

- A TaqMan® Mutation Detection Assay. The assay contains two primers and a FAM™ dye-labeled MGB probe to detect a mutant allele or reference gene target. Mutant allele assays also contain a MGB oligonucleotide blocker.
- TaqMan® Genotyping Master Mix. The master mix contains AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), dNTPs, and buffer required for the qPCR reactions
- (Optional) TaqMan® Mutation Detection IPC Reagent Kit. The kit contains an internal positive control (IPC) template, two primers, and a VIC® dye-labeled TAMRA™ probe. You can add the IPC reagents to any mutation detection assay reaction to distinguish true target negatives from PCR failure or inhibition.

The reactions are run on an Applied Biosystems® Real-Time PCR System, using a universal mutation detection thermal-cycling protocol. After the run, the real-time PCR system analysis software determines the C_T values for the mutation detection assays and (optional) IPC reagent reactions. Results or Results Table files (*.csv or *.txt files) containing the sample C_T values are exported from the real-time PCR instrument software; these are then imported into the Mutation Detector™ Software for post-PCR data analysis of mutation detection experiments.

About the Mutation Detector Software analysis

In mutation analysis calculations, the difference between the C_T value of the mutant allele assay and the C_T value of the gene reference assay is calculated for all mutant allele assays run on the sample. This ΔC_T value represents the quantity of the specific mutant allele detected within the sample. This ΔC_T is used to determine the sample mutation status by comparison to a predetermined detection ΔC_T cutoff value. If assay calibration ΔC_T values are available, the software can also calculate the percent mutation in a sample.

Note: The first released set of TaqMan® Mutation Detection Assays underwent extensive validation. This validated set covers a subset of the EGFR, KRAS, and BRAF mutation targets. The mutant allele assays in this set have corresponding wild type allele assays in addition to corresponding gene reference assays. The calibration ΔC_T values, which represent the inherent C_T difference between mutant allele assays and corresponding reference assays, have been determined, enabling quantitative analysis

of percent mutation in a sample. Additionally, detection ΔC_T cutoff values have also been predetermined for the validated assay set. For more detail on the validated assay set and data analysis using wild type allele assays, refer to Appendix C, "Validated Assay Set" on page 57

About the two experiment types

Two types of experiments are required for mutation detection analysis:

1. **Detection ΔC_T cutoff determination.** Run the mutant allele assay and the corresponding gene reference assay on wild type gDNA samples that are from the same sample type as the test sample (e.g. gDNA from FFPE tissue samples). Run at least three different wild type samples and three technical replicates of each sample. ΔC_T values are calculated for each sample run with a mutant allele assay - gene reference assay pair. The average ΔC_T value for all samples is then calculated and is used to derive the detection ΔC_T cutoff value for the mutant allele assay.
2. **Mutation detection**. Run the test sample with a mutant allele assay(s) and corresponding gene reference assay. The ΔC_T value for the mutant allele assay - gene reference assay pair is calculated and this value is compared to the predetermined detection ΔC_T cutoff value to determine the sample mutation status.

Note: The two experiment types can be run either sequentially or at the same time. All wild type gDNA samples used for determination of ΔC_T cutoff values can be run together on one plate or on separate plates; the sample ΔC_T values from multiple plates can be combined to generate an assay ΔC_T cutoff value.

Once the mutant allele assay detection ΔC_T cutoff values are determined, these can be used for all subsequent mutation detection experiments run with the same sample type.

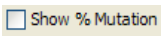
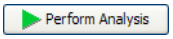
About the software interface

The major elements of the Mutation Detector™ Software interface are described in this section.

Menu bar

Item	Allows you to...
File menu	• Create, open, edit, close, or save studies. Studies contain instrument results files (*.txt or *.csv files) exported from the real-time PCR system software and a default or custom Assay Attributes file that contains calibration ΔC_T and detection ΔC_T cutoff values. • Print tables that contain study data. • Exit the software.
Help menu	• Open the product documentation in portable document format (PDF). • Contact Life Technologies. • View information for the current software version.

Toolbar

Item		Allows you to...
Create new study		• Load instrument results files into a study and open the study. • (Optional) Load a custom Assay Attributes file.
Open study from saved .cast.study file		Open an existing study (*.cast.study) file.
Edit study		• Add instrument results files to the current study. • Remove instrument results files from the current study. • Change the Assay Attributes file.
Close study		Close the currently active study. If you have made changes to the study, the software prompts you to save the changes.
Save As		• Save results to a new *.cast.study file. • Save data as individual tables to a spreadsheet or simple text application. **Note:** When you initially create a study by importing *.csv and *.txt files from the real-time PCR system software, the study does not become a *.cast.study file until it has been saved using the Save As function.
Save to existing files		Save changes to the currently active study: • If a study has already been saved (that is, it is already a *.cast.study file), the Save function saves changes to the study. Note that no dialog box or message will appear to confirm the save. • If the study has not yet been saved, the Save As dialog box appears. See the "Save As" function above.
Help		Open this user guide in portable document format (PDF).
Current Study drop-down list		Select a currently open study from the drop-down list.
Show % Mutation checkbox		Display the calculated percent mutation (that is, the percentage of mutant allele detected within a sample). This feature is available when assays with calibration ΔC_T values are included in the analysis.
Perform Analysis		Perform mutation analysis calculations. The Perform Analysis button turns green whenever the data should be reanalyzed.

Data inputs section

Item	Allows you to...
Well Data tab	• View the C_T values for all wells in the study. • Assign controls to wells. • View and edit the omit status of a well. • View any flags.
Replicates Average tab	View the: • Number of valid replicates • Technical replicates C_T averages • Standard deviations • Omit status of a sample. • Sample and assay level failures with flags
Assay Attributes tab	• View and edit predetermined calibration ΔC_T values and detection ΔC_T cutoff values. • (If applicable) View calibration ΔC_T values and detection ΔC_T cutoff values that have been calculated in real-time by the software (from study control data). • (If applicable) Select the real-time calculated values for use in analysis. • Import Assay Attributes files.

Study section

Item	Allows you to...
General tab	• View and edit the study name, operator, and date • Enter comments for the study
Current Study Settings tab	View and edit the settings that the software uses for the mutation analysis.

Results section

Item	Allows you to...
Results table	• View results as yes/no mutation detected or percent mutation in a sample when a ΔC_T value is available • View sample analysis details for each mutant allele assay and corresponding gene reference assay pair tested
ΔC_T cutoff calculations	View details of the calculated detection ΔC_T cutoff value analysis.

Workflow

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6. (Optional) Save or edit the study (page 46)

2 Obtain the real-time PCR data

Prepare and run the reactions

To generate real-time PCR instrument results files (*.csv or *.txt files) that can be analyzed with the Mutation Detector™ Software:

- Prepare the TaqMan® Mutation Detection Assay reactions
- Run the reactions on a supported real-time PCR system

IMPORTANT! Be sure to follow the guidelines in this section. For detailed procedures, refer to the *TaqMan® Mutation Detection Assays Protocol* (see "Product documentation" on page 65)

Supported real-time PCR systems

The Mutation Detector™ Software can import instrument results files from the real-time PCR systems and software versions listed in the table below.

Applied Biosystems® real-time PCR system	Block module	Software version
7500 system	Standard 96-well Block Module	SDS Software v1.x and v2.x
7500 Fast system	Fast 96-well Block Module	SDS Software v1.x and v2.x
7900HT Fast system	Standard 96-well Block Module, Fast 96-well Block Module, 384-well Block Module	SDS Software v2.x
StepOnePlus™ system	Fast 96-well Block Module	StepOne™ Software v2.x
ViiA™ 7 system	Standard 96-well Block Module, Fast 96-well Block Module, 384-well Block Module	ViiA™ 7 Software v1.x
QuantStudio™ 12 Flex system	Standard 96-well Block, Module, Fast 96-well Block Module, 384-well Block Module	QuantStudio™ 12K Flex Software v1.x

Guidelines for setting up the plate document or experiment

To perform downstream analysis with the Mutation Detector™ Software, set up the plate document or experiment in the real-time PCR system software as follows:

1. For the experiment type, select **Absolute Quantitation** or **Quantitation - Standard Curve**.
2. For each well that contains a reaction, apply a sample name, assay name, and target or detector name. Note the following:

Parameter	Comments
Sample Name	If you enter the sample name as **NTC**, the Mutation Detector™ Software treats the sample as a No Template Control.
	Apply the same sample name to all wells containing the sample and mutation detection assays that will be analyzed together. If the sample name is not identical for each well across the reaction plate, the Mutation Detector Software treats these as different samples.
	If you are using technical replicates, apply the same sample name to the wells of each technical replicate group. The Mutation Detector Software combines data from replicate wells only if the wells share the same sample name. If the replicate wells are named differently, the software analyzes the wells as different samples.
Target Name or Detector Name	In order for the Mutation Detector Software to correctly analyze the data, you must use TaqMan® assay names. Using TaqMan® assay names ensures that a mutant allele assay will be paired with the appropriate gene reference assay in the analysis. See "Assay naming conventions" on page 15.
	If the IPC reagents are duplexed in the reaction, enter **IPC** as the detector name.
Reporter and Quencher Names	For wells that contain: • TaqMan® Mutation Detection Assays, FAM™ dye is the reporter and Non Fluorescent or NFQ-MGB is the quencher • IPC reagents (from the TaqMan® Mutation Detection IPC Reagent Kit), VIC® dye is the reporter and TAMRA™ dye is the quencher **Note:** Instrument results files exported from the 7500 SDS v1.x software do not contain a Reporter column with dye names. In this case, the Mutation Detector Software uses the assay name (from the Detector column) to determine the dye name. If the assay name is *IPC*, the software assumes that the reporter is VIC® dye; for all other assay names, the software assumes that the reporter is FAM™ dye.

3. Select **Standard** as the task for each well of interest, then enter a numeric value. We recommend that the numeric values you enter are relevant to the ng amount of gDNA or copies of DNA input. Note the following:

Parameter	Comment
Amount of sample DNA	For all mutation detection assay results that will be used to calculate a mutation status, load the same amount of sample DNA into the wells. If the sample quantity is not specified in the real-time PCR system software, then the Mutation Detector Software assumes that the sample amounts in each well are equivalent.
Sample quantity value	The sample quantity value must be equal and must be provided in the instrument results file (*.csv or *.txt file) for all samples that will be analyzed together if you are calculating calibration ΔC_T values in real-time (comparing positive control sample C_T values between a mutant allele assay and a corresponding gene reference assay). For more information, see "Calibration ΔC_T values" on page 50.

Note: In order for the Mutation Detector™ Software to compute ΔC_T cutoff values or sample mutation status, you must run each mutant allele assay, the corresponding gene reference assay, and (if applicable) technical replicates on the same reaction plate with the same sample.

For detection ΔC_T cutoff determination, different wild type gDNA samples can be run on one plate or on different plates. The software can combine sample ΔC_T values across plates to generate detection ΔC_T cutoff values.

Assay naming conventions

The naming conventions for the TaqMan® Mutation Detection Assays are described in the table below.

For...	The assay name contains the...	Example
Mutant allele assays	• Gene symbol • COSMIC ID† • Suffix *_mu*	KRAS_517_mu
Gene reference assays	• Gene symbol • Suffix *_rf*	KRAS_rf

† The COSMIC ID is from the Catalogue of Somatic Mutations in Cancer (**www.sanger.ac.uk/genetics/CGP/cosmic/**).

The Mutation Detector™ Software first looks in the Assay Attribute file to get the pairing between mutant assay and corresponding gene reference assay. If the mutant allele assay does not exist in the Assay Attribute file, then it uses the assay names to pair the mutant allele assays with corresponding gene reference assays. The assay pairing information is used for mutation detection analysis, and to associate the assays with assay-specific attributes (for example, calibration ΔC_T and detection ΔC_T cutoff values).

Analyze the experiments using the real-time PCR system software

Use the real-time PCR system software to perform a preliminary analysis of the TaqMan® Mutation Detection Assay experiment data. The preliminary analysis generates the threshold cycle (C_T) values that the Mutation Detector™ Software uses in the mutation detection analysis.

1. In the real-time PCR system software, define the analysis settings:
   a. Open the Analysis Settings dialog box.
   b. Set the following:
      - Manual C_T (threshold cycle): **0.2**
      - Automatic Baseline: **On**
   c. Click **Apply**, then close the window.
2. Click **Analyze** to reanalyze the data with the new analysis settings.
3. View the amplification plots and/or C_T values for all reaction wells, as follows:

Reaction type	What to look for
Samples tested with gene reference assays (FAM™ dye signal)	Verify that the amplification curves have a distinct, linear amplification phase and that the C_T values are within a range of ~18 to 28 for a 20-µL reaction and ~17 to 27 for a 10-µL reaction.
Samples tested with mutant allele assays (FAM™ dye signal)	Review the amplification curves and C_T values. The presence or absence of a distinct, linear amplification phase and C_T values depend on the amount of mutant allele present in the sample.
Positive control samples	Verify that the amplification curves have a distinct, linear amplification phase and that the C_T values are within the expected range for the quantity of target present in the sample. **Note:** Positive control samples that are used to calculate calibration ΔC_T values must contain 100% target sequence for the mutation detection assay that is run with the positive control.
Negative control samples	Verify that the negative control samples *either* do not amplify *or* have very high C_T values.
No template control (NTC) samples	Verify that the NTC samples do not amplify.
Technical replicates	Verify that the C_T values are similar between replicates. **Note:** Some variance is expected between replicates for samples that contain low amounts of target allele and have high C_T values.
(If using) IPC reagents, from the TaqMan® Mutation Detection IPC Reagent Kit	Verify that the amplification curves (VIC® dye signal) in all samples have a distinct, linear amplification phase and that the C_T values are similar for all wells that contain the same sample

4. Check for any data quality flags, then review the real-time data for the associated samples.

Export the data

In the real-time PCR system software, export the instrument results (from the Results or Results Table) for each TaqMan® Mutation Detection Assay experiment. Export the instrument results as one of the following file types:

- Tab-delimited (*.txt) files
- Comma-separated (*.csv) files

IMPORTANT! Do not modify the exported instrument results files. The Mutation Detector™ Software may not be able to add files to a study or analyze files that have been modified.

For more information

For more information on performing, analyzing, or exporting a PCR experiment, refer to the appropriate user guide for your instrument. For document titles and part numbers, see "Documentation and Support" on page 65.

3 Create a Study and Analyze the Data

About studies

Studies contain:

- Instrument results files (*.txt or *.csv files) exported from the real-time PCR system software; each instrument results file corresponds to a single reaction plate

  and

- An Assay Attributes file that contains predetermined calibration ΔC_T values and detection ΔC_T cutoff values (for assays for which these have been calculated)

In the Mutation Detector™ Software, you can:

- Load one or more instrument results files into a single study.
- Determine mutant allele assay detection ΔC_T cutoff values for your sample types
- Use predetermined calibration ΔC_T values (optional) and detection ΔC_T cutoff values (required). The values have been predetermined by Life Technologies for a validated subset of the assays. For more information see, Appendix C, "Validated Assay Set" on page 57.
- Use real-time calculated calibration ΔC_T values and detection ΔC_T cutoff values. The values are calculated in real-time by the software, when the required control data for the calculations exist in the current study.
- Use default or edited data analysis settings (for example, C_T thresholds). The analysis setting are applied to all reaction plates in the study.
- Save studies as *.cast.study files. The *.cast.study files contain the analyzed study data and can be opened directly in the software.

Create a study

Guidelines for creating studies

- Assign a unique name to each reaction plate in the study; the Mutation Detector Software analyzes each plate individually.

  Note: If no reaction plate name is provided in the instrument results file, the *.csv or *.txt file name is used.

- Be sure that all technical replicates are on the same reaction plate and have the same sample name and sample quantity.
- Be sure that all samples to be analyzed together contain the same amount of DNA. Note the following:
  - If sample DNA quantities are indicated in the instrument results file, the quantities must be equal for all samples and technical replicates.
  - If no DNA quantities are indicated, the Mutation Detector Software assumes the samples contain the same quantity.
  - DNA quantity values must be in the instrument results file for samples that will be used to calculate new calibration ΔC_T values.
- Run mutant allele assays and the corresponding gene reference assays with the same sample in the same reaction plate.

  Note: Wild type samples for detection ΔC_T cutoff determination experiments can be run on one or more plates within a study.

Create a study

Create a study that contains the instrument results files (*.csv or *.txt files) exported from the real-time PCR system software.

In the Mutation Detector™ Software:

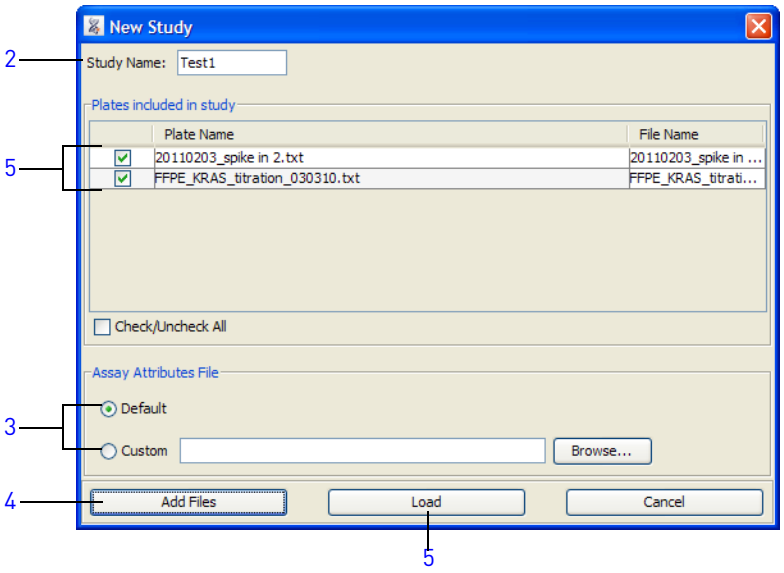
1. Select **File** ▸ **New Study** or click  (**Create new study**).
2. In the New Study window, enter a study name.
3. Select the type of Assay Attributes file to use:
   - **Default** – A file supplied by Life Technologies that contains all TaqMan® Mutation Detection Assays and predetermined calibration ΔC_T values and detection ΔC_T cutoff values calculated for the validated assay set. The default file is named '*TMDA_default_assay_attributes.txt*' and is located in the 'MutationDetector' software installation folder on your hard drive.
   - **Custom** – A file that you created. A custom file should contain your own predetermined calibration ΔC_T values (optional) and detection ΔC_T cutoff values (required) for all TaqMan® Mutation Detection Assays in your study. To import a custom file, select **Browse**, then browse to and open your file. (For information on creating a custom Assay Attributes file, see "Create and import a custom Assay Attributes file" on page 34.)

     Note: Detection ΔC_T cutoff values are required for mutation analysis calculations. These values need to be provided to the software in the Assay Attributes file, or by calculation of the values in real-time in your study, or by entering predetermined values into the Assay Attributes table. See , "(Optional) Edit data in the Assay Attributes table" on page 33.

4. Click **Add Files**, then browse to and open the instrument results files that you exported from the real-time PCR system software. The reaction plate name is displayed in the software. If a reaction plate name is not available in the instrument results file, the software assigns the *.csv or *.txt file name as the reaction plate name.

5. Select the checkbox next to each *.csv and/or *.txt file to include in the study, then click **Load**.

   The software opens a new study; the real-time data (including sample names, quantity, and C_T values) appear in the Well Data, Replicates Average, and Results tables, and the assay attributes appear in the Assay Attributes table.



Analyze the data

Analyze the data

1. Select the appropriate study from the Current Study drop-down list. The data for the selected study appear.

2. Click Perform Analysis. The software analyzes the data using the current analysis settings.

Note: The software analyzes each reaction plate separately; however, the results for all plates in the study are displayed together in the data tables. For detection ΔC_T cutoff determination experiments, wild type sample ΔC_T data from different plates within a study will be combined to generate the assay ΔC_T cutoff value.

4 View the Analysis Results and Edit Input Data and Settings

View data in the Well Data table

About the Well Data table

The Well Data table displays well-level data for all reaction plates included in the study. The table below describes each column in the Well Data table.

Column	Description	Edits allowed?
Plate	The reaction plate name **Note:** If no reaction plate name is provided in the instrument results file, the *.csv or *.txt file name is used.	No
Well	The well number from the reaction plate	No
Assay	The name of the assay in the well	No
Sample	The name of the sample in the well	No
Control	(If applicable) The control assigned to the well: NTC, POSITIVE, or NEGATIVE If you change the control status of a well, you must reanalyze the data to account for the change. Click **Perform Analysis**. **Note:** IPCs are assigned according to the instrument results files exported from the real-time PCR system software. If you included IPC reagents in your PCR reactions (from the TaqMan® Mutation Detection IPC Reagent Kit), the Mutation Detector Software simultaneously analyzes the C_T values in the reaction well for the mutation detection assay reaction (FAM™ dye) and the IPC reaction (VIC® dye). IPCs cannot be edited in the Mutation Detector Software.	Yes; see page 27
Sample Ct	The C_T value of the sample	No
IPC Ct	The C_T value of the IPC	No
Quantity	The numeric value amount of sample in the well (typically in reference to ng amounts or number of copies).	No
Omitted	The omit status of the well When the checkbox is selected, the well is omitted from the analysis. The Well Flag column contains a flag that explains why the well was omitted. If you change the omit status of a well, you must reanalyze the data to account for the change. Click **Perform Analysis**.	Yes; see page 28
Well Flag	A flag that explains why the well was omitted from the analysis In the Well Data table, the software assigns only one flag per well. The flag hierarchy is: NOFAM, ACTEC, IPCEC, OFILE, CTOUT, OUSER.	No

View data

To view the data in the Well Data table:

1. Click the **Well Data** tab.
2. (Optional) Click a column header to arrange the data in ascending or descending order.
3. If the Perform Analysis button is green, click it to reanalyze the data.
4. Check for flags in the Well Flag column and determine the source of the warning. The software assigns only one flag per well.

Flag	Description
NOFAM	The well contains VIC® dye C_T values, but no assay FAM™ dye C_T values are detected.
ACTEC	The FAM™ dye C_T value for the assay exceeds the cutoff value.
IPCEC	Both the VIC® dye C_T value for the Internal Positive Control and the FAM™ dye C_T value for the assay exceed the cutoff values.
OFILE	There was an omitted well in the instrument results file (*.csv or *.txt file).
CTOUT	The software omitted a C_T outlier in a replicate set.
OUSER	A user omitted the well in the Mutation Detector Software.

5. Compare the data in the Well Data table to the related sample-level data in the Replicates Average table.

   Note: If you select a row in any of the tables (Well Data, Replicates Average, Assay Attributes, and Results tables), the associated assay sample data is selected in the other tables.

6. Check the C_T values:
   - Lower C_T values indicate a larger amount of amplified target in a sample than higher C_T values.
   - Wells that contain undetermined C_T values or C_T values that exceed the maximum C_T cutoff (ACTEC flag) do not contain the target or a detectable amount of target.
   - If you used technical replicates, verify that the C_T values are similar between replicates. If a replicate has been omitted by the software (CTOUT flag), review the C_T data for the entire replicate group.

     Note: Some variance is expected between replicates for samples that contain low amounts of target allele and have high C_T values.

7. (If applicable) View the IPC reaction data. In the table below, results are provided for the default limit C_T values of 30 for the IPC VIC dye signal and the mutation detection assay FAM dye signal:

IPC (VIC® dye) C_T value	Mutation detection assay (FAM™ dye) C_T value	Result
<30	<30	PCR is valid
<30	>30 or Undetermined	PCR is valid
>30 or Undetermined	<30	PCR is valid
>30 or Undetermined	>30 or Undetermined	PCR failure or inhibition

If both the mutation detection assay reaction and IPC reaction results are mutation negative in a well, the PCR did not occur (IPCEC flag). In this instance, the mutation-negative result is not valid.

Note: The IPC reaction may fail to generate a C_T value less than the default limit (default = 30) when the mutation detection assay reaction generates a low C_T value, corresponding to a large quantity of target present in the sample. This failure is acceptable because the IPC reaction result is intended to evaluate PCR failure or inhibition in a reaction.

See:

- "View the data" on page 40 for troubleshooting information.
- "(Optional) Edit the analysis settings" on page 44 to include samples that failed the IPC reactions in the mutation detection analysis.

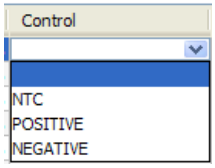
(Optional) Edit data in the Well Data table

In the Well Data table, you can edit the data as follows:

- Assign or change a control (this page)
- Change the omit status of the well (page 28)

Assign or change a control

1. Click the **Well Data** tab.
2. For the well that you want to change, click inside the Control column cell.
3. Select **NTC**, **POSITIVE**, or **NEGATIVE** from the drop-down list. All related replicate wells are automatically populated with the same control name. The controls are defined in the table below.



Note: IPCs are assigned according to the instrument results files exported from the real-time PCR system software. IPCs cannot be edited in the Mutation Detector Software.

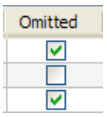
4. Click Perform Analysis to reanalyze the data.

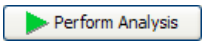
Control	Description
NTC	No Template Control By default, samples that were named **NTC** in the instrument results files exported from the real-time PCR system software are labeled as NTCs in the Mutation Detector Software. However, you can change the NTC assignment in the Mutation Detector Software.
POSITIVE	A positive control is an assay run on a sample containing 100% on-target templates (i.e.,gene reference assays run on a wild type sample or mutant allele assays run on a sample that contains 100% mutant allele). There are two uses for positive controls: • Test for proper assay amplification of the assay target. If the positive control fails the C_T cutoff value, all wells with that assay are excluded from mutation detection calculations. • Real-time calculation of calibration ΔC_T values. If positive control samples are selected for both a mutant allele assay and a corresponding gene reference assay, and the sample quantity is identical between controls, then the positive control samples are used to automatically calculate a calibration ΔC_T value for the paired mutant allele assays and gene reference assays. Note that the mutant allele and gene reference positive controls must be the same sample type. **Note:** For more information on calibration ΔC_T values, see "View data in the Assay Attributes table" on page 32 and "Assay Attributes Table Calculations" on page 49.

Control	Description
NEGATIVE	A negative control is a mutant allele assay run on a sample containing 100% wild type allele. Negative controls are used for real-time calculation of detection ΔC_T cutoff values. Note the following: • When a mutant allele assay negative control sample is selected, all wells containing other mutant allele assays run on the same sample are automatically selected as Negative controls by the software. • If a mutant allele assay negative control sample is selected, and a gene reference assay was run with the same sample, then the software automatically calculates a detection ΔC_T cutoff value for the mutant allele assay. If a calibration ΔC_T value for the assay pair is available, then this value will be used to calculate a normalized detection ΔC_T cutoff value for the mutant allele assay. • You cannot assign negative controls to wells that contain gene reference assays. **Note:** For more information on detection ΔC_T cutoff values, see "View data in the Assay Attributes table" on page 32 and "Assay Attributes Table Calculations" on page 49.

Change the omit status

1. Click the **Well Data** tab.
2. For the well that you want to change, in the Omitted column:
   - Select the checkbox to omit the well from the analysis
   - Deselect the checkbox to include the well in the analysis



3. Click  to reanalyze the data.

Note: If you deselect the checkbox for a well that you previously omitted (a well that exceeds the maximum C_T cutoff value (ACTEC flag)), and reanalyze the data, the software will use the maximum C_T cutoff value as the C_T value for the sample in the mutation detection calculations.

View data in the Replicates Average table

About the Replicates Average table

The Replicates Average table displays the sample-level data for all reaction plates included in the study. The table below describes each column in the Replicates Average table.

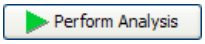
Note: You cannot edit the data in the Replicates Average table.

Note: Technical replicates are not required for mutation detection analysis, but we strongly recommend that you use technical replicates for calculation of detection ΔC_T cut-off values.

Column	Description
Plate	The reaction plate name **Note:** If no reaction plate name is provided in the instrument results file, the *.csv or *.txt file name is used.
Assay	The name of the assay run with the sample
Sample	The sample name
Quantity	The numeric value amount of sample in the well (typically in reference to ng amounts or number of copies)
Control	The control assigned to the sample To edit the controls, see "Assign or change a control" on page 27.
#Replicates	The number of technical replicates The software determines the number of technical replicates by counting all wells on the same reaction plate that have the same sample name, assay name, and DNA quantity (if not provided, the DNA quantity is assumed to be equal).
#Valid Replicates	The number of valid technical replicates The software determines the number of valid technical replicates by counting only those wells that have not been omitted from the analysis. View the Well Data table Omitted and Well Flag columns to discern which replicate(s) have been omitted and why (see "View data in the Well Data table" on page 24).
Avg Ct	The average C_T value for the valid technical replicates
Std Dev	Standard deviation of the valid technical replicates
Omitted	Samples that have been omitted from the analysis because all wells for the sample were omitted To include omitted wells in the analysis, you can: • Change the omit status in the Well Data table (see page 28). • Edit the appropriate analysis settings in the Current Study Settings tab (see page 44).
Flag	A flag that explains why the sample was omitted from the analysis **Note:** Multiple flags may appear for a sample if more than one flag applies.

View the data

To view the data:

1. Click the **Replicates Average** tab.
2. (Optional) Click a column header to arrange the data in ascending or descending order.
3. If the 

button is green, click it to reanalyze the data.
4. Check for flags in the Flag column and determine the source of the warning. Multiple flags may appear for a sample if more than one flag applies.

Flag	Description
NTCCT	The assay C_T value for the No Template Control is below the cutoff value (amplification was detected).
PCTEC	The assay FAM™ dye C_T value for the positive control sample exceeds the positive control C_T cutoff value.
RCTOR	The FAM™ dye C_T value for the reference assay is outside of the defined range.
NTYPE	The assay type is unknown. The assay name does not have the correct format or is not specified in the Assay Attributes file.

5. Compare the data in the Replicates Average table to the related well-level data in the Well Data table.

   Note: If you select a row in any of the tables (Well Data, Replicates Average, Assay Attributes, and Results tables), the associated assay sample data is selected in the other tables.

6. Check the C_T values for:
   - **No template control (NTC) samples** – Verify that the NTC samples do not contain valid C_T values (NTCCT flag), which could indicate that wells or reagents were contaminated.
   - **Samples run with a gene reference assay** – Verify that the C_T values are within a range of 18 to 28 for a 20-µL reaction and 17 to 27 for a 10-µL reaction. The recommended C_T valid range ensures that as low as 1% mutation can be detected. For detection as low as 0.1% mutation, ensure that the gene reference assay C_T value is 26 or less. If your samples have significantly higher amounts of mutation in them, then a higher C_T value for the gene reference assay may be acceptable.
   - **Positive control samples** – Verify that the C_T values are within the expected range for the quantity of target present in the sample. Positive control samples that are used to calculate calibration ΔC_T values must contain 100% target sequence for the mutation detection assay that is run with the positive control.
   - **Negative control samples** – Verify that the negative control samples do not amplify. The negative control samples should have either undetermined C_T values or very high C_T values. Some mutant allele assays are expected to give low level non-specific amplification of wild type gDNA samples. The TaqMan® Mutation Detection Assay Index file (download from: **www.lifetechnologies.com/castpcr**) provides off-target amplification C_T values determined for each mutant allele assay that can be used to evaluate an assay's performance. Note that the off-target C_T value may differ for different sample types.

If samples have been omitted from the mutation detection analysis because of NTC or C_T cutoff failures, you can edit the analysis settings in the Current Study Settings tab (see page 44).

View data in the Assay Attributes table

About the Assay Attributes table

The Assay Attributes table displays the *predetermined* and (if applicable) the *real-time calculated* calibration ΔC_T values and detection ΔC_T cutoff values that are used in the mutation detection calculations. The table below describes each column or button in the Assay Attributes table.

Note: For more information on predetermined and real-time calculated values, see Appendix A, "Assay Attributes Table Calculations" on page 49.

Column/button	Description	Edits allowed?
Mutant Assay	The name of the mutant allele assay	No
GeneRef Assay	The name of the gene reference assay that is paired with the mutant allele assay	No
Calibration ΔCt(-gr)	The predetermined calibration ΔC_T values for paired mutant allele assays and gene reference assays	Yes; see page 33
Cal.Calibr. ΔCt(-gr)	The calculated calibration ΔC_T values for paired mutant allele assays and gene reference assays, when the required positive control data for the calculation exists in the current study	No
Detection ΔCt Cutoff	The predetermined detection ΔC_T cutoff values The cutoff value is the limit of detection that is used to determine the presence or absence of a mutant allele in a sample. For assays having calibration ΔC_T values and therefore normalized detection ΔC_T cutoff values, the detection ΔC_T cutoff values are converted to % in the Results table when the 'Show % Mutation' analysis option is selected.	Yes; see page 33
Cal. Detection ΔCt Cutoff	The calculated detection ΔC_T cutoff values, when the required negative control and gene reference assay data for the calculation exists in the current study. The cutoff value is the limit of detection that is used to determine the presence or absence of a mutant allele in a sample. If you select real-time calculated detection ΔC_T cutoff values, these values are shown in the results table. If a calibration ΔC_T value is available for the assay, then these values are converted to % in the Results table.	No
Reset button	Resets all edited values in the table to the predetermined values previously used in the current study	N/A
Import button	Imports a custom Assay Attributes file that contains user-defined predetermined values (see "Create and import a custom Assay Attributes file" on page 34)	N/A
Default button	Restores all edited values in the table to the default predetermined values from the '*TMDA_default_assay_attributes.txt*' file stored in your Mutation Detector Software installation folder.	N/A

View data

To view the data in the Well Data table:

1. Click the **Assay Attributes** tab.
2. (Optional) Click a column header to arrange the data in ascending or descending order.
3. If the Perform Analysis button is green, click it to reanalyze the data.

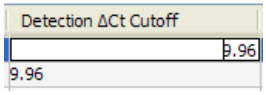
(Optional) Edit data in the Assay Attributes table

In the Assay Attributes table, you can edit the data as follows:

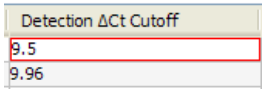
- Manually edit predetermined ΔC_T values (this page)
- Create and import a custom Assay Attributes file (page 34)
- Select real-time calculated values for use in the analysis (page 35)

Manually edit predetermined ΔC_T values

1. Click the **Assay Attributes** tab.
2. For the predetermined value that you want to change:
   a. In the appropriate column Calibration ΔCt(-gr) or Detection ΔCt Cutoff, double-click to activate the cell.



   b. Enter a new value.



   The cell is outlined in red to indicate that the value has been edited. The new value will be used for future mutation detection calculations.
3. Click Perform Analysis to reanalyze the data with the new values.

Create and import a custom Assay Attributes file

Create and import a custom Assay Attributes file that contains your own predetermined calibration ΔC_T values and detection ΔC_T cutoff values.

Note: To create a template for custom Assay Attributes files, see page 35.

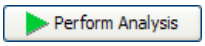
IMPORTANT! The *TMDA_default_assay_attributes.txt* file that is installed with the Mutation Detector Software contains TaqMan® Mutation Detection Assay names and any Life Technologies predetermined values. By default, the software uses these predetermined values to populate the Assay Attributes table and to perform the mutation detection calculations. Do not alter this file or you will lose the default values.

1. Using a spreadsheet application (such as Microsoft® Excel® software), open a new *.txt file and create the following columns:
   - Mutant Assay
   - GeneRef Assay
   - Calibration dCt(-gr)
   - Detection dCt Cutoff

   In the *.txt file, enter the column headings as they appear in the Mutation Detector Software, *except* type **dCt** instead of **ΔCt**.
2. Enter the appropriate values in each column.
3. In the Mutation Detector Software, click the **Assay Attributes** tab, click **Import**, then browse to and open the *.txt file.

   The calibration ΔC_T values and the detection ΔC_T cutoff values from the imported file appear in the Assay Attributes table. The imported values will be used for future mutation detection calculations.

	A	B	C	E
1	Mutant Assay	GeneRef Assay	Calibration dCt(-gr)	Detection dCt Cutoff
2	TP53_10722_mu	TP53_rf		9.96
3	KRAS_516_mu	KRAS_rf	1.728	9.96
4	PTEN_5219_mu	PTEN_rf		9.96
5	PTEN_5033_mu	PTEN_rf		9.96
6	TP53_10808_mu	TP53_rf		9.96
7	KRAS_518_mu	KRAS_rf	2.055	9.96
8	NRAS_584_mu	NRAS_rf		9.96

4. Click 

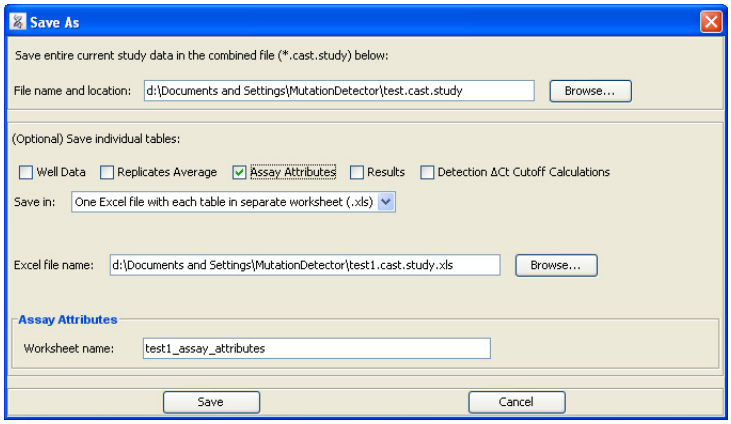
to reanalyze the data with the new values.

Note: Regarding missing calibration ΔC_T values for paired assays or missing ΔC_T cutoff values:

- If calibration ΔC_T values are not available for an assay pair, the software calculates the mutation status of a sample qualitatively (yes/no answer). Calibration ΔC_T values are required for calculation of the percent mutation in mutation positive samples.
- If detection ΔC_T cutoff values are not available, the mutation status of a sample will not be calculated. Predetermined or real-time calculated ΔC_T cutoff values are required for mutation analysis.

Create a template for custom Assay Attributes files

1. In the Mutation Detector Software, open a study, then select **Save As**.
2. Complete the Save As dialog box:
   a. Select the **Assay Attributes** checkbox.
   b. Select **Separate text files with each table in one file (.txt)**.
   c. Browse to and open a save location.
   d. (Optional) Change the default file name.
   e. Click **Save**.



3. Using a spreadsheet application (such as Microsoft® Excel® software), open the saved Assay Attributes *.txt file. Edit, add, or remove assay names and ΔC_T values, then save and close the file.
4. Import the saved Assay Attributes file as described on page 34.

   Note: If Cal.Calibr. dCt(-gr) or Cal.Dectection dCt Cutoff columns are present in the saved Assay Attributes file, any values in these columns are not imported into the Mutation Detector Software.

Select real-time calculated values for use in the analysis

The software does not use real-time calculated calibration ΔC_T values or detection ΔC_T cutoff values in the mutation detection analysis unless predetermined values do not exist or a user selects the calculated values.

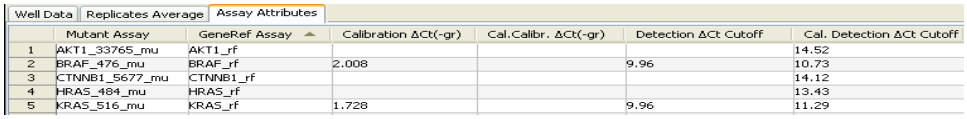
In general, you must determine mutant allele assay detection ΔC_T cutoff values for your specific sample types as these values are required for mutation analysis. If no assay ΔC_T cutoff value is provided to the software, no mutation detection analysis can be performed. For the subset of validated assays for which Life Technologies provides predetermined ΔC_T cutoff values, we highly recommend that you determine the cutoff values for your own sample types.

It is not necessary to calculate calibration ΔC_T values as these are not required for basic mutation detection analysis. However, these values, which reflect the inherent amplification efficiency difference between assays, are needed for quantitative analysis (i.e. percent mutation in a sample). Life Technologies currently provides predetermined calibration ΔC_T values for the validated assay subset. See Appendix C, "Validated Assay Set" on page 57.

Note: See "Assay Attributes Table Calculations" on page 49 to learn more about detection ΔC_T cutoff values and calibration ΔC_T values and to review the guidelines for generating real-time calculated ΔC_T values.

Select real-time calculated detection ΔC_T cutoff values

1. Assign negative control status to wells containing negative control samples (that is, a mutant allele assay run with a wild type sample).
   a. Click the **Well Data** tab.
   b. For the well that you want to change, click inside the Control column cell, then select **NEGATIVE** from the drop-down list. All related replicate wells, and any wells containing other mutant allele assays run with the same sample, are automatically assigned as negative controls.
2. Click Perform Analysis, then click the **Assay Attributes** tab to view the new values in the Cal. Detection ΔCt Cutoff column. The details of this analysis are displayed in the 'Detection ΔCt Cutoff Calculations' table that appears in the lower half of the software window.


Well Data | Replicates Average | Assay Attributes

	Mutant Assay	GeneRef Assay	Calibration ΔCt(-gr)	Cal.Calibr. ΔCt(-gr)	Detection ΔCt Cutoff	Cal. Detection ΔCt Cutoff
1	AKT1_33765_mu	AKT1_rf				14.52
2	BRAF_476_mu	BRAF_rf	2.008		9.96	10.73
3	CTNNB1_5677_mu	CTNNB1_rf				14.12
4	HRAS_484_mu	HRAS_rf				13.43
5	KRAS_516_mu	KRAS_rf	1.728		9.96	11.29

3. The real-time calculated ΔC_T cutoff values will automatically be used for mutation analysis if no predetermined values are present. If both predetermined and real-time calculated ΔC_T cutoff values are present, the predetermined values will be used unless a calculated value is selected. In the 'Cal. Detection ΔCt Cutoff' column, select the appropriate option:

Option	Description
Select	Selects a specified value The box is highlighted in green and this value is used in the mutation detection calculations. If not selected, the default predetermined detection ΔC_T cutoff values are used in the calculations.
Deselect	Deselects the value from the mutation detection analysis
Select All	Selects all values in the Cal. Detection ΔCt Cutoff column for use in mutation detection analysis
Deselect All	Deselects all values from the mutation detection analysis
Copy to Predetermined	Moves the value into the adjacent predetermined column to be used in mutation detection analysis
Copy All to Predetermined	Moves all values in the column into the adjacent predetermined column to be used in mutation detection analysis

4. Click Perform Analysis.

The software will populate the Detection Cutoff column of the Results table with the selected real-time calculated detection ΔC_T cutoff values. For assays with calibration ΔC_T values, the detection ΔC_T cutoff value will be converted to % when the 'Show % mutation' analysis option is selected. The software evaluates the mutation status of samples in comparison to the values in the Detection Cutoff column.

Select real-time calculated calibration ΔC_T values

1. Assign positive control status to wells containing positive control samples (that is, a mutant allele assay run on a 100% mutant allele template and a corresponding gene reference assay run on 100% wild type template sample).
   a. Click the **Well Data** tab.
   b. For the well that you want to change, click inside the Control column cell, then select **POSITIVE** from the drop-down list. All related replicate wells are automatically assigned as positive controls.
2. Click Perform Analysis, then click the **Assay Attributes** tab to view the new values in the Cal. Calibr. ΔCt(-gr) column.
3. In the Cal. Calibr. ΔCt(-gr) column, select the appropriate option:

Option	Description
Select	Selects a specified value The box is highlighted in green and this value is used in the mutation detection calculations. If not selected, the default predetermined calibration ΔC_T values are used in the calculations.
Deselect	Deselects the value from the mutation detection analysis
Select All	Selects all values in the Cal. Calibr. ΔCt column for use in mutation detection analysis
Deselect All	Deselects all values from the mutation detection analysis
Copy to Predetermined	Moves the value into the adjacent predetermined column to be used in mutation detection analysis
Copy All to Predetermined	Moves all values in the column into the adjacent predetermined column to be used in mutation detection analysis

4. Click Perform Analysis. The software uses the real-time calculated calibration ΔC_T values to calculate normalized ΔC_T values in the mutation detection calculations. When calibration ΔC_T and normalized ΔC_T cutoff values are available, the software can calculation percent mutation for mutation positive samples.

View data in the Results table

About the Results table

Each row in the Results table displays the analysis results for a sample run with a mutant allele assay paired with a gene reference assay. The software compares the calculated ΔC_T value to the detection ΔC_T cutoff value for the mutant allele assay. The table below describes each column in the Results table.

Note: You cannot edit the data in the Results table.

Column	Description
Plate	The reaction plate name **Note:** If no reaction plate name is provided in the instrument results file, the *.csv or *.txt file name is used.
Sample	The sample name
Quantity	The numeric value amount of sample in the well (typically in reference to ng amounts or number of copies)
Assay	The name of the mutant allele assay
Detection Cutoff	The detection cutoff value for the mutant allele assay, expressed as expressed as a ΔC_T value (from the Assay Attributes table) The detection cutoff value is expressed as a percentage for assays having calibration ΔC_T and normalized detection ΔC_T cutoff values, when the 'Show % Mutation' analysis option is selected. *% detection = $1/2^{\Delta C_T}$ × 100%* The software allows a minimum % detection cutoff value of 0.001%. The software does not limit the maximum detection C_T cutoff value used for a 'yes/no' mutation detected answer. For more information, see "Assay Attributes Table Calculations" on page 49. **Note:** A detection ΔC_T cutoff value is required for mutation detection analysis. If no detection ΔC_T cutoff value is provided in the Assay Attributes table for the analysis, then the mutation detection calculation cannot be performed and a NOCUT flag is displayed in the results table.
Detected?/%Mutation **Note:** Select/deselect the **Show % Mutation** button to toggle between the Detected? view and the % mutation view. The Show % Mutation option is available only for assays with calibration ΔC_T values.	**Detected?** – The calculated mutation detection results The results are displayed as follows: • Y = yes; the amount of mutation detected is below the mutation detection cutoff ΔC_T value and/or exceeds the percent mutation detection cutoff value for the mutant allele assay. That is, the sample ΔC_T value is less than the detection ΔC_T cutoff value. • N = no; the sample is either mutation negative, or below the limit of detection for the mutant allele assay. That is, the sample ΔC_T value is greater than the detection ΔC_T cutoff value. • I = invalid result; a flag or flags indicating the reason for the failure are displayed in the Flag column.
	% Mutation – The calculated amount of mutant allele within a mutation-positive sample for assays with calibration ΔC_T values. Quantitative analysis is not an option for mutant allele assay and gene reference assay pairs that have not been calibrated. For a sample in which a mutation was not detected, the software does not calculate a % mutation, but assigns 0%.

Column	Description
#Valid Replicates (mu)	The number of valid technical replicates for the mutant allele assay The software determines the number of valid technical replicates by counting only those wells that have not been omitted from the analysis. View the Well Data table Omitted and Well Flag columns to discern which replicate(s) have been omitted and why (see "View data in the Well Data table" on page 24).
Avg Ct (mu)	The average C_T value of the valid technical replicates for the mutant allele assay
Std Dev (mu)	Standard deviation of the valid technical replicates for the mutant allele assay
Ref Assay	The name of the gene reference assay that is paired with the mutant allele assay for mutation detection analysis
#Valid Replicates (rf)	The number of valid technical replicates for the gene reference assay The software determines the number of valid technical replicates by counting only those wells that have not been omitted from the analysis. View the Well Data table Omitted and Well Flag columns to discern which replicate(s) have been omitted and why (see "View data in the Well Data table" on page 24).
Avg Ct (rf)	The average C_T value of the valid technical replicates for the gene reference assay
Std Dev (rf)	Standard deviation of the valid technical replicates for the gene reference assay
ΔCt	The ΔC_T value of the mutant allele assay minus the gene reference assay in this sample
Calibration ΔCt	The calibration ΔC_T value of the paired mutant allele assay and reference assay if available; (from the Assay Attributes table) that is used to calculate the normalized ΔC_T value. This column is visible only when assays with calibration ΔC_Ts are available in the Current Study.
ΔCt_{norm}	Normalized ΔC_T value (the sample ΔC_T value minus the calibration ΔC_T) The normalized ΔC_T value is compared to the detection ΔC_T cutoff value for the mutant allele assay to determine sample mutation status. This column is visible only when assays with calibration ΔC_Ts are available in the Current Study.
Flag	Flags that explain why a valid mutation detection result could not be calculated for the sample **Note:** Multiple flags may appear for a sample if more than one flag applies.

View the data

1. Click the Perform Analysis button to analyze the data.
2. (Optional) Maximize the Results table by clicking the ▲ arrow above the table.
3. (Optional) Click a column header to arrange the data in ascending or descending order.
4. Check for flags in the Flag column and determine the source of the warning. Multiple flags may appear for a sample if more than one flag applies.

   Note: If changes are made in the Current Study Settings or Assay Attributes table that impact the analysis results, the Perform Analysis button will turn green. Click it to reanalyze the data.

Flag	Description
MUPOS	A mutant allele assay run on a 100% mutant allele sample is selected as a positive control in the Well Data table. **Note:** The MUPOS flag appears when the corresponding assay samples are selected as positive controls in the Well Data table.

Flag	Description
NOREF	There is no gene reference assay that pairs with the mutant allele assay for the same sample on the plate.
NOCUT	No Detection ΔC_T cutoff value (predetermined or real-time calculated) is available in the Assay Attributes table for the mutation analysis.
Additional flags	After the software performs the analysis, all flags relevant to the mutation detection calculations are shown in the Results table, including: • Replicates Average table flags, except NTYPE (see page 30) • Well Data table flags, if all wells for a sample were omitted (see page 25)

5. View the results as yes/no mutation detected (this is the default setting):

   a. In the Detected?/% Mutation column, check that each sample run with mutation detection assays displays a **Y** (mutation detected) or **N** (mutation not detected). If there are any **I** (invalid) results, troubleshoot as follows:

      - Review the Flag column to identify the cause of the failure.
      - Review the associated assay sample data in the Well Data, Replicates Average Data, and Assay Attributes.

        Note: If you select a row in any of the tables (Well Data, Replicates Average Data, Assay Attributes, and Results tables), the associated assay sample data is selected in the other tables.

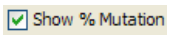
   Causes for the mutation detection analysis failure include:

Cause	Recommended action
No Detection ΔC_T cutoff value is available for the mutation analysis.	The Assay Attributes table must contain either a predetermined or real-time calculated detection ΔC_T cutoff value for the software to calculate the sample mutation status. Either include the appropriate mutant allele assay detection ΔC_T cutoff value determination experiment in the Current Study (See Appendix A, "Assay Attributes Table Calculations" on page 49}, or add a predetermined detection cutoff value to the Current Study by *either* editing the data in the Assay Attributes table *or* by uploading a custom Assay Attributes file containing the value (see, "(Optional) Edit data in the Assay Attributes table" on page 33).
The sample C_T value for the gene reference assay exceeds the maximum C_T cutoff value.	Repurify and quantify your sample preparation, then repeat the experiment using an appropriate amount of functional template.
The C_T value for the gene reference assay is outside of the defined C_T valid range but does not exceed the maximum C_T cutoff value.	The default C_T valid range of 18–28 is for a 20-µL reaction. If you are using a 10-µL reaction, set the range to 17–27, then reanalyze the data.
	Select to ignore the gene reference assay C_T valid range (see "(Optional) Edit the analysis settings" on page 44), then and reanalyze the data. The recommended C_T valid range is to ensure that as low as 1% mutation can be detected. For detection of down to 0.1% mutation, ensure that the gene reference assay C_T value is 26 or less. If your samples have significantly higher amounts of mutation in them, then a higher C_T value for the gene reference assay may be acceptable.

Cause	Recommended action
The positive control sample assay C_T value exceeds the positive control C_T cutoff value.	Check that the positive control sample contains 100% target. If it does not, you may choose to ignore this failure and reanalyze your data (see "(Optional) Edit the analysis settings" on page 44).
Both the mutation detection assay reaction and IPC reaction results are negative in a well, which indicates that the PCR did not occur.	Repurify and quantify your sample preparation, then repeat the experiment using an appropriate amount of functional template.

6. View the results as percent mutation (that is, the percent of mutant allele detected within a sample), if this option is available:

   a. Select the **Show % Mutation** checkbox to display the %Mutation column.

   b. Compare the sample % mutation results in the %Mutation column to the mutant allele assay % detection cutoff in the adjacent Detection Cutoff column.

   c. Review samples that have a value greater than 0% mutation, which indicates that a mutation was detected. Both the mutant allele assay and paired gene reference assay run with the sample will have passing C_T values. Check that the % mutation calculated for any control samples is within the expected range.

   d. Review samples that have a value of 0% mutation, which indicates that no mutation was detected. The gene reference assay run on this sample will have a passing C_T value. The mutant allele assay will have either: (1) no passing C_T value (the ACTEC flag is displayed in Well Data table) and no normalized ΔC_T will be calculated or (2) a passing C_T value, but the normalized ΔC_T value exceeds the assay detection ΔC_T value.

Note: Quantitative analysis (percent mutation calculation) is available only for assays in the Current Study that have calibration ΔC_T and normalized ΔC_T cutoff values. Quantitative analysis is not an option for mutant allele assay and gene reference assay pairs that have not been calibrated.

About the Detection ΔC_T Cutoff Calculations table

The Detection ΔC_T Cutoff Calculations table is dynamically displayed when a detection ΔC_T cutoff value determination experiment is included in the Current Study. Each row in the ΔC_T Cutoff Calculations table displays all the data used to determine a ΔC_T cutoff value. In a typical experiment, three or more wild type gDNA samples are run with mutant allele assays (negative controls selected in the Well Data table) and paired gene reference assays. The software calculates a mutant allele assay ΔC_T cutoff value when a calibration ΔC_T value is not available and a normalized ΔC_T cutoff value when a calibration ΔC_T value is available. The table below describes each column in the ΔC_T Cutoff Calculations table.

Note: You cannot edit the data in the ΔC_T Cutoff Calculations table.

Column	Description
Assay	The name of the mutant allele assay
Sample	The wild type sample name(s). Multiple values are separated by commas
C_T(mu)	The C_T value(s) for the mutant allele assay (from the Replicates Average table). Multiple values are separated by commas. For any C_T values that are undetermined or greater than the Maximum C_T cutoff value (set in the Current Study Settings), the Maximum C_T cutoff value is shown and is used for the ΔC_T cutoff calculations
Ref Assay	The name of the gene reference assay that is paired with the mutant allele assay for the detection ΔC_T cutoff determination analysis
C_T(ref)	The C_T value(s) for the gene reference assay (from the Replicates Average table). Multiple values are separated by commas
ΔC_T	The ΔC_T value(s) of the mutant allele assay minus the gene reference assay in this sample. Multiple values are separated by commas
Ave ΔC_T	The average ΔC_T value for all wild type samples run with the same paired mutant allele assay and gene reference assay
Std Dev	Standard deviation of the sample ΔC_T values
ΔC_T cutoff	$\Delta C_T = [C_{T(\text{mutant allele assay negative control})} - C_{T(\text{gene reference assay})}]$ - (3 × *the standard deviation or 2* C_T*, whichever is greater).* See, "Detection ΔC_T cutoff values" on page 51.
Calibration ΔC_T cutoff (—gr)	The calibration ΔC_T value of the paired mutant allele assay and gene reference assay (from the Assay Attributes table), if available, that is used to calculate the normalized ΔC_T value
ΔC_T norm	*Normalized* $\Delta C_T = [C_{T(\text{mutant allele assay negative control})} - C_{T(\text{gene reference assay})}]$ - *Calibration* ΔC_T - (3 × *the standard deviation or 2* C_T*, whichever is greater).* See, "Detection ΔC_T cutoff values" on page 51.

View the data

1. (Optional) Maximize the Results table by clicking the arrow icon above the table.
2. (Optional) Click a column header to arrange the data in ascending or descending order.

(Optional) Edit the analysis settings

The Current Study Settings tab displays the analysis settings that are applied to all reaction plates within the currently open study at the well or sample level. You can edit the default values and/or choose to ignore specific failures in the mutation analysis.

Note: Certain failures (described in the table below) cause all wells or samples that are run with the same assay to be omitted from the mutation detection analysis. Wells or samples that have been omitted from the analysis are indicated in the Well Data and Replicates Average tables, and flagged according to the failure type.

Edit the analysis settings

1. Click the **General** tab, then enter or edit the study name, operator, date, and any comments about the study.
2. Click the **Current Study Settings** tab, then edit the analysis settings as needed.

   Note: To return to the default settings at any time, click **Default**.

The table below describes each item in the Current Study Settings tab.

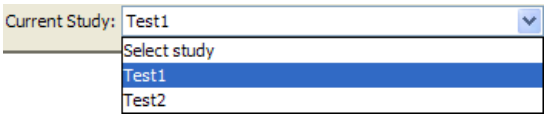
Item	Description
Maximum C_T cutoff	Applies to all assays in all wells, and to all reaction plates in a study. If the FAM™ or VIC® dye C_T value of a well is greater than the specified C_T cutoff value, the well is considered an amplification failure and the software omits the well from the mutation detection calculations. The default C_T cutoff value is 37; you can set your own C_T cutoff value within a range of 30 to 40. **Note:** C_T cutoff values greater than 37 may lead to false positive results in some cases. **Note:** For mutant allele assay negative control samples that have undetermined C_T values or C_T values greater than the Maximum C_T cutoff in the Well Data table, the Maximum C_T cutoff value is shown in the Assay Replicates table and is used for the detection ΔC_T cutoff value calculations.
Exclude outlier from replicates	If more than two replicates are run per sample, the software performs one round of outlier calculation. To determine outliers, the software uses Grubb's outlier test and the following calculation: absolute value of the (well C_T – mean C_T) ≥ 0.25 With this test/calculation, only one well can be an outlier. You can select to exclude or include the outlier in the calculation.
Ignore NTC failure	If No Template Controls (NTCs) are used in a study, the NTC status is based on the maximum C_T cutoff value. If an assay run in an NTC well has a C_T cutoff value that is less than the maximum C_T value (for example, if the C_T value is less than the default maximum C_T cutoff value of 37), the NTC fails; the software excludes all wells run with the assay from the analysis. If you select to ignore the NTC failure, the software calculates the mutation status of all samples run with the assay regardless of NTC failures.

Item	Description
Positive Control C_T cutoff	Positive control samples are selected in the Well Data table. A positive control is an assay run on a sample containing 100% on-target template (that is, gene reference assays run on a wild type sample or mutant allele assays run on a sample containing 100% mutant allele). If a positive control C_T value is greater than the C_T cutoff value (default = 30), the software omits all wells that contain that assay from the mutation status calculations.
Ignore Positive Control failure	If you select to ignore positive control failures, the software calculates the mutation status for all samples run with the assay.
IPC failure: VIC Ct> [x] FAM Ct>[x]	The optional IPC reaction (VIC® dye label) is run in duplex PCR with TaqMan® Mutation Detection assays (FAM™ dye label). For an IPC reaction to fail, both the VIC® and FAM™ dye C_T values must be greater than the C_T cutoff value (default = 30). If the IPC reaction fails, the software excludes the well data from the mutation detection analysis.
Ignore IPC failure	If you select to ignore IPC failures, the software calculates the mutation status for samples in wells that fail the IPC control.
Gene Ref Ct valid range	To enable detection of low levels of mutated DNA in a sample, be sure to add sufficient sample to the reaction wells. A sample run with a gene reference assay should have a C_T value within the following ranges: • ~18 to 28 for a 20-µL reaction (default values) • ~17 to 27 for a 10-µL reaction If a sample run with the gene reference assay has a C_T value outside of this range, the software excludes the C_T values for the gene reference assay from the mutation detection calculations. **Note:** The range may vary for different sample types and for different instruments. You can select a different C_T range.
Ignore Gene Ref Ct valid range	If you select to ignore gene reference assays that are outside of the valid range, the software calculates the mutation status for samples run with a mutant allele assay paired with a gene reference assay that failed the C_T cutoff values.
Default button	Restores all of the analysis settings to the default values.

(Optional) Save or edit the study

Saving a study

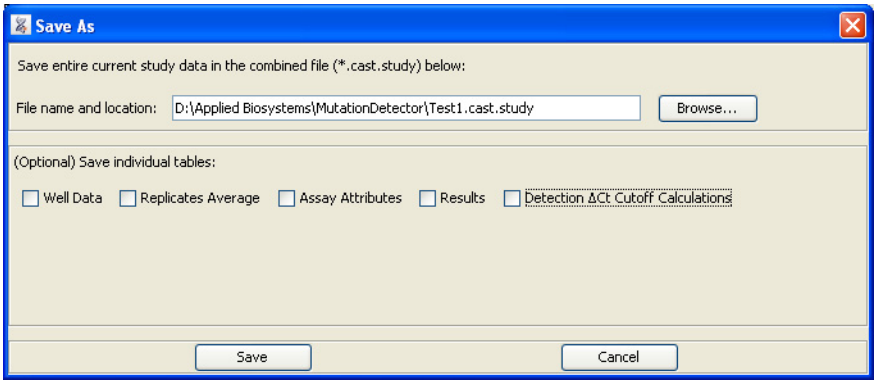
1. If multiple studies are open, be sure to select the appropriate study from the Current Study drop-down list.



2. Select the appropriate save option:

To save...	Perform these steps
A newly created study (that is, you are saving the study for the first time as a *.cast.study file)	1. Select one of the following: • **File ▸ Save As** or click (**Save As**) • **File ▸ Save** or click (**Save to existing files**) 2. Continue with step 3.
An existing *.cast.study file to a new *.cast.study file	1. Select **File ▸ Save As** or click (**Save As**). 2. Continue with step 3.
Changes to an existing *.cast.study file	Select **File ▸ Save** or click (**Save to existing files**). The Save function saves changes to the study. Note that no dialog box or message will appear to confirm the save. No further steps are required.

3. Save the entire study:
   a. (Optional) Change the default file name.
   b. Browse to and open a save location.
   c. Click **Save**.



4. (Optional) Save individual tables:
   a. Select the checkbox for each table you want to save: **Well Data**, **Replicates Average**, **Assay Attributes**, **Result** and/or **Detection ΔC_T Cutoff Calculations** (if available).

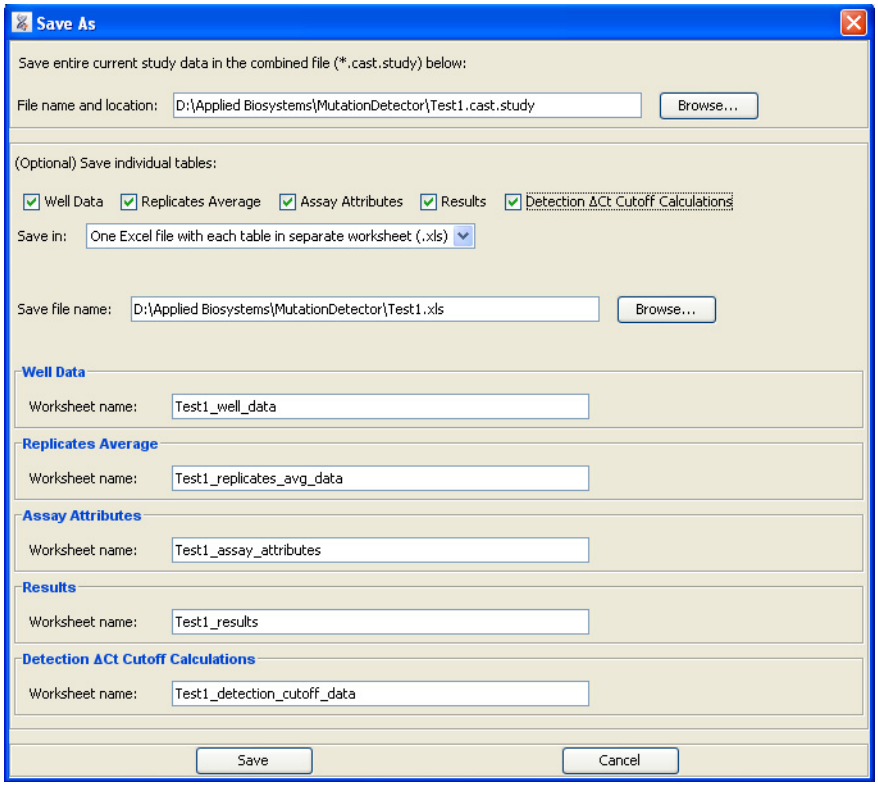
b. Select a format to save the file(s) in:

- **One Excel file with each table in a separate worksheet (.xls)**
- **Separate text files with each table in one file (.csv)**
- **Separate text files with each table in one file (.txt)**

c. Browse to and open a save location.

d. (Optional) Change the default file name(s).

e. Click **Save**.

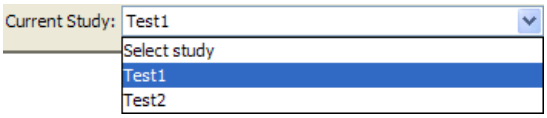


Note: The save 'Detection ΔC_T Cutoff Calculations' table option is dynamically displayed. It is available only if a detection ΔC_T cutoff determination experiment was included in the Current Study.

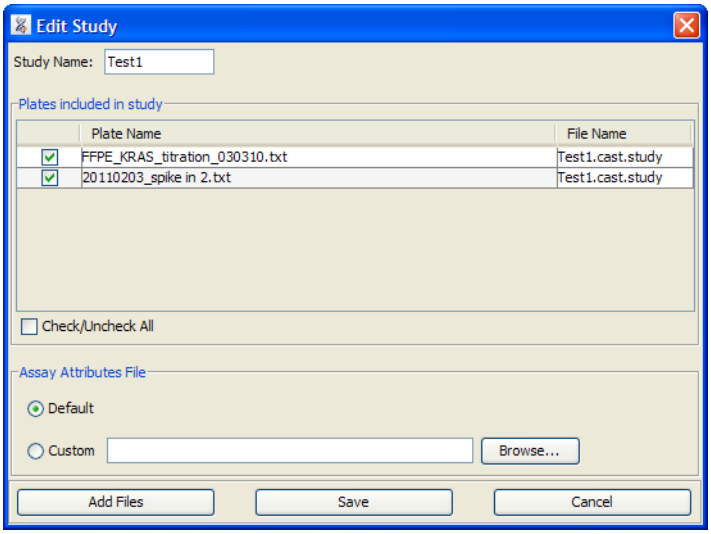
Editing a saved study (*.cast.study file)

1. In the Mutation Detector Software, open the study to edit:
   a. In the Mutation Detector Software, select **File ▸ Open Study** or click  (**Open study from saved .cast.study file**).
   b. Browse to and open the appropriate *.study.cast file.

   Note: If the study is already open, be sure to select it in the Current Study drop-down list.



2. Select **File ▸ Edit Study** or click  (**Edit study**).
3. Edit the study as needed. In the Edit Study dialog box, you can:
   - Edit the study name
   - Add or remove instrument results files (*.csv and *.txt)
   - Change the Assay Attributes file



4. Click **Save**.

A Assay Attributes Table Calculations

About predetermined and real-time calculated values

The Mutation Detector™ Software can use *predetermined* or *real-time calculated* calibration ΔC_T values and detection ΔC_T cutoff values. The two terms are described in the table below.

Term	Description
Predetermined	The calibration ΔC_T values and detection ΔC_T cutoff values for the TaqMan® Mutation Detection Assays are predetermined by Life Technologies (for the validated assay subset) or by the user. The software uses these values to: • Populate the Assay Attributes table • Perform the mutation detection calculations By default, the software uses the Life Technologies predetermined values. You can edit the predetermined values, either manually (see page 33) or by importing a custom Assay Attributes file (see page 34). **Note:** The Life Technologies predetermined values are contained in the *TDMA_default_assay_attributes.txt* file that is installed with the Mutation Detector™ Software.
Real-time calculated	The calibration ΔC_T values and detection ΔC_T cutoff values for the TaqMan® Mutation Detection Assays are calculated in real-time by the software. The software uses these values to: • Populate the Assay Attributes table • Perform the mutation detection calculations The software uses the calculated values only when predetermined values do not exist or when a user selects the calculated values (see page 35). You cannot edit the calculated values.

Calibration ΔC_T values

The calibration ΔC_T value is the C_T difference between the mutant allele assay and the corresponding gene reference assay when assays are run on the same amount of pure target DNA for the assay. Calibration ΔC_T values for an assay pair in an analysis are required for quantitation of the percent mutation in a sample. If calibration ΔC_T values are not available, then only the qualitative yes/no mutation status can be determined.

Theoretically, if assays have 100% amplification efficiency, then mutant allele assays and the corresponding gene reference assays should generate the same C_T value for the same amount of on-target templates. In reality, different assays have different amplification efficiencies; therefore, they do not always generate the same C_T values for equal quantities of DNA. This difference is determined by amplifying equal amounts of DNA templates containing 100% mutant allele (for the mutant allele assay) and 100% wild type DNA (for the gene reference assay). Life Technologies calculated the predetermined calibration ΔC_T values using 10^4 copies of synthetic plasmids as positive control templates for the validated subset of the TaqMan® Mutation Detection Assays. See, Appendix C, "Validated Assay Set" on page 57.

Calibration ΔC_T values reflect the inherent difference in the mutant allele assay and the corresponding gene reference assay amplification efficiencies. The calibration ΔC_T values theoretically should not vary significantly between different sample types, thus they can be determined using plasmid DNA templates in well-controlled experiments. These values can also be generated for your own sample types, if desired, though it may be difficult to acquire the requisite 100% mutant allele samples. If you are interested in generating calibration ΔC_T values, see "Guidelines for generating calculated values" below.

Guidelines for generating calculated values

For the Mutation Detector Software to generate meaningful calibration ΔC_T values for the real-time calculations, we recommend the following:

- Run at least three technical replicates per sample.
- Samples must be of the highest quality and must be accurately quantified for functional template.
- The mutant allele assay positive control is the assay that is run on a DNA sample containing 100% mutant allele.
- The corresponding reference assay positive control is a gene reference assay that is run on a DNA sample containing 100% wild type gene.
- The positive control samples are labeled as such in the Control column of the Well Data table.
- The positive control samples contain the same amount of DNA (from the same sample type), and the amount is indicated in the Quantity column of the Well Data table.

How the software performs the real-time calculations

The Mutation Detector Software calculates the calibration ΔC_T value for each mutant allele assay and its corresponding gene reference assay. The calculation takes into account the difference between assay amplification efficiencies, as follows:

$$Calibration\ \Delta C_T = C_{T\ (mutant\ allele\ assay\ positive\ control)} - C_{T\ (gene\ reference\ assay\ positive\ control)}$$

Detection ΔC_T cutoff values

The detection ΔC_T cutoff value is used to determine the limit of the percent mutation in a sample that the mutant allele assay can detect. A ΔC_T cutoff value is required for mutation detection analysis and must be present in the Assay Attributes table for the software to perform the analysis.

You can determine the required mutant allele assay detection ΔC_T cutoff values prior to running a mutation detection experiment, or you can include both experiment types within the same study. ΔC_T cutoff values calculated ahead of time can be entered into the software Assay Attributes Table, or alternatively added to a custom Assay Attributes file that can be uploaded into any study that used the assays.

Note: Life Technologies has generated predetermined detection ΔC_T Cutoff values for the validated subset of mutation detection assays using cell line DNAs (see Appendix C, "Validated Assay Set," on page 57). Given that ΔC_T cutoff values can vary for different sample types, we highly recommend that you generate your own ΔC_T cutoff values, on your own samples, for any of the validated assays that you use in your studies.

Guidelines for generating calculated values

Genomic DNA extracted from different sample types and preparation methods may generate different detection ΔC_T cutoff values. We recommend that you generate detection ΔC_T cutoff values for the sample type that you will use in your mutation detection experiments; run the mutant allele assay and the corresponding gene reference assay on wild type gDNA samples that are of the same sample type as the test sample (e.g. gDNA from FFPE tissue samples).

For the Mutation Detector Software to generate meaningful detection ΔC_T cutoff values for the real-time calculations, we recommend the following:

- Run the mutant allele assay and the corresponding gene reference assay on wild type gDNA samples that are from the same sample type as the test sample.
- Run three different wild type samples and at least three technical replicates of each sample.
- Samples must be of highest quality and must be accurately quantified for functional template.
- The mutant allele assay negative control is the assay that is run on a DNA sample containing 100% wild type allele.
- The corresponding gene reference assay is run on the same DNA sample.
- The negative control samples for the mutant allele assays are labeled as such in the Control column of the Well Data table. (The gene reference assay samples are automatically identified by the software.)
- The negative control and gene reference assay samples contain the same amount of DNA (from the same sample type).
- Each wild type gDNA sample tested must be run with the mutant allele assay, corresponding gene reference assay, and all technical replicates on the same plate. However, different samples may be run on different plates within a study.

How the software performs the real-time calculations

To perform the real-time calculations for the detection ΔC_T cutoff values, the Mutation Detector Software:

1. Averages the C_T values from the technical replicates.

2. Calculates the ΔC_T value between amplification reactions for the mutant allele assay and the corresponding gene reference assay, as follows:

   $\Delta C_T = [C_{T(\text{mutant allele assay negative control})} - C_{T(\text{gene reference assay})}]$

   or

   $\text{Normalized } \Delta C_T = [C_{T(\text{mutant allele assay negative control})} - C_{T(\text{gene reference assay})}] - \text{Calibration } \Delta C_T$

   where: The calibration ΔC_T value is the inherent C_T difference between the mutant allele assay and the corresponding gene reference assay. These values are currently available for a subset of the mutation detection assays. See, Appendix C, "Validated Assay Set" on page 57.

3. Averages the ΔC_T value from each sample.

4. Calculates the detection ΔC_T cutoff value, as follows:

   Detection ΔC_T cutoff = Average ΔC_T – (3 × the standard deviation or 2 C_T, whichever is greater)

   or

   Detection ΔC_{Tnorm} cutoff= Average Normalized ΔC_T – (3 × the standard deviation or 2 C_T, whichever is greater)

   Note: We recommend adjusting any calculated detection ΔC_T cutoff values that are greater than 9.96 to 9.96 ΔC_T , which is equivalent to a 0.1% detection cutoff.

For assays with calibration values, the quantitative calculation for the percent mutation in a sample can be done. For these assays, the cutoff value can be expressed as a percentage. The conversion formula between % and ΔC_T is:

$\%\ \text{detection} = 1/2^{\Delta C_T} \times 100\%$

The software allows a minimum % detection cutoff value of 0.001% (there is no maximum ΔC_T cutoff restriction).

Note: When using artificial template DNAs to calculate detection ΔC_T cutoff values, some TaqMan® Mutation Detection Assays can detect extremely rare amounts of mutant allele in a high background of wild type allele that exceed the 0.001% lower limit detection cutoff value for quantitation results that is set in the software.

B Results Table Calculations

Overview

This appendix describes how the Mutation Detector™ Software performs the mutation detection calculations, using either predetermined or real-time calculated calibration ΔC_T values and detection ΔC_T cutoff values. The mutation analysis results are displayed in the Results table.

Note: For more information on the tables, see "View data in the Assay Attributes table" on page 32 and "View data in the Results table" on page 39.

Mutation status

The Mutation Detector Software determines the mutation status of a sample by first calculating the ΔC_T value between amplification reactions for a mutant allele assay and a corresponding gene reference assay, as follows:

$$\Delta C_T = C_{T(mutant\ allele\ assay)} - C_{T(gene\ reference\ assay)}$$

or

$$Normalized\ \Delta C_T = [C_{T(mutant\ allele\ assay)} - C_{T(gene\ reference\ assay)}] - Calibration\ \Delta C_T$$

where: The calibration ΔC_T value is the inherent C_T difference between the mutant allele assay and the corresponding gene reference assay. The calibration ΔC_T values are displayed in the Assay Attributes table, if available.

The software then compares the ΔC_T or normalized ΔC_T value to a predetermined detection ΔC_T cutoff value to determine if a mutation is detected within the sample, as follows:

If the ΔC_T value is...	Then...
Greater than the detection ΔC_T cutoff value	The software classifies the gDNA sample as **mutation not detected**. The sample is either mutation negative, or below the limit of detection for the TaqMan® Mutation Detection Assays.
Less than the detection ΔC_T cutoff value	The software classifies the gDNA sample as **mutation detected**, and calculates the percent mutation in the sample if the calibration ΔC_T value is available.

Percent mutation

For assay pairs having calibration ΔC_T values, quantitative mutation analysis is possible. The Mutation Detector Software determines the percent mutation present in a mutation-positive sample based on the normalized ΔC_T value between amplification reactions for a mutant allele assay and a corresponding gene reference assay. The software uses the following calculations:

$$\% \ mutation = 1/2^{\Delta C_T} \times 100\%$$

Note: The software does not calculate a percent mutation for ΔC_T results greater than the detection ΔC_T cutoff value. The detection ΔC_T cutoff values are displayed in the Assay Attributes table and copied to the Results table.

C

Validated Assay Set

About the TaqMan® Mutation Detection Assays validated assay set

The first set of TaqMan® Mutation Detection Assays underwent extensive validation. This set of validated assays covers 14 KRAS targets, 29 EGFR targets, and the BRAF V600E mutation targets and is referred to as the validated assay set. The main differences between the validated assays compared to other TaqMan® Mutation Detection Assays are:

1. The mutant allele assays have corresponding wild type allele assays in addition to corresponding gene reference assays. These assays enable more accurate quantitation of the percent mutation in a sample.
2. The inherent ΔC_T difference between mutant allele assays and the corresponding gene reference assay or wild type allele assay were predetermined, enabling quantitative analysis of percent mutation in a sample.
3. Detection ΔC_T cutoff values were predetermined for the validated assay set.

Wild type allele assays

Wild type allele assays for corresponding mutant allele assays use castPCR™ technology. Each wild type allele assay contains:

- an allele-specific primer that detects the wild type allele
- a MGB oligonucleotide blocker that suppresses the mutant allele
- a locus specific primer
- a locus specific TaqMan® FAM™ dye-labeled MGB probe

Wild type allele assay names are composed of the following 3 elements separated by underscore characters: Gene symbol, COSMIC ID, and Suffix _wt (for example: KRAS_517_wt).

Validated mutant allele assays can be paired with corresponding wild type allele assays, versus gene reference assays, in a mutation detection experiment for more highly accurate quantitation of the percent mutation in a sample. The basic mutation detection protocol can be used; however, it is recommended that three technical replicates are run for highest quantitation accuracy.

Calibration ΔC_T value

The calibration ΔC_T value is the inherent C_T difference between a mutant allele assay and a wild type allele assay or a gene reference assay. This difference is determined by amplifying equal amounts of DNA templates containing 100% mutant allele (for the mutant allele assay) and 100% wild type DNA (for the wild type allele assay or gene reference assay). Life Technologies determined the calibration ΔC_T values for the validated assay set in experiments using 10^4 copies of synthetic DNA templates.

Detection ΔC_T cutoff value

The detection ΔC_T cutoff value is used to determine the lower limit amount of mutation in a sample that the mutant allele assay can detect. The detection ΔC_T cutoff values were determined for the validated assay set in studies where each mutant allele assay was run with its corresponding wild type allele assay and gene reference assay on multiple genomic DNA (gDNA) samples isolated from wild type cell line samples. The ΔC_T value between amplification reactions for the mutant allele assay and the corresponding wild type allele assay or gene reference assay was used to calculate the detection ΔC_T cutoff values (see page 58 for the equations used). Calculated detection ΔC_T cutoff values were close in value for each mutant allele assay run with corresponding wild type allele assays and gene reference assays; the smaller calculated detection ΔC_T cutoff value was used for the mutant allele assay. All detection ΔC_T cutoff values greater than 9.96 were adjusted to 9.96, equivalent to a 0.1% detection cutoff.

To confirm that the predetermined mutant allele assay 0.1% sensitivity value was relevant to mutation detection analysis in gDNA isolated from cellular and FFPE samples, Life Technologies conducted TaqMan® Mutation Detection Assays experiments with:

- **Cellular gDNA samples** – 10 copies of plasmid DNAs carrying mutant alleles spiked into 30 ng (equivalent to 10^4 copies) of normal cell-line gDNA. In these studies, the mutant allele assay sensitivity was as low as 0.1%.
- **gDNA isolated from FFPE samples** – Limited testing done with available gDNA isolated from FFPE samples demonstrated equivalent levels of assay sensitivity.

IMPORTANT! Genomic DNA extracted from alternate sample types and preparation methods may have different cutoff values. We recommend that you determine the detection ΔC_T cutoff values for your specific sample types and preparation methods (see page 58).

For more information on the validated assay set, please see the Application Note *'Accurate and sensitive mutation detection and quantitation using TaqMan® Mutation Detection Assays for disease research'* (Part no. CO31540 01011), which can be downloaded from **www.lifetechnologies.com**.

Mutation analysis using mutant allele assays run with wild type allele assays

Mutation analysis calculations for mutant allele assays run with wild type assays

When mutant allele assays are run with wild type allele assays in a mutation detection experiment, variations in the assay attribute and mutation analysis calculations will be performed by Mutation Detector Software, as detailed in the following sections.

Assay Attributes Value Calculations

Determine the calibration ΔC_T value

Calibration ΔC_T values have been predetermined by Life Technologies for the validated set mutant allele assays and corresponding wild type allele assays. It is neither required, nor straightforward, to generate these for a give sample type. However, if you choose to calculate your own calibration ΔC_T values, follow the basic guidelines in , "Calibration ΔC_T values" on page 50, using wild type allele assays run with 100% wild type allele samples along with the corresponding mutant allele assays run with 100% mutant allele assay samples. The following calculation is used by the software to generate a calibration ΔC_T value:

$$Calibration\ \Delta C_T\ cutoff = C_{T(mutant\ allele\ assay\ positive\ control)} - C_{T(wild\ type\ allele\ assay\ positive\ control)}$$

Determine the detection ΔC_T cutoff value

To determine detection ΔC_T cutoff values for your own sample type (highly recommended), follow the basic protocol for Detection ΔC_T cutoff values determination in Appendix A, with the exception that each mutant allele assay will be paired with its corresponding wild type allele assay. After sample amplification, the ΔC_T value between amplification reactions for the mutant allele assay and the wild type allele assay will be calculated as follows:

$$Normalized\ \Delta C_T = [C_{T(mutant\ allele\ assay\ negative\ control)} - C_{T(wild\ type\ allele\ assay)}] - Calibration\ \Delta C_T$$

Then calculate the ΔC_T cutoff value:

$$Detection\ \Delta C_T\ cutoff = Average\ Normalized\ \Delta C_T - [3 \times the\ standard\ deviation\ or\ 2\ C_T,\ use\ whichever\ is\ greater]$$

Note: If in a detection ΔC_T cutoff experiment, more than one corresponding wild type allele assay or gene reference assay was run on the same plate as the mutant allele assay negative control, the smaller calculated ΔC_T cutoff value for the mutant allele assay appears in the Assay Attributes table.

Assay Attributes table values

When wild type allele assays are used in a mutation detection, real time calibration ΔC_T value, or real time detection ΔC_T cutoff value experiment, the following columns will show up in the Assay Attributes table, along with the Mutant Assay, Detection ΔC_T Cutoff, and Cal. Detection ΔC_T Cutoff columns (see , "View data in the Assay Attributes table" on page 32).

Column	Description
Wild type assay	The name of the mutant allele assay
Calibration ΔC_T (—wt)	The predetermined calibration ΔC_T values for paired mutant allele assay and wild type assays
Cal. Calibr ΔC_T (—wt)	The calculated calibration ΔC_T values for paired mutant allele assays and wild type assays, when the required positive control data for the calculation exists in the current study

Column Assay Attributes files

To create and import a custom Assay Attributes file that contains wild type allele assay values, follow the basic guidelines in, "Create and import a custom Assay Attributes file" on page 34, but use the following column headers for the Wild type allele assay:

- Mutant Assay
- Wild Type Assay
- Calibration dCt(-wt)
- Detection dCt Cutoff

In the *.txt file, enter the column headings as they appear in the Mutation Detector Software, except type **dCt** instead of **ΔCt.**

Note: If you are using both wild type allele assays and gene reference assays in a study, include all of the required columns for each assay type in the same *.txt file

Results table calculations

Determine the mutation status

The Mutation Detector™ Software determines the mutation status of a sample by first calculating the ΔC_T value between amplification reactions for a mutant allele assay and a corresponding wild type allele assay as follows:

$$Normalized\ \Delta C_T = [C_{T(mutant\ allele\ assay)} - C_{T(wild\ type\ allele\ assay)}] - Calibration\ \Delta C_T$$

The software then compares the ΔC_T value to a predetermined detection ΔC_T cutoff value to determine if a mutation is detected within the sample. If a sample is mutation positive, then the quantity of mutated DNA present relative to the total quantity of DNA is calculated.

Determine the percent mutation

The Mutation Detector™ Software determines the percent mutation present in a mutation-positive sample based on the normalized ΔC_T value between amplification reactions for a mutant allele assay and a corresponding wild type allele assay using the following calculation:

$$\%mutation = [1/2^{\Delta C_T} \div (1/2^{\Delta C_T} + 1)] \times 100\%$$

D Error messages

File type	Error message	Recommended action
Assay Attributes file (*.txt file), when importing the file	Assay Attributes file format wrong.	Check that the Assay Attributes file contains all required columns, and that the column headings are identical to those in the *TDMA_default_assay_attributes.txt* file.
	[file name] has no Assay Attributes value.	In the Assay Attributes file, check that the Calibration dCt(-wt), Calibration dCt(-gr), and Detection dCt Cutoff columns contain ΔC_T values for each paired mutant allele assay and wild type allele assay or gene reference assay.
Instrument results file (*.csv or *.txt file), when creating a study	[file name] is missing column data and cannot be added to the analysis. Verify that the file contains all required columns.	Verify that the file contains all of the required columns and that each column contains the appropriate data. The required columns include: • **Well Position** – Well identifier • **Sample Name** – User-defined text • **Detector/Target Name** – TaqMan® assay name or (if used) IPC • **C_T** – C_T value
	[file name] is not a compatible file and cannot be added to the analysis. Verify that the file format is compatible with the software.	Verify that the file format is compatible with the Mutation Detector™ Software. The instrument results files must be exported as *.csv or *.txt files. If they are exported as an *.xls file and then saved as a *.csv or *.txt file, they may not be compatible.
	Reporter field must be FAM or VIC	Verify that the Reporter field displays **FAM** or **VIC**.
	There is no sample FAM Ct data in the input file.	Verify that the file contains assay FAM™ dye C_T values in the appropriate reaction wells.
Study file (*.cast.study), when: • Opening a saved study file • Creating a new study file	Warning: A study with this name is already open. You can change the study name in the General tab Study name field or through the File menu Edit Study option.	Avoid having two or more studies with the same name open during an analysis session. Apply unique names to your studies.
	A study with this name is already open. Please choose another study name.	Choose a unique study name for the new study. Alternatively, you can close the open study of the same name before creating a new study with this name. Two studies of the same name should not be open during an analysis session.

File type	Error message	Recommended action
Saved study file (*.cast.study file), when reopening the study	[file name] has incorrect data format. The study file must come from the save function of the software and have the starting line: MutationDetector	The *.cast.study file must: • Be generated by the Save function in the Mutation Detector™ Software • Include the starting line: **MutationDetector**
	Control field must be either empty or contain the value: NTC, POSITIVE, or NEGATIVE	Verify that the Control field is empty (blank) or contains one of the following values: NTC, POSITIVE or NEGATIVE.

E

Installing the Software

System requirements

The Mutation Detector™ Software is compatible with the following operating systems:

- Windows® XP
- Windows Vista®
- Windows® 7

Download and install the software

The Mutation Detector™ Software is available as a free download from the Life Technologies website.

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

1. Go to:
   www.lifetechnologies.com
2. Select **Support** ▸ **Software Downloads**; under the Real-Time PCR category, select Mutation Detector™ Software.
3. In the Mutation Detector™ Software page, complete the registration as directed by the website, click **Download the software now**, then follow the prompts to download the software.
4. On your desktop, close all open applications, double-click Mutation Detector™ Software **Installer**, then follow the prompts to install the software. When the installation is complete, click **Finish**.

Documentation and Support

Documentation

Portable document format (PDF) versions of the documents listed in this section are available from the Life Technologies website. See "Obtaining support" on page 66.

Note: To open the PDF versions, use the Adobe® Acrobat® Reader software available from **www.adobe.com**

Product documentation

Document	Part no.
TaqMan® Mutation Detection Assays Protocol	4467011
TaqMan® Mutation Detection Assays Quick Reference Card	4467012
Mutation Detector™ Software Quick Reference Card	4467104
Mutation Detector™ Software User Guide	4467102

Instrument documentation

Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4387779
Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Comparative C_T/Relative Standard Curve Experiments	4387783
Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4364014
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Relative Standard Curve and Comparative C_T Experiments Getting Started Guide	4376785
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4376784
Applied Biosystems ViiA™ 7 Real-Time PCR System Getting Started Guides	4441434
Applied Biosystems ViiA™ 7 Real-Time PCR System User Guide	4442661

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/sds**

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

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

www.lifetechnologies.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

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit www.appliedbiosystems.com/support

www.lifetechnologies.com