August 2011

PyroMark Q96 Software User Guide

For use with the PyroMark Q96 ID

Sample & Assay Technologies

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Contents

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Use PyroMark Q96 Software only with the PyroMark Q96 ID System.

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Introduction

About this user guide

This user guide provides information about the functions and features of PyroMark Q96 Software. Please refer to the *PyroMark Q96 ID User Manual* for complete information about the proper care, maintenance, and use of the PyroMark Q96 ID Instrument and PyroMark Q96 Vacuum Workstation.

This user guide describes the features of the software and associated tools and enables the user to manage and modify files and analyses.

This user guide provides information about PyroMark Q96 Software in the following sections:

- Introduction
- PyroMark Q96 Software
- Start the software
- Set up an AQ, SNP, or CpG assay
- Set up an SQA assay
- Set up a run
- Process the run on the PyroMark Q96 ID Instrument
- Analyze the run
- View, print, and save analysis reports
- Manage instrument methods
- General hints and tips
- Troubleshooting guide

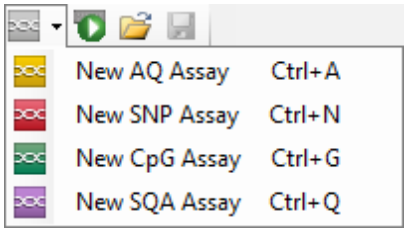
PyroMark Q96 Software

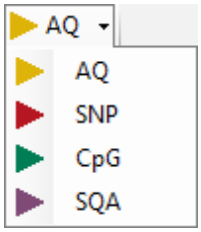
The PyroMark Q96 ID System is a complete solution comprising instrument, vacuum workstation, chemistry, and software.

The main advantages of the system are:

- High-resolution quantification of di-, tri-, or tetra-allelic mutations
- Genotyping and quantification of InDels
- AQ, SNP, and CpG assays use sequence context as built-in quality control
- Analysis of methylation in the presence of SNPs
- Built-in quality control for bisulfite treatment in methylation assays
- Base-calling with quality assessment

Analysis modes





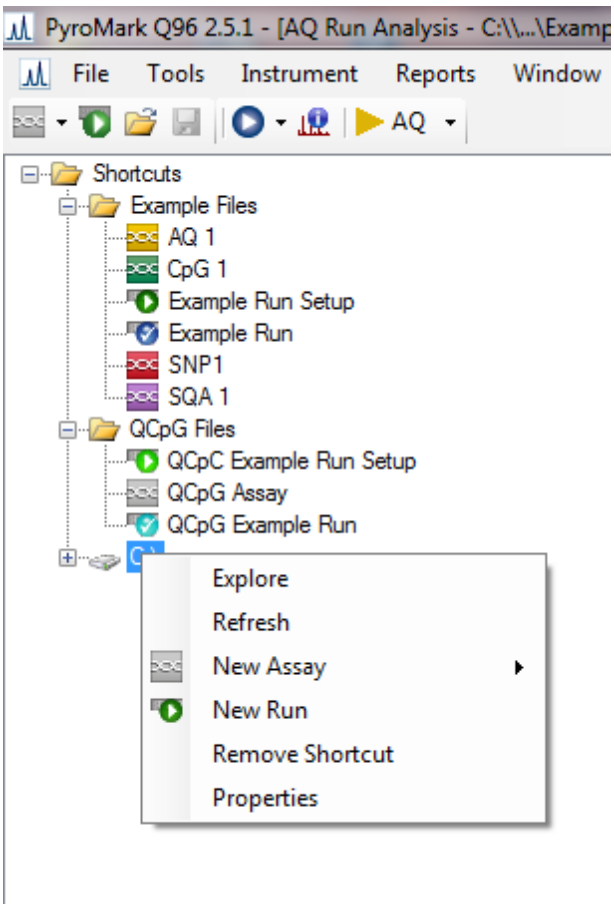
PyroMark Q96 Software has four analysis modes:

- AQ: A variety of quantification studies of SNPs and InDels
- SNP: Genotype analysis of SNPs and InDels. This mode does not provide quantification of alleles as in AQ mode, but enables more flexibility in number of sites to be analyzed.
- CpG: Methylation analysis of multiple consecutive CpG sites
- SQA: Base-calling of unknown sequences

The four different types of analysis can be performed on the same PyroMark Q96 Plate Low. To toggle between the analysis modes in the "Analysis" view, select “AQ”, “SNP”, “CpG”, or “SQA” in the toolbar.

Shortcut browser

The shortcut browser provides a quick and easy way to access folder contents and commonly used assay and run files.



The following icons are used to display information about the files:

- AQ assay file
- SNP assay file
- CpG assay file
- SQA assay file
- A run file that has not been processed
- A run file that has been processed
- Q-CpG assay file
- Q-CpG run file that has not been processed
- Q-CpG run file that has been processed
- Broken shortcut. This may be due to a network server that is temporarily inaccessible or that the file or the folder has been moved, renamed, or deleted outside the software.

Adding and removing shortcuts, updating the contents of a folder, and viewing file and folder properties:

- Add a shortcut to a folder or drive by clicking “Add Folder Shortcut” or right-click the “Shortcuts” folder and select “Add Folder Shortcut” from the context menu.
- Add a shortcut to a file by clicking “Add File Shortcut” or right-click the “Shortcuts” folder and select “Add File Shortcut” from the context menu.

- Remove a shortcut by right-clicking the shortcut and selecting "Remove Shortcut" from the context menu. (The files and subfolders in a shortcut folder cannot be removed separately.)
- Update the contents of a folder by right-clicking it and selecting "Refresh" from the context menu.
- View file or folder properties (e.g., run parameters) by right-clicking the file or folder and selecting "Properties" from the context menu.

  Note: If the mouse pointer is positioned over a file or a folder in the shortcut browser, a tooltip displays the file or folder pathway, the file name, the assay note for assay files, and the plate ID and run note for run files (if entered).

Creating, opening and copying files, and viewing the run log for a processed run:

- Create a new assay file by right-clicking the desired folder and selecting "New Assay" and the desired assay type from the context menu. Enter the filename and press "Enter". To set up the assay, see Set Up an AQ, SNP, or CpG Assay or Set Up an SQA Assay.
- Create a new run file by right-clicking the desired folder and selecting "New Run" from the context menu. Enter the filename and press "Enter". To set up the run, see Set Up a Run.

  Copy a processed run file and rerun it by right-clicking the run file and selecting "Copy and Rerun" from the context menu.

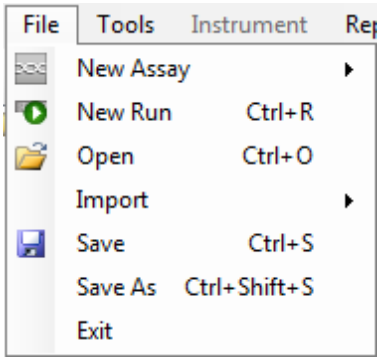
  Note: Only the run setup, not the run and analysis data, will be copied.
- Copy a file by right-clicking the folder containing the file and selecting "Explore" from the context menu. Windows® Explorer opens. For more information, press the "F1" key to open the online help for Windows Explorer.

  Note: To avoid losing data, do not copy a file that is open in PyroMark Q96 Software.
- Open a file by double-clicking it or right-click the file and select "Open" from the context menu. To open a processed run file, select "Open with" followed by the analysis mode ("AQ", "SNP", "CpG", or "SQA").
- View the run parameters and a run log for a processed run file by right-clicking the file and selecting "Run Information" from the context menu.

  Note: Run parameters and run log for Q-CpG assays cannot be viewed in PyroMark Q96 Software. The run file must first be converted to a new CpG run file.

Main menu and toolbars

File menu and toolbars

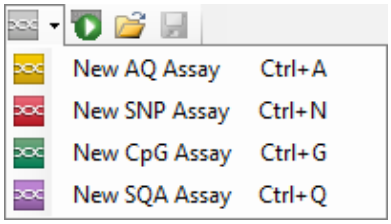


Select "New Assay" or click in the toolbar and select the desired assay type to create a new assay file. To set up the assay, see Set Up an AQ, SNP, or CpG Assay or Set Up an SQA Assay.

Select "New Run" or click in the toolbar to create a new run file. To set up the run, see Set Up a Run.

Select "Open" or click in the toolbar to open a saved assay or run file.

Select "Create New Run from Sample Layout File" from the "Import" submenu to create a new run using a plate layout for sample IDs and notes (optional) defined in a tab- or comma-delimited text file (*.tsv, *.txt, or *.csv); see Using the import/insert sample layout file feature.



Select "Create New AQ/SNP/CpG Assay from Assay Design File" from the "Import" submenu to create a new AQ, SNP, or CpG assay based on an assay file (*.xml) created with PyroMark Assay Design Software. The software will import the sequence to analyze and the names of the variable positions.

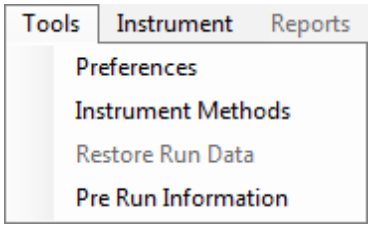
Select "Save" or click in the toolbar to save the changes in the current file. If the file has never been saved, select the location and enter the filename in the dialog box that opens.

Select "Save As" to save a copy of the current file. Select location and enter the filename in the dialog box that opens.

Select "Exit" to shut down the software.

Tools menu for unprocessed run files

Select "Preferences" to set up a connection to the PyroMark Q96 ID instrument. Serial number and URL for the instrument can be entered manually or detected automatically by clicking "Find Instrument", if the instrument is connected to the computer. The connection and information entered in the "Preferences" window can be tested by clicking "Test Connection".

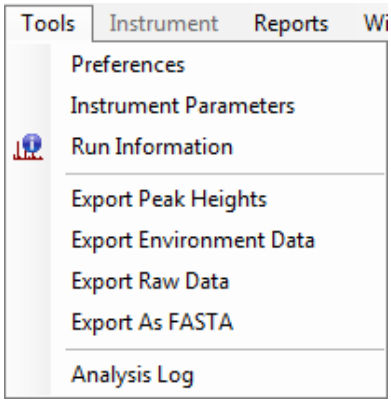


Select “Instrument Parameters” to view the settings for the instrument parameters and, if necessary, import or set up new instrument parameter files according to settings supplied by QIAGEN (see Manage Instrument Parameters).

Select “Pre Run Information” to view the plate setup and a list of required volumes of enzyme mix, substrate mix, and nucleotides for the current run file. To print the report, click  .

Note: To print the “Pre Run Information” report in color, turn on the “Print background colors and images” option in the Internet Explorer (“Tools/Internet Options/Advanced/ Printing”).

Tools menu for processed run files



Select “Run Information” to view the run parameters and a run log for the current run file. To print the report, click  .

Select “Export Peak Heights” to save the peak heights of all used wells as a text file.

Select “Export Environment Data” to save the mixer speed, block temperature, and pressure readings as a text file. The temperatures of the environment, the process chamber lid, and the cooler are also listed.

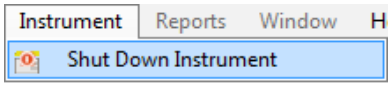
Select “Export Raw Data” to save the intensities and dispensation data as a text file.

Select “Export As FASTA” to save base-called sequences in FASTA format (SQA assays only). In the dialog that opens, select the wells to be included (all or selected), the sorting order of the wells (row or column), and the bases in the sequences to be included (all, passed, passed + check, or only quality control window).

Select “Analysis Log” to view or save the log with all analyses performed on the selected well as an HTML file. Each analysis is logged with the used analysis settings, analysis mode (AQ, SNP, CpG, or SQA), analysis version, results (including warnings), date and time, and the Windows user account used to perform the analysis (see General Hints and Tips).

Text files (*.tsv or *.csv) can be imported into Microsoft® Excel® or other applications that can handle data that is separated by semicolons (;) or tabs. This is useful when doing further calculations on the data.

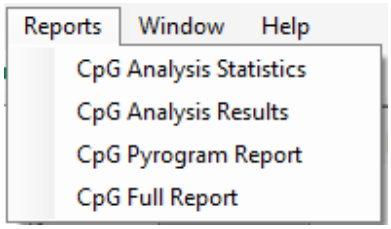
Instrument menu



The instrument menu is enabled only when a configured instrument is connected to the computer.

Select “Shut Down Instrument” to turn off the instrument.

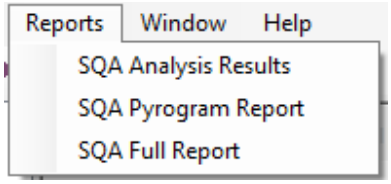
Reports menu for CpG runs



The “Analysis Statistics” report includes analysis statistics for all or selected wells.

The “Analysis Results” report includes well information and analysis results for all or selected wells.

Reports menu for SQA runs



The “Pyrogram Report” includes well information and a Pyrogram® for all or selected wells.

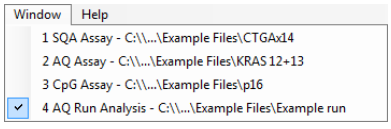
The “Full Report” includes run parameters, run log, well information, and analysis results (including Pyrogram) for all or selected wells.

Note: The "Reports" menu for AQ runs contains the same items as the "Reports menu" for CpG runs, while the reports menu for SNP runs matches that for SQA runs.

The report options are only available for processed runs. For more information on the reports, see View, Print, and Save Analysis Reports.

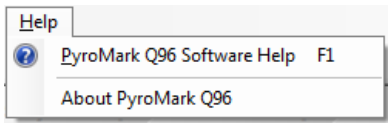
Note: To view reports in PDF format, a PDF reader must be installed on the computer. Adobe® Reader® can be downloaded at www.adobe.com.

Window menu



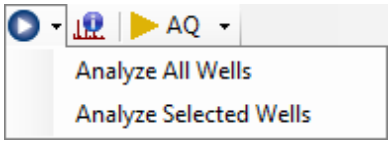
Toggle between open files in the software using the “Window” menu.

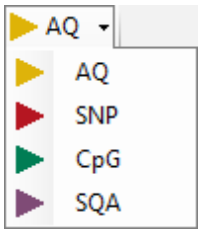
Help menu



Select “PyroMark Q96 Software Help” or press the “F1” key to open this user guide.

Analysis toolbar





Click and select “Analyze All Wells” or “Analyze Selected Wells” (see Select wells) for the current run file.

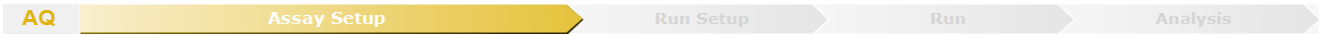
Click to view the run parameters and a run log for the current run file. To print the report, click .

Select “AQ”, “SNP”, CpG”, or “SQA” in the toolbar to toggle between the analysis modes.

Workflow views

PyroMark Q96 Software is organized into views that reflect the Pyrosequencing workflow: Assay Setup, Run Setup, Run, and Analysis. The active view is indicated in the status bar at the top of the window.

Assay Setup view



This view becomes active when creating a new assay. The color of the workflow arrow “Assay Setup” in the status bar reflects the type of assay selected. In this view, the user specifies the assay name, the sequence to analyze, and can optionally enter an assay note. A nucleotide dispensation order is generated by the software. The “Variable Positions” tab displayed upon generating the dispensation order lists the variable positions in the sequence entered and allows the user to name the positions, and indicate which should be analyzed. By default, all variable positions supported in the used analysis mode are selected for analysis. The “Analysis Parameters” tab is used to specify parameters for the data analysis (see Edit analysis parameters in the "Analysis Parameters" tab). The “Revert to Default” button resets default assay parameters, and the “Lock Assay” button locks entered assay parameters so they cannot be altered during analysis (see Analyze a Run). All changes made to an assay file are recorded in a Change Log, which can be accessed by clicking the “Show Change Log” button.

Run Setup view

A new run file is created in the "Run Setup" view. This view serves to enter run-specific information such as instrument parameters, Plate ID, Reagent ID, and a note about the run. Using the “Plate Setup”, assays are added to individual or a group of plate wells.

Run view

The PyroMark Q96 ID is controlled from the PyroMark Q96 Software on the operator’s computer. The "Run" view becomes active upon starting the processing of a run on the instrument, and allows the user to monitor the run. A diagram of the plate setup highlights the wells being processed and the color of the well indicates the most recently dispensed nucleotide or reagent. Real-time information regarding mixer frequency, temperature, and pressure in the instrument processing chamber is displayed, as well as any warning messages triggered by the instrument during the run. The progress of the run is displayed as a progress bar. Well-specific information and the real-time Pyrogram are displayed when a well is selected in the plate diagram.

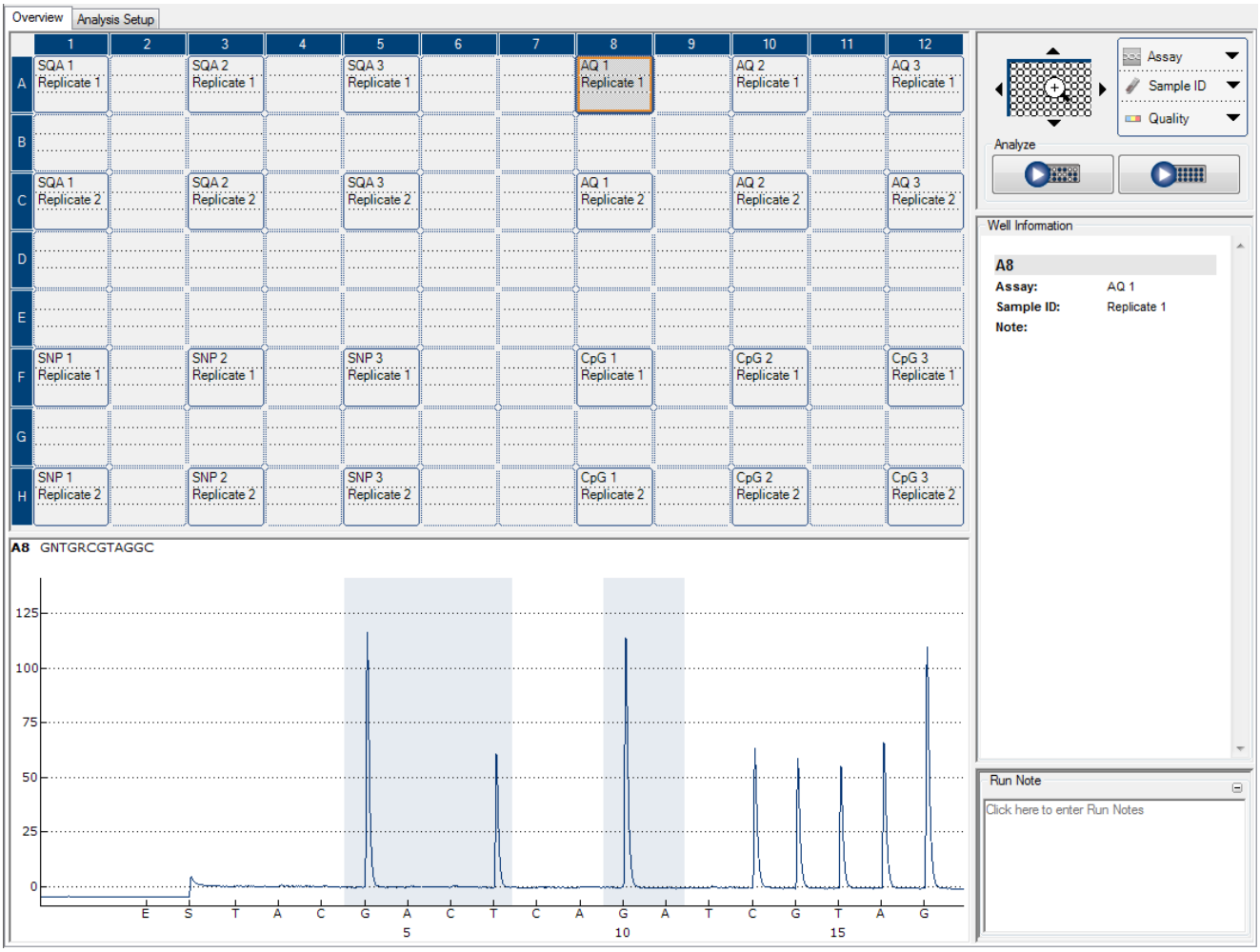
Analysis view

When a run is completed on the PyroMark Q96 ID, the "Analysis" view opens. Alternatively, this view becomes active when any processed run file is opened. The color of the workflow arrow “Analysis” in the status bar reflects the selected analysis mode. This view is used to manage the analysis of individual or groups of plate wells, including making changes to analysis parameters (for unlocked assays). Information for analysis is displayed in two tabs, the "Overview" tab (see Overview tab) and the "Analysis Setup" tab (see Analysis Setup tab).

Note: A dialog box will request confirmation of any modifications made in the “Overview” or “Analysis Setup” tabs prior to switching from one tab to another, or upon selecting (orange outline) another well.

Overview tab

The "Overview" tab in the "Analysis" view displays the plate overview with well-specific information. Directly below, in the "Pyrogram" pane, is the Pyrogram of the well selected in the plate overview. To view the corresponding histogram, zoom in to a plate quadrant. To the right is the "Well Information" pane and the "Run note", which display information specific to the well selected in the plate overview.

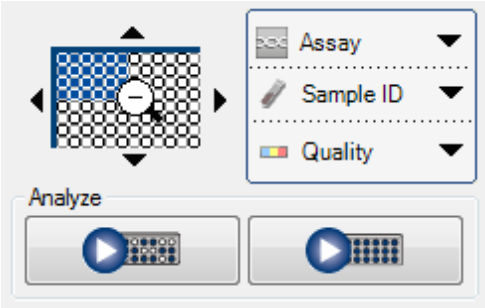


Above the "Well Information" pane is the “zoom tool”, which allows the user to zoom into quadrants of the 96-well plate. Click to zoom in to the plate. This action focuses the display on the 24 wells of the upper left plate quadrant, and makes room for both Pyrogram and histogram of the selected well to appear in the "Pyrogram" pane. The Pyrogram of the selected well always appears in the upper area of the "Pyrogram" pane and the histogram (for AQ, SNP, and CpG assays) or compensated Pyrogram (for SQA

assays) appears in the lower area. It is also possible to replace the histogram in the lower area with one or more Pyrograms of selected wells (see Simultaneously view Pyrograms of different wells).

Use the arrows around the zoom tool or click the desired quadrant of the plate to change which 24 wells are displayed. To zoom out, click .

Next to the zoom tool are dropdown menus to specify the type of information to be displayed for each well, and easy access buttons to initiate analysis of all wells or of selected wells.

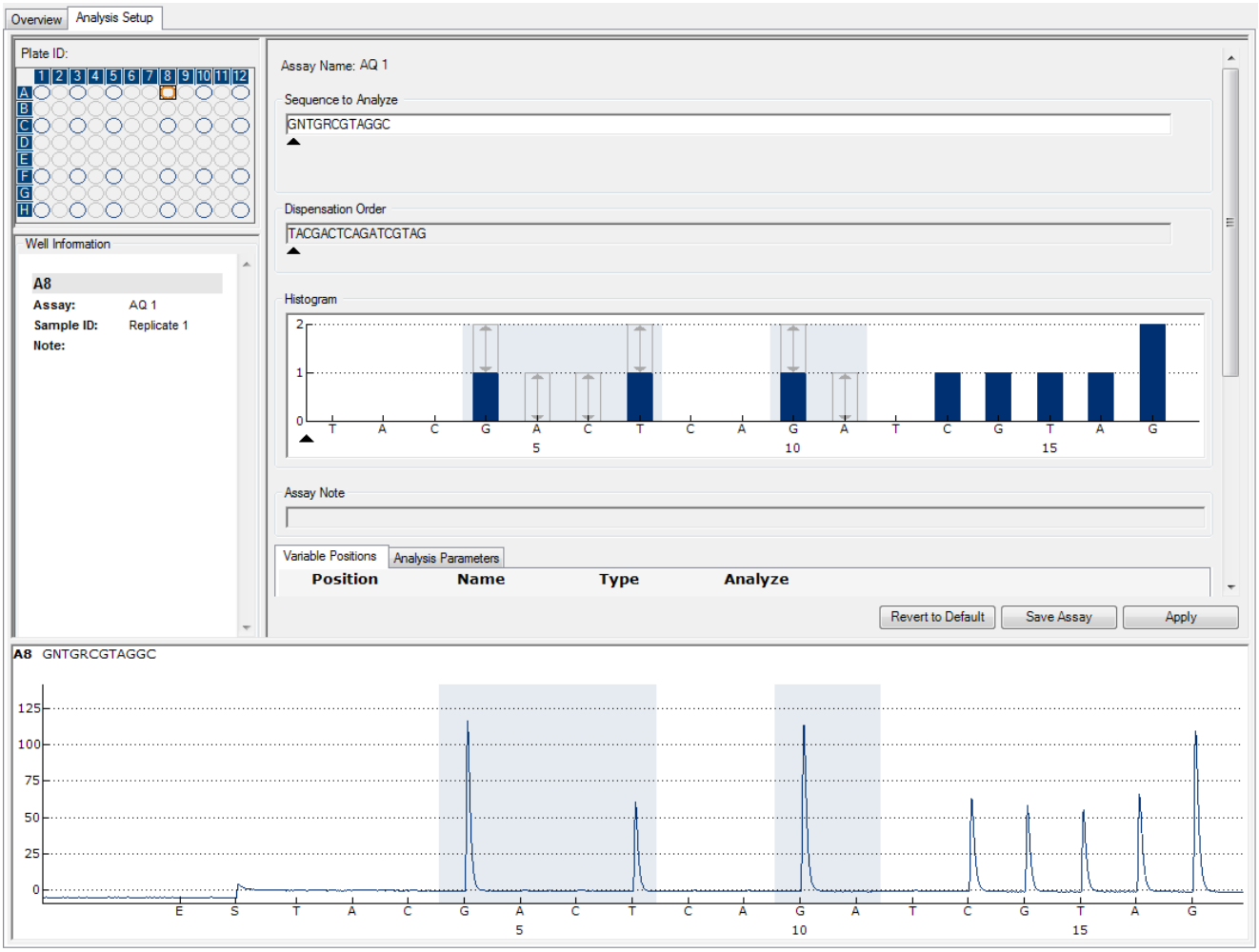


Right-clicking on the plate overview in this tab opens a context menu with the following options:

- Analyze Selected: initiates analysis of all selected wells
- Copy as Image: an image of the full plate layout is copied to the clipboard
- Print: an image of the full plate layout is formatted for printing
- Run Information: the "Run Information" window is displayed (see Main menu and toolbars)
- Open in Lower Area: replaces the histogram with one or more Pyrograms of selected wells. This function is enabled only when zoomed into a quadrant of the plate (see Simultanously view Pyrograms of different wells).

Analysis Setup tab

The "Analysis Setup" tab displays information specific to the analysis performed on a specific well or selection of wells.



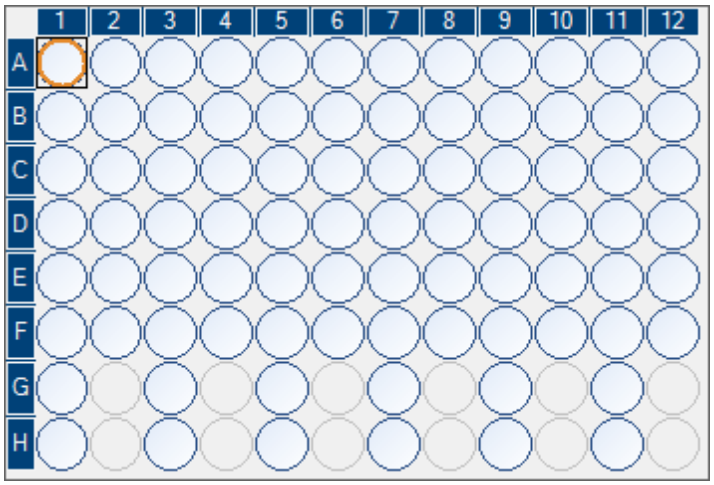
In the upper left pane is a schematic of the plate overview that displays the plate ID and permits the user to select wells (see Select wells). The "Well Information" pane summarizes all information associated with the well selected in the plate overview. The Pyrogram of the selected well is displayed at the bottom of the tab.

Details about the assay of a selected well are visible in the large main pane, including assay name, sequence to analyze, nucleotide dispensation order, histogram, and notes entered during assay setup. In addition, two tabs display the analyzed variable positions and the analysis parameters used.

Select wells

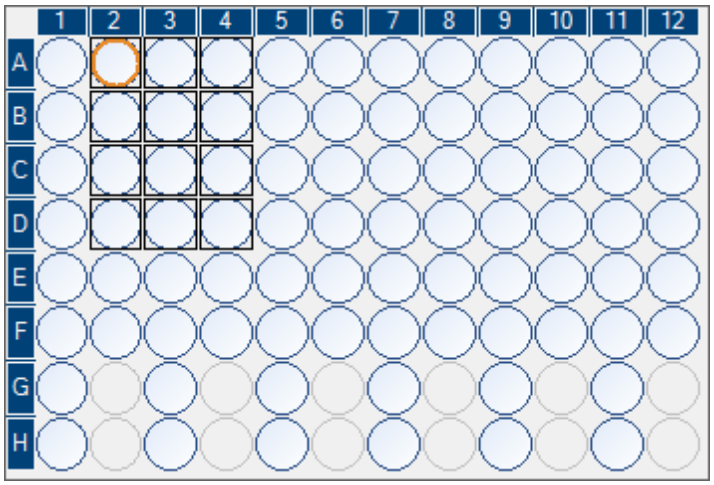
The following methods for selecting wells can be used in the plate overview of both the "Overview" and the "Analysis Setup" tabs. The color schematic for wells also applies to both tabs.

To select a single well, simply click on it.

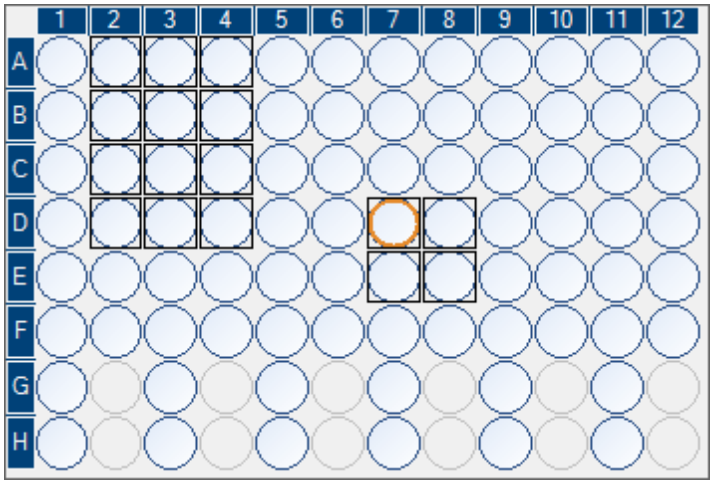


To select a rectangular group of wells, for example A2-A4, B2-B4, C2-C4 and D2-D4:

- Press and hold down the left mouse button while dragging the mouse pointer from well A2 to D4, or
- Select well A2 and press and hold down the “Shift” key while selecting well D4, or
- Select well A2 and press and hold down the “Shift” key while pressing the “Right Arrow” key two times and the “Down Arrow” key three times.



To add wells to the selection above, for example wells D7-D8 and E7-E8, press and hold down the “Ctrl” key while selecting the wells.



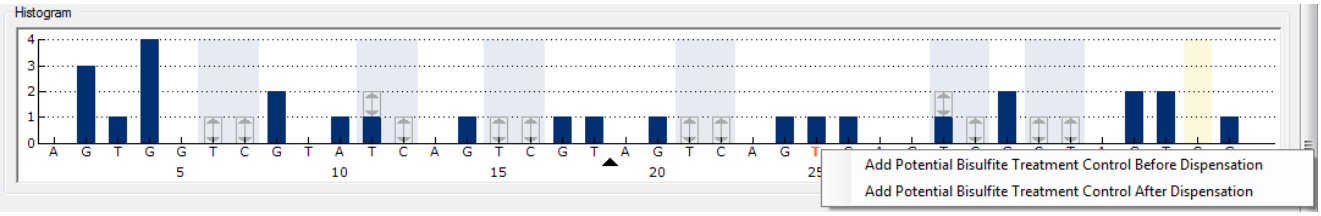
To deselect a well, press and hold down the “Ctrl” key while selecting the well.

Note: If several wells are selected in the plate, information for the well with the orange selection frame (in the "Analysis" view) is shown in the “Well Information” area, etc.

Colors of wells in plate overview diagrams

- Selected well: a dark blue outline (in the "Overview" tab) or a black outline (in the "Assay Setup" tab)
- Most recently selected well: an orange outline
- Active, analyzed wells: a light blue outline and pale blue background
- Active, unanalyzed wells: a light blue outline and gray background
- Inactive wells: a gray outline and background. Inactive wells cannot be selected.
- Well with an error: a red cross

Histogram



Histogram showing a theoretical CpG assay result.

When setting up an AQ, SNP, or CpG assay, the theoretical representation of the expected Pyrosequencing® peak pattern is presented in the "Histogram" area. The following icons and colors are used in the histogram:

- Variable regions (which contain one ore more variable positions) are highlighted with a blue-gray background color
- When showing reference peaks, blue diamonds are displayed above the reference peaks
- Bisulfite treatment controls are highlighted with a yellow background color. When showing reference peaks, orange diamonds are displayed above the bisulfite treatment controls (CpG assays only).

Zoom histogram

It is possible to zoom in on the histogram by selecting a stretch of it with the left mouse button.

Zoom out either by right-clicking the histogram area and selecting "Zoom Out" from the context menu (the zoom is set to the previous level) or by double-clicking the histogram area (the zoom is set to 100%).

It is possible to adjust the heights of histogram bars.

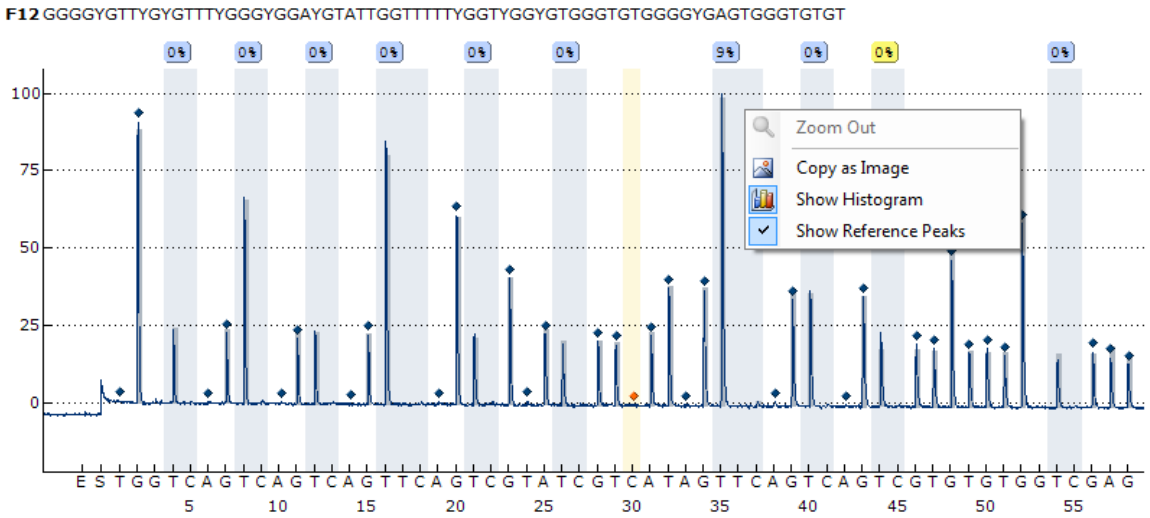
Export the histogram as an image

The histogram can be copied as an image to the clipboard by right-clicking the histogram and selecting "Copy as Image" from the context menu. The image can be pasted into applications that support Enhanced Metafile (EMF) images.

Pyrogram

The Pyrogram is the graph resulting from a sequencing reaction performed using Pyrosequencing technology. Incorporated nucleotides are shown as peaks in the Pyrogram.

AQ, SNP, and CpG assays



The following information, icons, and colors are displayed and used in the "Pyrogram" pane for an AQ, SNP, or CpG assay:

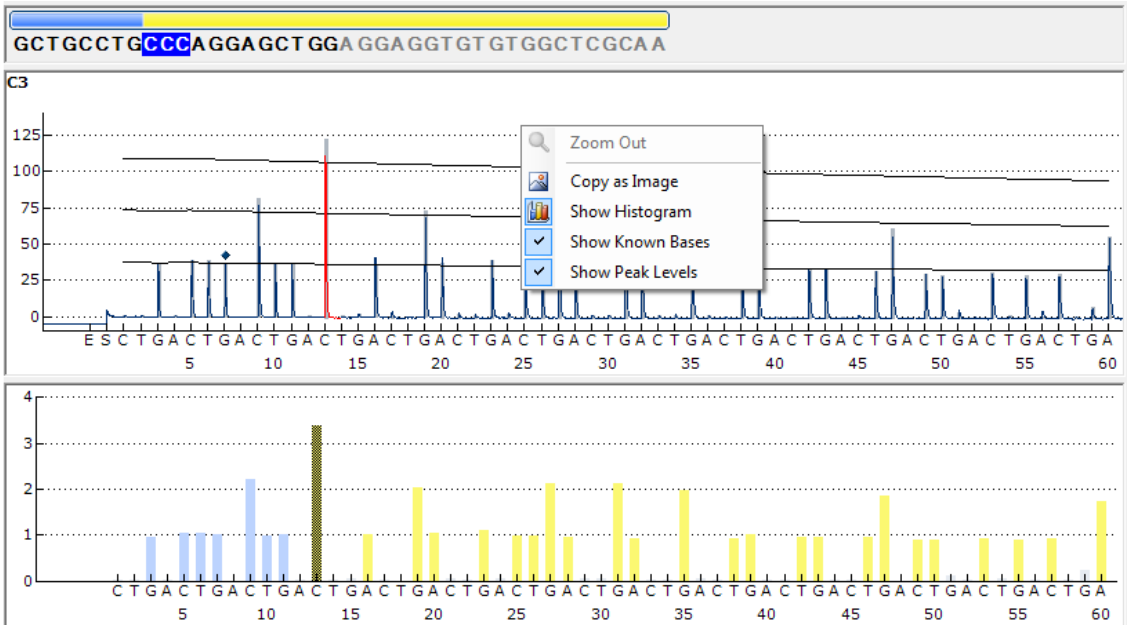
- The well name and the sequence to analyze are shown in the upper left corner
- The analysis result (allele frequencies in AQ mode A: 4% T: 96%, genotype in SNP mode T/T, or methylation percentage in CpG Mode 23%) is displayed above each variable position. The background color shows the quality assessment of the analysis result; see Quality colors. If a quality assessment has been edited by the user, this is displayed by a border around the analysis result, for example 44%. If the mouse pointer is positioned over the analysis result, a tooltip displays the position number, the position name in brackets, and any analysis warnings.

  Note: -- (in white) indicates that a site was deselected by the user. n.a. (in white) indicates that the software does not support the analysis, e.g., analysis of SNP in the CpG mode. n.a. (in red) indicates that the analysis was not possible due to lack of data.
- Variable regions are highlighted with a blue-gray background color
- When showing reference peaks, blue diamonds are displayed above the reference peaks
- Bisulfite treatment controls are highlighted with a light yellow background color. When showing reference peaks, orange diamonds are displayed above the bisulfite treatment controls (CpG assays only).

- To view the height of a peak, position the mouse pointer over the top of the peak. A tooltip displays the height.
- When showing the histogram, the histogram is displayed in gray over the peaks. It is best viewed when zoomed in.

  Note: By right-clicking the "Pyrogram" pane, it is possible to toggle between viewing and hiding the histogram and reference peaks.

SQA assays



When a base is selected in the base-called sequence, the corresponding peak is highlighted in both the upper and lower areas within the "Pyrogram" pane, and vice versa.

The following information and colors are displayed and used in the "Pyrogram" pane for an SQA assay:

- The well name is shown in the upper left corner
- To view the height of a peak, position the mouse pointer over the top of the peak. A tooltip displays the height
- When showing the histogram, a compensated Pyrogram is displayed in gray over the peaks in the Pyrogram. It is best viewed when zoomed in.
- When showing known bases, peaks with known bases are marked with blue diamonds in Pyrogram
- When showing peak levels, calculated peak levels are displayed in the Pyrogram

- Colors used in the Graph area correspond to quality assessments (see Quality colors)
- By positioning the mouse pointer on a base in the base-called sequence, a tooltip displays the position number

Note: By right-clicking the "Pyrogram" pane, it is possible to toggle between viewing and hiding the histogram, known bases, and peak levels.

Zoom Pyrogram

It is possible to zoom in on the Pyrogram by selecting a stretch with the left mouse button. Zoom out either by right-clicking the Pyrogram area and selecting “Zoom Out” from the context menu (the zoom is set to the previous level), or by double-clicking the Pyrogram area (the zoom is set to 100%).

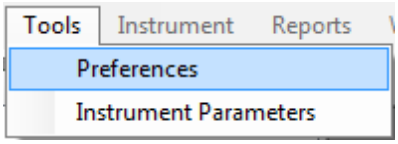
Export Pyrogram as an image

The Pyrogram can be copied as an image to the clipboard by right-clicking the Pyrogram area and selecting “Copy as Image” from the context menu. The image can be pasted into applications that support Enhanced Metafile (EMF) images.

Start the Software

In the Windows "Start" menu, select "(All) Programs/PyroMark/PyroMark Q96". The *PyroMark Q96 Software User Guide* (this publication) can be accessed at any time by pressing the "F1" key when in the software.

Connect the Instrument

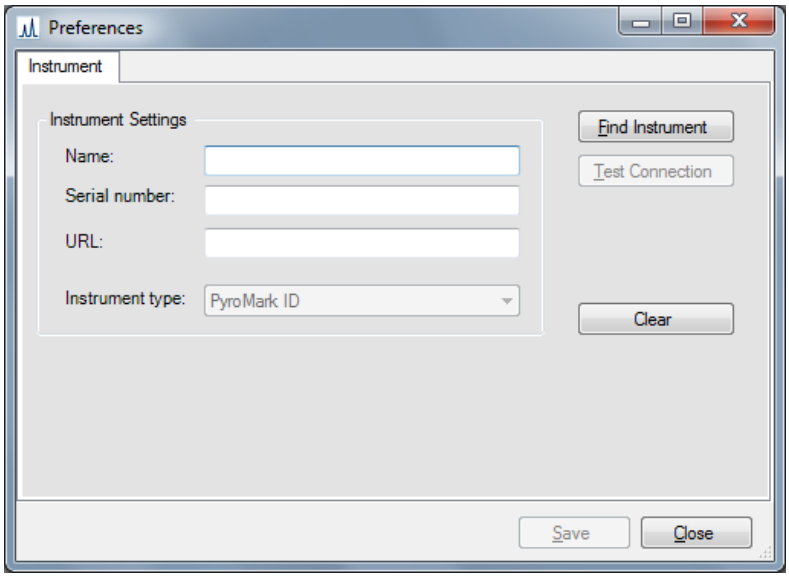


The "Preferences" item under the "Tools" menu is used to establish or test the connection to a PyroMark Q96 ID Instrument. Before establishing the connection to an instrument, ensure that the cable is connected to both instrument and operator's computer, and that the instrument is turned on.

1. **Select "Preferences" from the "Tools" menu. The Preferences dialog box will open.**
2. **Click "Find Instrument" to have the software detect the connected PyroMark Q96 ID Instrument. Upon detecting the instrument, the software will enter the instrument serial number and URL.**

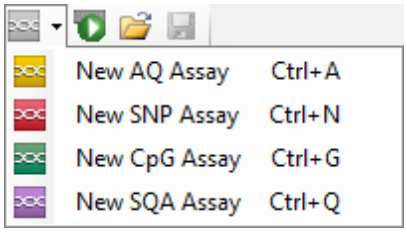
   If the software fails to detect the instrument, make sure the cable between instrument and operator's computer is properly connected.

   Alternatively, manually enter the serial number shown on the back of the instrument and the URL of the connection.
3. **To test the connection, click "Test connection".**
4. **Assign a name to the instrument and click "Save".**



Set Up an AQ, SNP, or CpG Assay

Workflow to set up an AQ, SNP, or CpG Assay



1. **Click in the toolbar and select "New AQ Assay", "New SNP Assay", or "New CpG Assay". A new assay file is created.**

   Alternatively, you can create a new assay file in the shortcut browser by right-clicking the folder you wish to place it in and selecting "New Assay" followed by "New AQ Assay", "New SNP Assay", or "New CpG Assay" from the context menu. Enter the filename and press "Enter". You cannot use the same name as another file saved in the same folder. To add a shortcut to a folder or drive, click "Add Folder Shortcut".

2. **Enter the sequence to analyze.**
3. **Click the "Generate Dispensation Order" button (see Generate the dispensation order).**
4. **Optional: If creating a CpG assay, enter the "Sequence Before Bisulfite Treatment". This information is useful when adding bisulfite treatment controls.**
5. **Recommended: If creating a CpG assay, add bisulfite treatment controls, preferably at the beginning of the sequence (see Add or remove bisulfite treatment controls).**
6. **Optional: Enter information about the assay in the "Assay Note" text box.**

   Note: An assay note can be displayed in a tooltip in the shortcut browser by positioning the mouse pointer over the assay file.

7. **Optional: Set up the variable positions.**
8. **Before running your samples, validate your assay using a reference DNA sample (see Appendix B of the *PyroMark Q96 ID User Manual*).**
9. **Optional: If applicable, during the assay validation, edit the analysis parameters.**

10. **Optional: Lock the assay for editing by clicking the "Lock Assay" button at the bottom of the assay setup window. A locked assay ( ) that has been run on the PyroMark Q96 ID Instrument cannot be unlocked (i.e., it will not be possible to edit the analysis parameters or results after the assay has been processed).**
11. **Click in the toolbar to save the file. If the file has never been saved, select location and enter the filename in the dialog box that opens.**

Enter the sequence to analyze

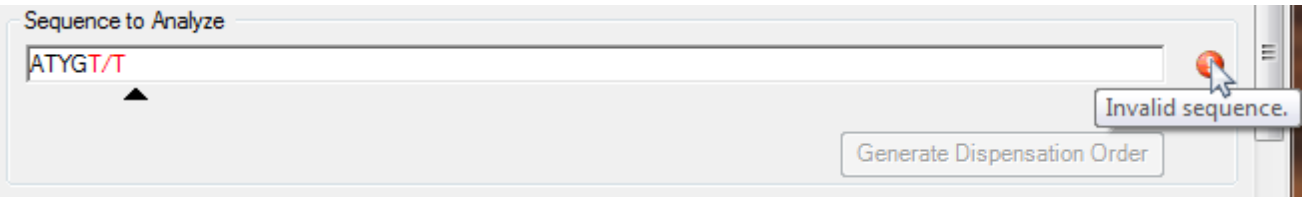
Type or paste the sequence to analyze into the "Sequence to Analyze" text box. If creating a CpG assay, enter the sequence after the bisulfite treatment.

Note: It is possible to add assays to a well in the plate layout without a sequence to analyze.

The following rules apply when entering the DNA sequence in the software:

- The allowed characters for sequence input are A, C, G, and T as well as IUPAC codes
- Variable positions can be entered using either IUPAC codes or a forward slash (/) as a separator between the two potential bases (e.g., C/T)
- InDels should be entered using square bracket notation "[ ]" (e.g., [AT])
- The sequence should not include more than 400 characters or 100 variable positions
- Variable positions involving a combination of SNPs and InDels should be entered using a combination of "/" or IUPAC codes and "[ ]". For example, [T/A] or [W] represents a tri-allelic polymorphism where the possible alleles are a T, an A, or neither (deletion)
- It is not possible to have a combination of a single nucleotide polymorphism and constant bases within an InDel (e.g., [A/TC]
- Nested InDels are not supported (e.g., [ATT[C]G])

If the sequence to analyze contains an error, this is displayed by a red exclamation mark at the end of the text box. Position the mouse pointer over the exclamation mark and a tooltip will display an explanation of the error. The character or characters that caused the error will be marked in red in the sequence to analyze.



As T/T is not a valid variable position, it causes an "Invalid sequence" error.

In SNP assays, a warning message will appear if the sequence to analyze contains multiple variable sites within the same variable region that will generate the same sequence pattern. This will make it difficult to discriminate genotypes.

Note: If analyzing "nonstandard" methylation patterns, for example methylations of Cs that are not followed by Gs, these patterns can be analyzed in the AQ mode. To analyze in the CpG mode, enter extra Gs in the "Sequence to Analyze" text box and set the expected heights of the extra Gs to zero (0); see Adjust heights of histogram bars.

IUPAC codes

Code	Description	Code	Description
A	Adenine	W	T or A
C	Cytosine	S	C or G
G	Guanine	B	C, T, or G (not A)
T	Thymine	D	A, T, or G (not C)
R	Purine (A or G)	H	A, T, or C (not G)
Y	Pyrimidine (C or T)	V	A, C, or G (not T)
M	C or A	N	Any base (A, C, G, or T)
K	T or G		

Note: S, B, V, and N are not valid after bisulfite treatment.

Valid patterns in a CpG assay

Patterns that cannot exist after bisulfite treatment are not valid in a CpG assay. For example, GC/TGAC/G is not valid since C/TG is a forward CpG site and C/G cannot exist after bisulfite treatment.

The following CpG site and SNPs can be included in a forward assay:

- CpG site: C/TG
- SNPs: A/T, A/G, G/T, and A/T/G (i.e., C cannot be included)

The following CpG site and SNPs can be included in a reverse assay:

- CpG site: CG/A
- SNPs: A/T, A/C, C/T, and A/T/C (i.e., G cannot be included)

Note: The software does not support analysis of CpG sites that include an additional variable position, for example A/C/TG. These kinds of SNPs can be analyzed by typing C/TG in the "Sequence to Analyze" text box and ATCG in the "Dispensation Order" text box. Proceed with the run as usual. After analysis of the CpG sites, switch to the AQ mode and change C/TG to A/C/TG (in the "Sequence to Analyze" text box) and analyze the variable position. In the same way, C/TG/A can be analyzed by typing C/TG in the "Sequence to Analyze" text box and TCGA in the "Dispensation Order" text box. After analysis of the CpG sites, switch to the AQ mode and change C/TG to C/TG/A (in the "Sequence to Analyze" text box) and analyze the variable position.

Generate the dispensation order

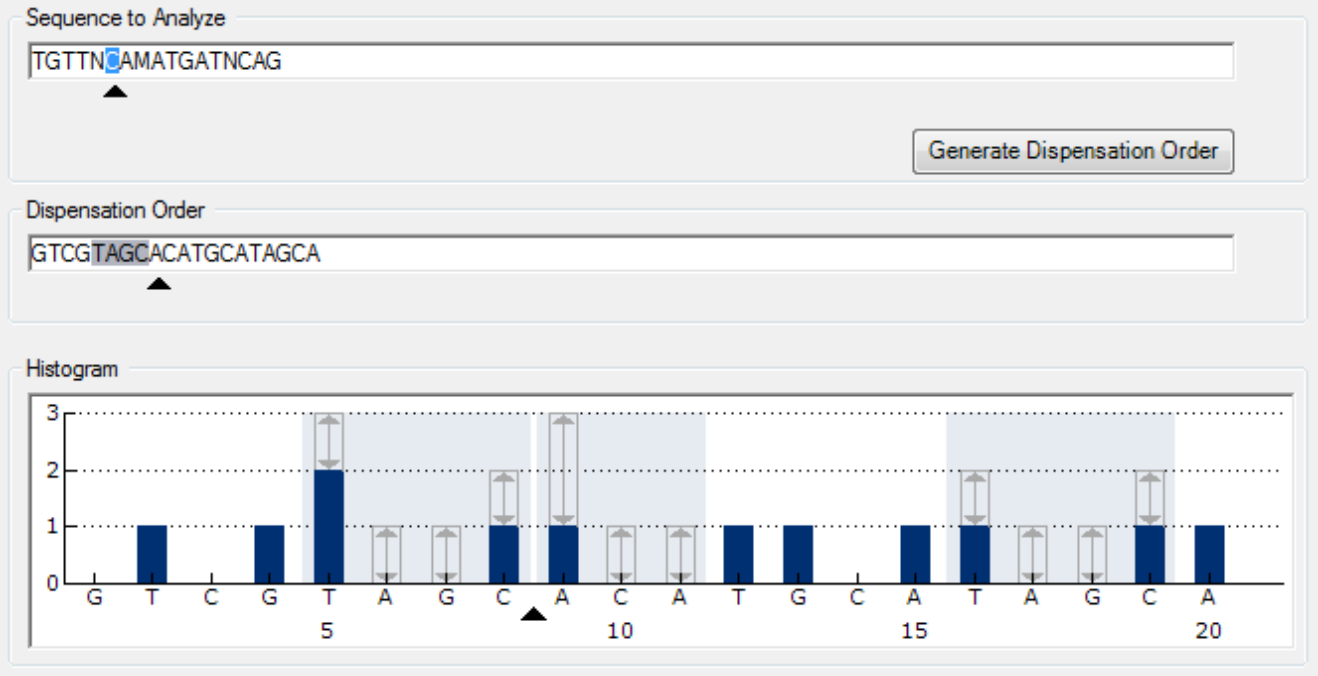
A dispensation order for the entered sequence to analyze is generated by the software by clicking the "Generate Dispensation Order" button. The generated dispensation order includes blank dispensations to ensure that the correct sequence has been obtained.

When creating CpG assays, the dispensation order should also include bisulfite treatment controls. These controls have to be added manually, by the user, after the dispensation order has been generated (see Add or remove bisulfite treatment controls (CpG assays)).

If desired, the dispensation order can be entered or adjusted manually.

Note: When clicking "Generate Dispensation Order", any existing dispensation order will be overwritten.

Note: When a base position is selected in the sequence to analyze, the corresponding dispensation is highlighted with a gray background color, and vice versa.



The arrow in the sequence to analyze, the dispensation order, and the histogram show the position of the cursor.

Note: If the last variable position in the sequence to analyze is a long InDel, dispensation will only be performed until three variable peaks are found and providing the requirement of five reference peaks is fulfilled. To dispense the whole InDel, add a variable position after the InDel or adjust the dispensation order manually.

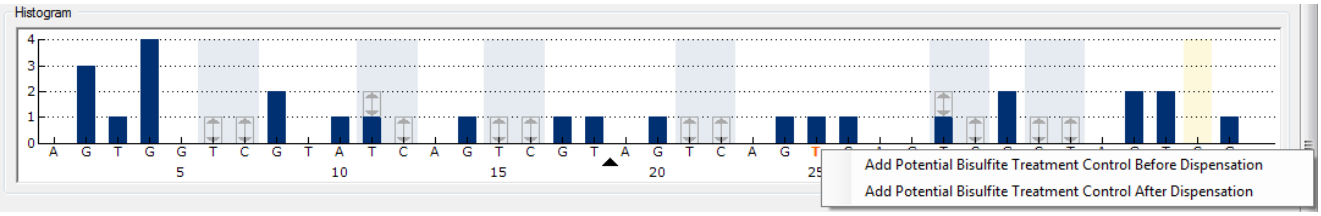
Note: If it is not possible for the sequence to come in phase before 32 alleles are dispensed, the dispensation order will not be completed. For example, the sequence ACTCDDDDG will have the dispensation order ACTC, since the four D polymorphisms will generate an out-of-phase stretch over too many alleles.

Dispensation warnings

If the dispensation order contains a warning, this is displayed by a red exclamation mark at the end of the "Dispensation Order" text box. It is possible to run an assay with a dispensation warning, but the warning must be considered when evaluating the analysis result. If you position the mouse pointer over the exclamation mark, a tooltip will display an explanation of the warning.

Warning	Suggested action
Sequence uncertain due to lack of terminal sequence information.	The problem may be resolved by either entering more sequence information or reducing the number of dispensations.
Sequence not in phase at the end of the dispensations.	The problem may be resolved by adjusting the dispensation order (manually or by clicking "Generate Dispensation Order") or entering more sequence information. **Note**: If the problem is not resolved, the out-of-phase stretch will not be analyzed.
The generated dispensation order contains less reference peaks than required.	If possible, enter more sequence information and increase the number of dispensations. For the best possible quality assessment of the results, five or more reference peaks with the height 1, 2, or 3 are recommended.
Some genotypes will generate the same sequence patterns and will not be distinguishable.	The sequence to analyze contains multiple variable sites within a variable region that will generate sequence patterns that are indistinguishable between genotypes. Review results carefully.

Add or remove bisulfite treatment controls (CpG assays)



CpG assays should contain at least one internal control to assess successful bisulfite treatment, preferably at the beginning of the sequence. C bases that are not followed by G in the sequence are usually not methylated, and should therefore be fully converted to T after bisulfite treatment and PCR. As a result of successful bisulfite treatment, all templates should show only Ts and no Cs in these positions. For reverse assays, all templates should show only As and no Gs in these positions.

The potential positions for bisulfite treatment controls are illustrated with a bold, orange letter in the histogram: **T** in a forward assay and **A** in a reverse assay.

A bisulfite treatment control can be added by left-clicking the bold, orange **T** or **A** and selecting the desired option from the context menu. It can also be added manually by adding a C before or after a T in the dispensation order.

A bisulfite treatment control can be removed by left-clicking the control (C in a forward assay or G in a reverse assay) and selecting "Remove Bisulfite Control" from the context menu.

Note: In the sequence before bisulfite treatment, check whether the suggested bisulfite treatment controls are Cs converted to Ts (read as Gs and As in a reverse assay) and suitable as controls or not.

Set up the variable positions

The variable positions can be set up in the "Variable Positions" tab. The available parameters are listed below.

Note: If the sequence to analyze is changed (and a new dispensation order is generated), the variable position parameters are reset to their default values.

Position	The location of the variable position in the sequence to analyze, counting from left to right.
Name	The name of the variable position. To change the name, either select the text box (the current contents will be selected) or double-click the text box.
Type	The type of variable position: SNP, InDel, or CpG site.
Analyze	If this option is checked, the variable position will be analyzed. **Note**: This option is not available for variable positions that cannot be analyzed for the current assay type.
Methylation ranges (CpG assays only)	The expected CpG methylation. Setting this parameter for all the CpG sites allows easy identification of sites (in the analysis results) that are outside the expected methylation range: ▪ The light green area is below the expected range

- The green area is within the expected range
- The dark green area is above the expected range

Note: The expected methylation cannot be set for CpG sites with the "Analyze" option unchecked.

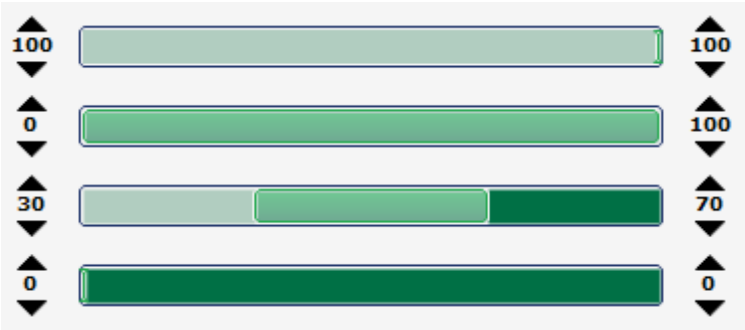
The expected range area can be moved to the left or to the right by holding down the left mouse button while moving the area with the mouse.

The arrows can be used to increase or decrease the expected range. You can also increase or decrease the expected range by:

1. Positioning the mouse pointer over the left or the right end of the green area, so that the pointer changes from a white arrow to ⇿.
2. Moving the mouse to the left or the right while holding down the left mouse button.

To edit all methylation ranges simultaneously, hold down the "Shift" key while changing one of the ranges.

Examples of methylation ranges



1. Expected methylation = 100%.
2. Expected methylation = 0–100%.
3. Expected methylation = 30–70% (default)
4. Expected methylation = 0%

To reset the parameters in the "Variable Positions" tab and the "Analysis Parameters" tab to their default values, click "Revert to Default".

Edit analysis parameters

The default analysis settings have been set to give optimal analysis results for most assays. If applicable, during the assay validation, the results may be improved by editing the analysis parameters:

- Edit analysis parameters in the "Analysis Parameters" tab
- Enable or disable reference peaks
- Enable or disable bisulfite treatment controls (only CpG assays)
- Adjust heights of histogram bars
- Enable or disable variable positions and/or change expected methylation ranges (only CpG assays); see Set up the variable positions

Ensure changes are validated; see Appendix B of the *PyroMark Q96 ID User Manual*.

Note: When using QIAGEN kits, use the settings stated in the kit handbooks.

Note: All saved changes are logged. To view a change log for an assay, open the assay file and click "Show Change Log".

Edit analysis parameters in the "Analysis Parameters" tab

The following analysis parameters can be edited in the "Analysis Parameters" tab.

Unsuccessful bisulfite treatment (CpG assays only)	These parameters state the highest acceptable percentage of unconverted sequence to achieve "Passed" quality assessment and "Check" quality assessment for the CpG sites. The entered values are compared to the single peak height value that the analysis algorithm determines.
Allowed percentage for passed quality	The highest acceptable percentage of unconverted sequence to achieve "Passed" quality assessment for the CpG sites. Above this value, the warning "Uncertain bisulfite conversion at dispensation: *number(s)*" is triggered during the analysis and a "Check" quality assessment is assigned. The default value is 5%. **Note**: The value cannot be higher than the allowed percentage value for check quality (see below).

Allowed percentage for check quality	The highest acceptable percentage of unconverted sequence to achieve "Check" quality assessment for the CpG sites. Above this value, the warning "Failed bisulfite conversion at dispensation: *number(s)*" is triggered during the analysis and a "Failed" quality assessment is assigned. The default value is 7%. Note: The value cannot be lower than the allowed percentage value for passed quality (see above).
Peak height threshold	These parameters define the lower intensity limit for the single peak height level at the beginning of the Pyrogram.
Required peak height for passed quality	The minimum signal value for a peak to achieve "Passed" quality assessment for the variable positions. Below this value, the warning "Uncertain due to low peak height" is triggered during the analysis and a "Check" quality assessment is assigned. The default value is 7. **Note**: The value cannot be lower than the required peak height value for check quality (see below).
Required peak height for check quality	The minimum signal value for a peak to achieve "Check" quality assessment for the variable positions. Below this value, the warning "Failed due to low peak height" is triggered during the analysis and a "Failed" quality assessment is assigned. The default value is 3. **Note**: The value cannot be higher than the required peak height value for passed quality (see above).
Stringency levels	The stringency of the warnings for "Pattern deviation in variable positions" and "Sum deviation in variable positions" can be set to "Low", "Normal" (default), or "High". A high stringency level narrows the allowed deviation.

Pattern deviation in variable positions (only in AQ and CpG modes)	The deviation between the measured peak pattern in the variable position and the theoretical peak pattern. If the deviation is higher than the set stringency level allows, the warning "Uncertain/Failed due to high pattern deviation in variable position" is triggered during the analysis. Whether the warning will yield a "Check" or "Failed" quality assessment for the analysis result depends on the magnitude of the deviation.
Sum deviation in variable positions	The deviation between the measured sum of all the peaks in the variable position and the theoretical sum (based on the single peak height). If the deviation is higher than the stringency level allows, the warning "Uncertain/Failed due to high sum deviation in variable position" is triggered during the analysis. Whether the warning will yield a "Check" or "Failed" quality assessment for the analysis result depends on the magnitude of the deviation.
Genotype deviation in variable positions (only in SNP mode)	The deviation between the best and second best genotype match for a variable position. If the deviation is higher than the stringency level allows, the warning "Uncertain/Failed due to high genotype deviation in variable position" is triggered during the analysis. Whether the warning will yield a "Check" or "Failed" quality assessment for the analysis result depends on the magnitude of the deviation.

Parameters

A-peak reduction factor	The factor by which the A-peak intensities are multiplied to account for the fact that A-peaks are higher than other peaks. The default value is 0.90.

To reset the parameters in the "Variable Positions" tab and the "Analysis Parameters" tab to their default values, click "Revert to Default".

Enable or disable reference peaks and bisulfite treatment controls

Nonvariable peaks, i.e. peaks that are not a part of a variable position (including blank dispensations), are referred to as "reference peaks". Reference peaks are used in the analysis both as references when calculating the single peak height level and as internal controls when assessing the quality. For the best possible quality assessment of the results, it is recommended that the reference peaks that are generated by the software are kept enabled.

By left-clicking a reference peak diamond in the histogram, the peak is either enabled or disabled as a reference peak, depending on the previous status. The diamond displays the status:

- Filled blue diamond: Enabled as a reference peak
- Hollow blue diamond: Disabled as a reference peak

By left-clicking a bisulfite treatment control diamond (CpG assays only), the control is either enabled or disabled as a control and/or a reference peak, depending on the previous status. The diamond displays the status:

- Filled orange diamond: Enabled both as a bisulfite treatment control and a reference peak
- Filled blue diamond: Enabled as a reference peak but disabled as a bisulfite treatment control
- Hollow orange diamond: Disabled both as a bisulfite treatment control and a reference peak

Position the mouse pointer over the diamond and a tooltip will describe the consequence of a click.

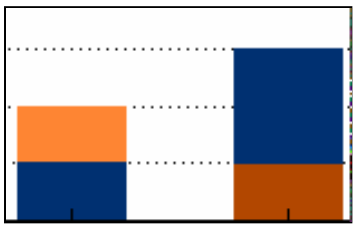
Note: To toggle between viewing and hiding reference peaks in the histogram, right-click the histogram and select "Show Reference Peaks" from the context menu.

Adjust heights of histogram bars

This feature can be used when previous experiences have shown a reproducible deviation in the measured pattern from the theoretical pattern. Use this feature with care.

1. **Press and hold down the "Ctrl" key while left-clicking the top of the histogram bar (left-click when the pointer changes from a white arrow to ☝).**
2. **Either enter the height in the text box that opens, or increase or decrease the height by using the arrows next to the text box.**
3. **To apply the new height, press "Enter".**

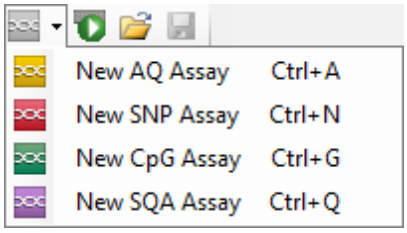
Note: Instead of removing "nonstandard" methylation patterns from the sequence to analyze, for example methylations of Cs that are not followed by Gs, set the expected heights of the Gs to zero (0).



Light orange = decreased height
Dark orange = increased height

Set Up an SQA Assay

Workflow to set up an SQA Assay



1. **Click in the toolbar and select "New SQA Assay". A new assay file is created.**

   Alternatively, you can create a new assay file in the shortcut browser by right-clicking the folder you wish to place it in and selecting "New Assay" and "New SQA Assay" from the context menu. Enter the filename and press "Enter". To add a shortcut to a folder or drive, click "Add Folder Shortcut".

2. **Enter the dispensation order.**
3. **Optional: Enter information about the assay in the "Assay Note" text box.**

   Note: An assay note can be displayed in a tooltip in the shortcut browser by positioning the mouse pointer over the assay file.

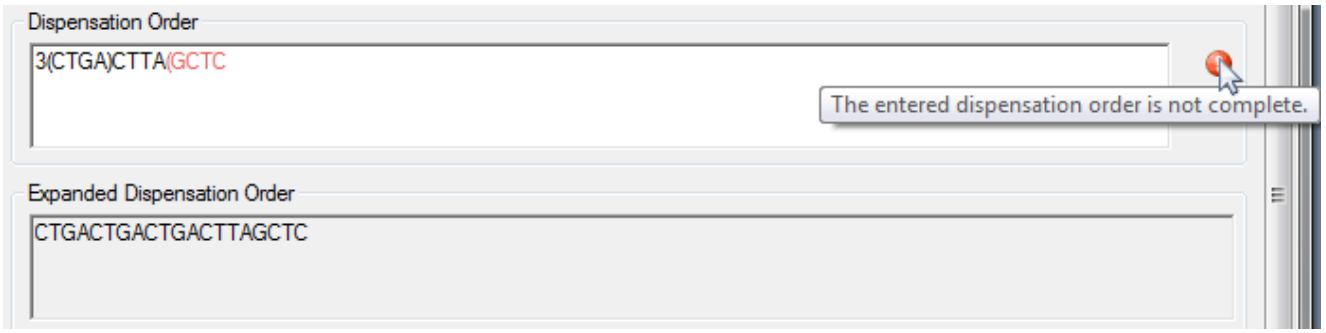
4. **Before running your samples, validate your assay using a reference DNA sample (see Appendix B of the *PyroMark Q96 ID User Manual*).**
5. **Optional: If applicable, during the assay validation, edit the analysis parameters.**
6. **Optional: Lock the assay for editing by clicking the "Lock Assay" button at the bottom of the assay setup window. A locked assay ( ) that has been run on the PyroMark Q96 ID Instrument cannot be unlocked (i.e., it will not be possible to edit the analysis parameters or results after the assay has been processed).**
7. **Click in the toolbar to save the file. If the file has never been saved, select location and enter the filename in the dialog box that opens.**

Enter the dispensation order

Type the dispensation order into the "Dispensation Order" text box. The following rules apply when entering the dispensation order in the software:

- The allowed characters for input are A, C, G, and T
- To repeat a group of bases, use numbers in combination with parenthesis, e.g., "3 (CTGA)" corresponds to "CTGACTGACTGA"

If the dispensation order contains an error, this is displayed by a red exclamation mark at the end of the text box. Position the mouse pointer over the exclamation mark and a tooltip will display an explanation of the error. The character or characters that caused the error will be marked red in the dispensation order.



The error "The entered dispensation order is not complete" is caused by a missing or incorrect positioned parenthesis. In this example, a closing parenthesis is missing.

Edit analysis parameters

The default analysis settings have been set to give optimal analysis results for most assays. If applicable, during the assay validation, the results may be improved by editing the analysis parameters:

- The "Quality Control Window" setting in the "Settings" tab is by default set to 20. If more or less bases are required, change accordingly.
- Edit analysis parameters in the "Analysis Parameters" tab

Ensure changes are validated; see Appendix B of the *PyroMark Q96 ID User Manual*.

Note: When using QIAGEN kits, use the settings stated in the kit handbook.

Note: All saved changes are logged. To view a change log for an assay, open the assay file and click "Show Change Log" at the bottom of the assay setup window.

Edit analysis parameters in the "Analysis Parameters" tab

The following analysis parameters can be edited in the "Analysis Parameters" tab.

Peak height threshold	These parameters define the lower intensity limit for the single peak height level at the beginning of the Pyrogram.
Required peak height for passed quality	The minimum signal value for a peak to achieve "Passed" quality assessment in the base-called sequence. Below this value, the warning "Uncertain due to low peak height" is triggered during the analysis and a "Check" quality assessment is assigned. The default value is 5. **Note**: The value cannot be lower than the required peak height value for check quality (see below).
Required peak height for check quality	The minimum signal value for a peak to achieve "Check" quality assessment in the base-called sequence. Below this value, the warning "Failed due to low peak height" is triggered during the analysis and the "Failed" quality assessment is assigned. The default value is 0. **Note**: The value cannot be higher than the required peak height value for passed quality (see above).
Parameters	
A-peak reduction factor	The factor by which the A-peak intensities are multiplied to account for the fact that A-peaks are higher than other peaks. The default value is 0.90.
Plus shift compensation	Plus shift: A small proportion of the template sequences that incorporates more than one type of nucleotide at a time (if, for example, there are residues left from the dispensation before) and will be sequenced ahead of the rest of template sequences. If this option is checked, the peaks are compensated for plus shift.

Minus shift compensation	Minus shift: A small proportion of the template sequences that fails to incorporate a nucleotide will be sequenced subsequent to the rest of template. If this option is checked, the peaks are compensated for minus shift.
Stringent homopolymer scoring	If this option is checked, stricter rules are used for the quality assessment of homopolymers. The warning "Peak height deviates from the expected peak level at dispensation: *number(s)*" is triggered during the analysis.
Known bases	If there are any known bases in the dispensation order, it is recommended that these are entered as this can improve the analysis: 1. Left-click the relevant dispensation and either enter the height in the text box that opens, or increase or decrease the height by using the arrows next to the text box. 2. To apply the height, press "Enter".

To reset the parameters in the "Settings" tab and the "Analysis Parameters" tab to their default values, click "Revert to Default".

Set Up a Run

Workflow to set up a run

1. **Create a new Run Setup by one of the following methods:**

- Click in the toolbar
- Select “New Run” from the "File" menu
- Press the "R" key while holding down the "Ctrl" key
- Right-click a folder in the shortcut browser and select “New Run” from the context menu. Enter a run name and press “Enter”. To add a shortcut to a folder or drive, click “Add Folder Shortcut”.
- To base your run on a previous run, right-click the processed run file in the shortcut browser and select “Copy and Rerun” from the context menu. Only the run setup, not the run and analysis data, will be copied.

2. **If the new run has not yet been saved, click to enter a run name and save the file in the desired folder.**
3. **In the “Instrument Parameters” drop-down list, select the instrument parameters file that corresponds to the PyroMark Q96 Cartridge to be used. To set up new instrument parameter files, see Manage Instrument Parameters.**
4. **Enter the remaining run parameters and an optional note (see Enter the run parameters).**
5. **Set up the plate in the plate layout of the run file by adding assays to wells and, if desired, entering a sample ID and note for each used well (see Add assay files to the plate).**
6. **When the run is set up and ready to run on the PyroMark Q96 ID Instrument, click to save.**
7. **Print the plate setup and a list of required volumes of enzyme mix, substrate mix, and nucleotides, by selecting “Pre Run Information” from the “Tools” menu and, when the report opens, clicking .**

   Note: To print the “Pre Run Information” report in color, turn on the “Print background colors and images” option in the Internet Explorer (“Tools/Internet Options/Advanced/Printing”).

Enter the run parameters

The following run parameters are available.

Run name	The name of the run is given when the file is saved. Renaming the file also changes the name of the run.
Instrument parameters	Select an instrument parameters file according to the reagents and cartridge that will be used for the run (see Manage Instrument Parameters). **Note**: It is recommended that only instrument parameter settings supplied by QIAGEN are used.
Plate ID	**Optional**: Enter ID of the PyroMark Q96 Plate Low. **Note**: If you position the mouse pointer over a run file in the shortcut browser, a tooltip displays the entered plate ID.
Barcode	**Optional**: Enter a barcode number for the plate or, if you have a barcode reader connected to your computer, place the mouse cursor in the "Barcode" text box and scan the barcode.
Reagent ID	**Optional**: Enter the lot number for the PyroMark Gold Q96 Reagents to be used. The lot number can be found on the product label. **Note**: It is recommended that the reagent ID is entered so that any unexpected problems with the reagents can be traced.
Estimated run time	The estimated run time. Calculated by software if instrument parameters are defined and at least one assay has been assigned to the plate.
Run note	**Optional**: Enter a note about the contents or purpose of the run.

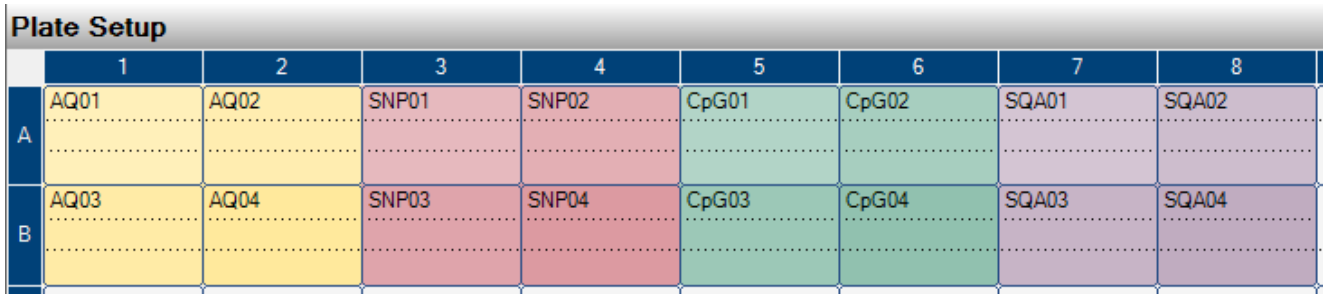
Add assay files to the plate

To add assays to wells, you can either:

- Select the assay in the shortcut browser and press and hold down the left mouse button while you drag the assay to the well
- Right-click the well and select "Load Assay" from the context menu (this option is only available when one well is selected)
- Add an assay to several wells by selecting the wells and dragging the assay to the selection. To select several wells, either click and drag the cursor across the wells to be selected, or hold down the Control key as you click wells.

Note: It is not possible to add an assay with no dispensation order or add two or more assays with the same assay name but have different dispensation orders.

Note: Assays created with PyroMark CpG Software or Q-CpG Software seamlessly load to a well by any of the methods described above. PyroMark Q96 Software converts the old assay into a CpG assay.



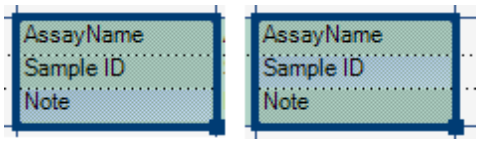
A well is colored according to the assay type loaded to the well. Wells with AQ assays are orange; wells with SNP assays are red; wells with CpG assays are green; wells with SQA assays are purple. Different shades of each color indicate wells with different assays of the same type.

Enter sample IDs and notes

- To enter a sample ID or note, select the corresponding cell (see image below) and enter the text
- To edit a sample ID or note, double-click the corresponding cell
- To import a sample and note layout defined in a text file (*.tsv or *.csv), right-click a well and select "Insert Sample Layout File" from the context menu. For more information, see Define sample ID and note externally.

- To paste a sample layout from the clipboard, right-click a well and select "Paste Sample Layout" from the context menu. For more information, see Define sample ID and note externally.

Note: Commas and semicolon are not supported.



A selected cell is highlighted with a blue background color.

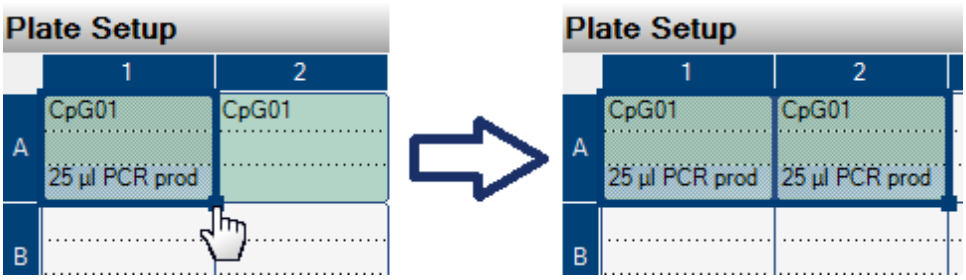
Copy or delete contents from cells

- To cut the contents of a cell to the clipboard, right-click the cell and select "Cut" from the context menu
- To copy the contents of a cell to the clipboard, either right-click the cell and select "Copy Cell" from the context menu or select the cell and press "Ctrl+C"
- To paste the clipboard to a cell or a selection of cells (see Select wells), either right-click the cell or the selection and select "Paste" from the context menu or select the cell(s) and press "Ctrl+V"
- To delete one or more assays, sample IDs, or notes, either right-click the cell or the selection and select "Delete" from the context menu or select the cell(s) and press "Delete"

Drag-copy the contents of a cell to other wells

To drag-copy the contents of a cell to other wells:

1. **Select the cell that you wish to copy.**
2. **Position the mouse pointer over the lower right square of the selection, and press and hold down the left mouse button while you move the mouse to change the selection.**
3. **When the left mouse button is released, the contents of the first selected cell are pasted into the selected cells.**



Drag-copy of the note "25 µl PCR prod".

Drag-copy and increment sample ID

If the last part of an entered sample ID is a number, the number can be incremented when drag-copying the sample ID:

1. **Select the sample ID cell.**
2. **To increment by row:**

   Position the mouse pointer over the lower right square of the selection.

   Press and hold down the "Ctrl" key + the left mouse button while moving the mouse to change the selection.

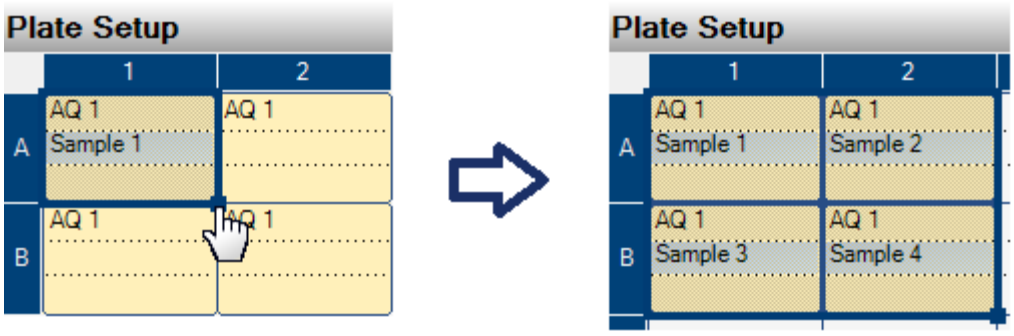
   First, release the left mouse button, then the "Ctrl" key. When the left mouse button is released, the sample ID of the first selected cell is incremented and pasted into the selected cells.

3. **To increment by column:**

   Position the mouse pointer over the lower right square of the selection.

   Press and hold down the "Shift" and "Ctrl" keys + the left mouse button while moving the mouse to change the selection.

   First, release the left mouse button, then the "Shift" and "Ctrl" keys. When the left mouse button is released, the sample ID of the first selected cell is incremented and pasted into the selected cells.



The sample ID "Sample 1" is copied and incremented by column.

Print or export plate setup as image

The "Plate Setup" can be printed or copied as an image (to the clipboard) by right-clicking the plate and selecting "Print" or "Copy as Image" from the context menu. The image can be pasted into applications that support Enhanced Metafile (EMF) images.

Define sample ID and note externally

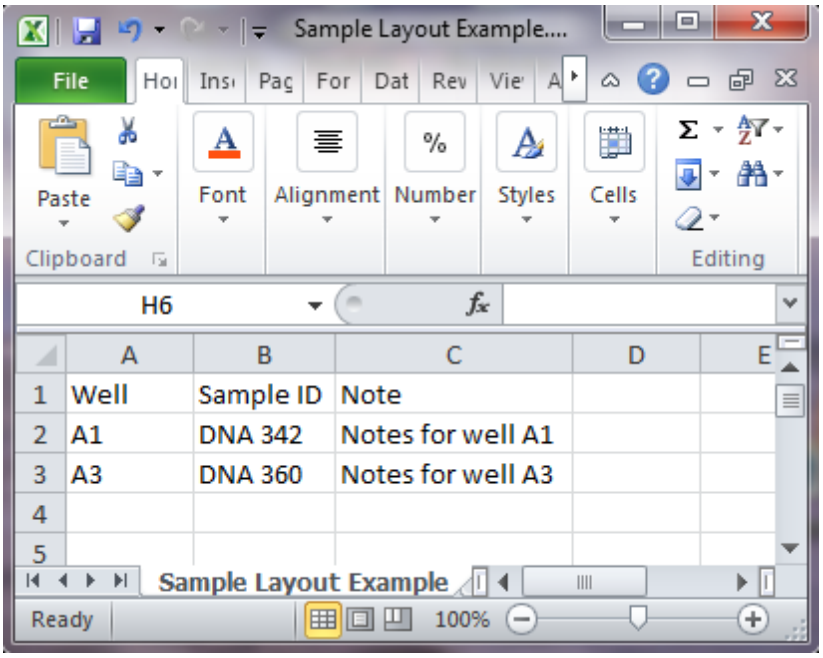
By using the "Import/Insert Sample Layout File" or "Paste Sample Layout" feature, you can easily use the same layout in several runs and reuse information available in existing documentation.

Using the import/insert sample layout file feature

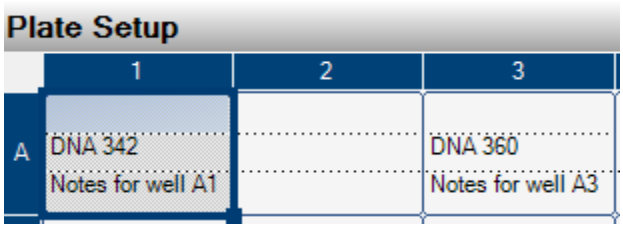
You can, for example, generate layout files from your Laboratory Information Management Systems (LIMS). Sample and note layout files can also be created in Microsoft Excel, Notepad, and similar applications. The layout file must have two or three columns: "Well", "Sample ID", and "Note" (optional). Each column must be separated by a tab, comma, or semicolon, and each line must be delimited by a line break. Save the file as a tab- or comma-delimited text file (*.tsv, *.txt, or *.csv).

The sample and note layout file can be imported into:

- An existing run file by right-clicking a well in the "Plate Setup" and selecting "Insert Sample Layout File" from the context menu
- A new run file by selecting "Import" followed by "Create New Run from Sample Layout File" from the "File" menu



An example of a sample and note layout file created in Microsoft Excel.



The result when importing the sample and note layout file above.

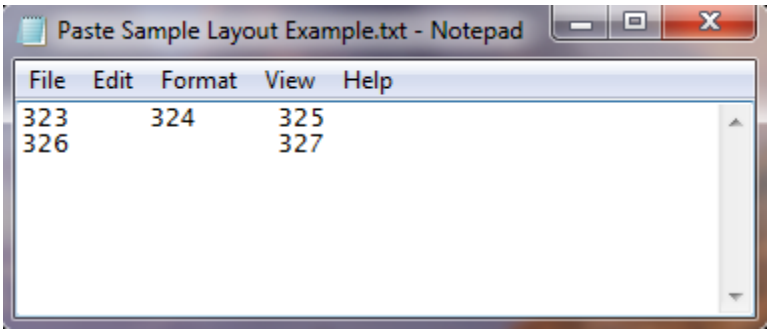
Using the paste sample layout feature

You can, for example, generate and copy layouts from your LIMS. Sample layouts can be copied from Microsoft Excel, Word, Notepad, and similar applications. In the source file, each column of sample IDs must be delimited by a tab and each row of sample IDs must be delimited by a line break.

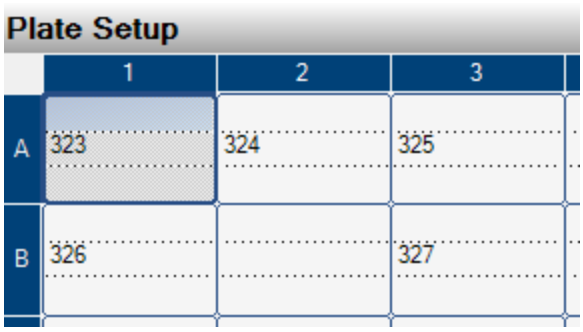
To paste a sample layout into an existing run file:

1. **Copy all the information in the source file.**
2. **Right-click a well in the "Plate Setup" and select "Paste Sample Layout" from the context menu.**

   The software will paste the sample IDs into the plate, starting at well A1. Well notes that have been entered into the wells are kept.



An example of a sample layout created in Microsoft Notepad.



The result when copying and pasting the sample layout created in Microsoft Notepad.

Review the plate setup

The "Well Information" area shows the following information about a well that is selected in the "Plate Setup":

- Well name
- Type of assay (AQ, CpG, SNP, or SQA)
- Assay name
- Sample ID (if entered)
- Sequence to analyze, if entered (AQ, CpG, and SNP assays)
- Dispensation order
- Well note (if entered)

If several wells are selected in the "Plate Setup", the information for the first selected well is shown. If the wells were selected by clicking while holding down "Ctrl", the information for the last well selected will be displayed.

Process a Run

Workflow to process a run

A finalized Run Setup can be processed on the PyroMark Q96 ID Instrument if the instrument has been configured and is not currently processing a run.

1. **Prepare your samples.**
2. **Fill the PyroMark Q96 Cartridge with the required volumes of reagents and nucleotides.**

   Reagent and nucleotide volumes are given on the "Pre Run Information" in the "Tools" menu.
3. **Open the instrument lid and load the reagent cartridge and PyroMark Q96 Plate Low into the instrument. Close the instrument.**
4. **Start the run by clicking . The "Run" view becomes active. The run can be monitored, paused, or stopped in this view.**

   Note: The "Start" icon is enabled only if an instrument connection has been configured, instrument parameters have been defined for the run file, and at least one well has an assay.
5. **Upon completing the run (or stopping a run; see Monitor and control a run), click "OK" and the "Overview" tab of the "Analysis" view will appear. The analysis of the plate will take place automatically and the analysis results will be saved.**

   Note: If upon completion a run contains one or more warnings, a message box appears automatically giving the user access to more information about the run.
6. **If the instrument will no longer be used, shut it down by selecting "Shut Down Instrument" from the "Instrument" menu.**

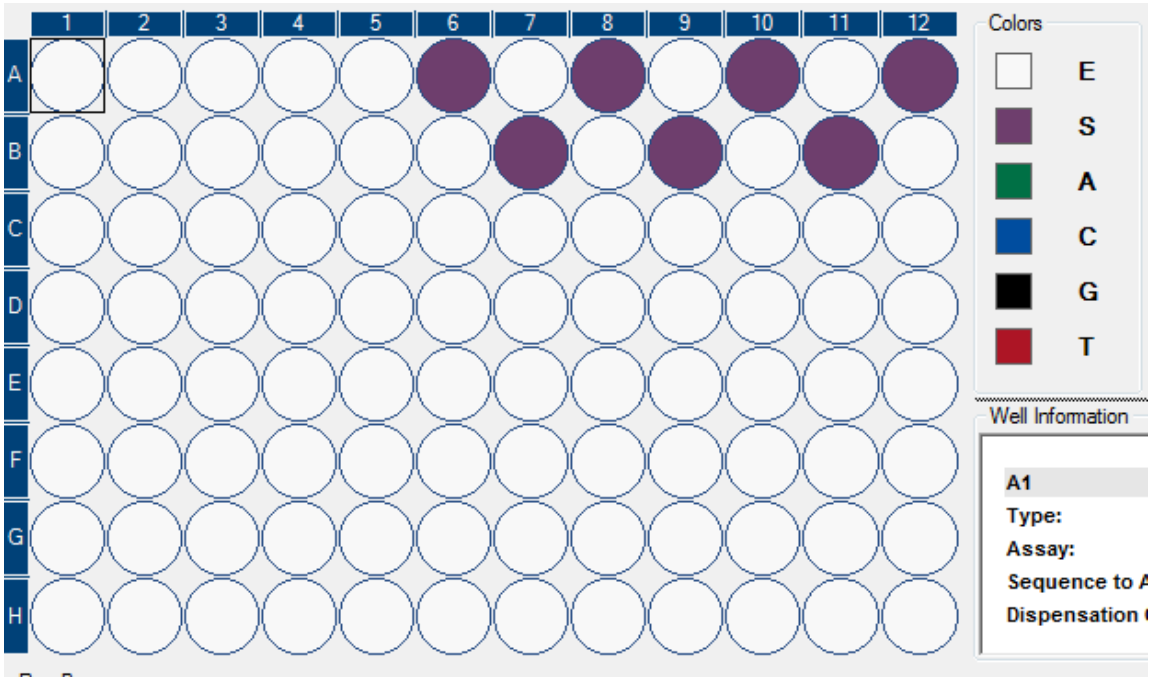
For more information, see Section 5.4 of the *PyroMark Q96 ID User Manual*.

Monitor and control a run

The "Run" view displays the following information regarding the progress of a run:

- Last nucleotide or reagent dispensation to each well
- Well-specific information
- Instrument-specific information in the Instrument Status Monitor

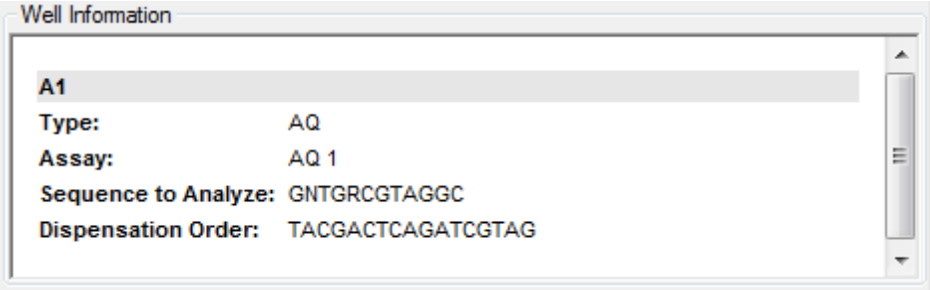
A diagram of a 96-well plate displays all wells active in the run. During the course of the run, each well is colored to reflect the last reagent (Enzyme Mix or Substrate Mix) or nucleotide (A, C, G, or T) dispensed to the well. A legend indicating the reagent that corresponds to each color is provided.



The color of the wells indicates that Substrate Mix was dispensed into these wells.

The user can select a well in the plate diagram. The well will be highlighted with an orange outline. Well information for the selected well is displayed in the "Well Information" pane. This information includes:

- Type of assay (AQ, SNP, CpG, or SQA)
- Name of assay
- Sample ID (if entered during Run Setup)
- Note (if entered during Run Setup)
- Sequence to analyze, if entered during Run Setup (not for SQA assays)
- Dispensation order

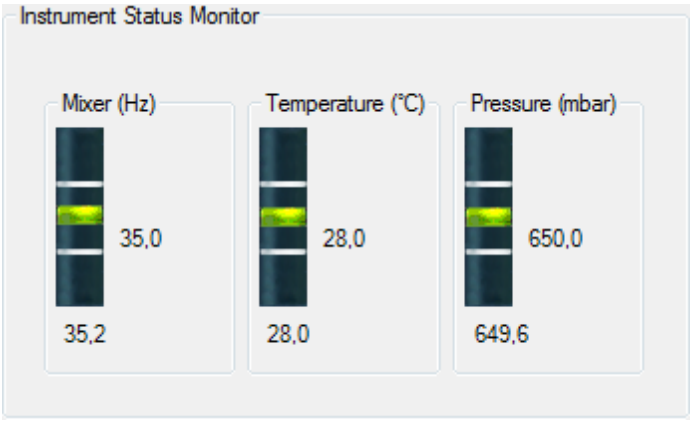


In addition, a Pyrogram appears at the bottom of the window, allowing the user to monitor the measured values for the selected well as the run progresses.

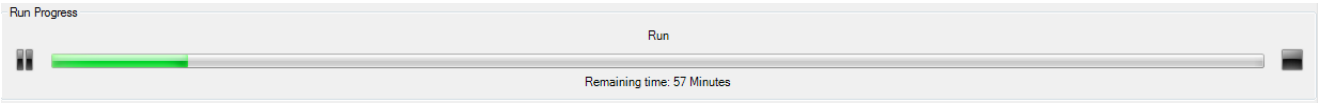
To zoom in on the Pyrogram, click and drag the mouse across the desired stretch.

To zoom out, either right-click the Pyrogram area and select “Zoom out” from the context menu, or double click the Pyrogram.

Mixer frequency, temperature of the process chamber, and pressure in the dispensing unit are displayed in real-time in the "Instrument Status Monitor". Each parameter is displayed on a gauge where white bars indicate the valid upper and lower limits for the instrument at the time of a dispensation, and a green bar shows the actual value upon dispensation if this value is within the limits. The bar turns red when the parameter value falls out of the limits.

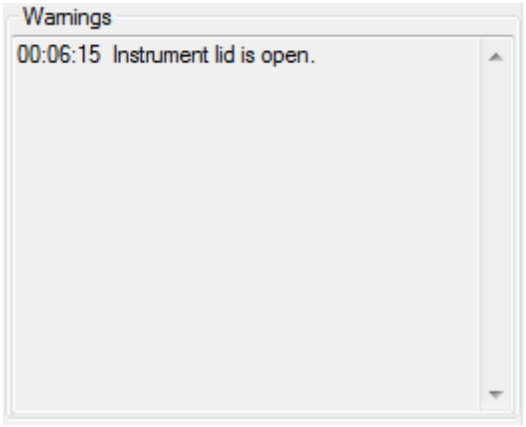


A run progress bar displays the current state of a run (e.g., preparing for run, adding reagents, run, end of run, connection lost) and the time remaining to complete the run. From the run progress bar it is also possible to pause, stop, resume, and start a run by clicking the icons flanking the progress bar.



Warnings during a run

Errors may occur during a run, which triggers warnings that will appear in the "Warnings" pane. The warning is also documented in the Run Log of the "Run Information". If an error occurs during a run, a dialog box that appears at the end of the run gives the user the option to review the Run Information.



Restoring a run

If the connection to the instrument is lost during a run, a message box will appear to inform the user. Upon clicking "OK", the "Run" view will close but the instrument will continue to process the run. The data collected will be stored on the instrument and the run file can be restored.

1. **Check cables and reestablish connection with the instrument.**

   Note: The instrument connection can be tested by selecting "Preferences" in the "Tools" menu and then clicking "Test Connection" in the dialog box.

2. **Double-click the interrupted Run Setup file.**
3. **Select "Restore Run Data" from the "Tools" menu.**
4. **Click "Yes" in the dialog box to proceed with restore. Data stored on the instrument will be transferred to the operator's computer and the "Analysis" view will open.**

   Note: The application cannot be closed while a run is processed by the instrument.

Analyze a Run

Workflow to analyze a run

1. **Either analyze all wells on the plate or select the wells to be analyzed and run the analysis (see below).**
2. **View the analysis results.**
3. **Optional: If applicable, modify how the analysis is performed (see Edit analysis parameters).**
4. **Optional: Enter an analysis note in the "Note" text box in the "Overview" tab.**

   Note: To expand or collapse the "Note" field, click ⊞ or ⊟.

5. **To save the analysis results, click in the toolbar.**

Note: A dialog box will request confirmation of any modifications made in the "Overview" or "Analysis Setup" tabs prior to switching from one tab to another, or upon selecting (orange outline) another well.

Note: It is not possible to edit the analysis parameters or enter an analysis note for a locked assay ( ).

Analyze all or the selected wells

In the "Overview" tab, there are two ways to perform the analysis:

Analyze all wells with a valid analysis setup for the current analysis mode.

Analyze the selected wells (see Select wells).

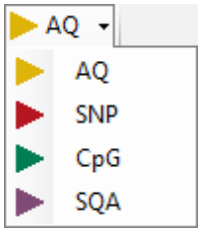
Note: It is also possible to right-click the selection and select "Analyze Selected" from the context menu.

During the analysis, a progress dialog box is shown. This dialog box contains a progress bar, a stop button, and the name of the well that is being analyzed. The analysis can be stopped by clicking “Stop”. The progress dialog box closes when the analysis is finished or by clicking “Stop”.

Note: When a well has been analyzed, the well color changes to light blue.

Note: If the analysis of a well resulted in an error, the well is marked with a red cross.

Analysis modes



PyroMark Q96 Software has four analysis modes: AQ, SNP, CpG, and SQA. To toggle between the modes, select “AQ”, “SNP”, “CpG”, or “SQA” in the toolbar.

A plate can include wells that must be analyzed in different modes. To complete all analyses:

1. **With the run file open in the "Analysis" view, toggle to the desired analysis mode.**
2. **Click "Analyze all".**

   All wells with an Assay Setup corresponding to the analysis mode will be analyzed.

   If none of the wells has a valid Assay Setup for the chosen analysis mode, the analysis is not performed.

AQ, SNP, and CpG assays can be analyzed in any analysis mode without modifying analysis parameters. To do so, select the relevant wells and click "Analyze selected". Multiple single nucleotide polymorphisms in a variable region can be analyzed in SNP mode, but not in AQ mode. These variable regions will be automatically disabled in AQ mode. InDels can be analyzed in AQ and SNP mode, but not in CpG mode.

Analyzing assays in CpG mode requires that the sequence to analyze be a valid CpG sequence. If necessary, the appropriate sequence to analyze can be added in the "Analysis Setup" tab. Since the CpG mode does not support automatic analysis of SNPs, methylation percentages and quality assessments are only determined for the CpG sites. SNPs in a CpG assay can be analyzed in the AQ mode using the sequence to analyze used in the CpG setup. To exclude the CpG sites in the SNP reports, select the "Analysis Setup" tab and uncheck the "Analyze" option for these positions in the "Variable Positions" tab.

To analyze SQA assays in any other analysis mode, a sequence to analyze must be entered in the "Analysis Setup" tab.

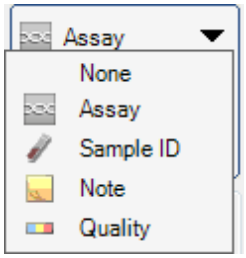
View the analysis results

By selecting an analyzed (light blue) well in the “Overview” tab, the corresponding Pyrogram is displayed in the Pyrogram area and the well information (including analysis warnings) is listed in the “Well Information” area. If several wells are selected in the plate overview, information for the well with the orange selection frame is shown.

Get an overview of the results

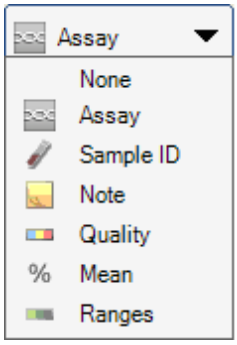
The following well information can be viewed in the plate overview in the “Overview” tab:

AQ and SNP assays



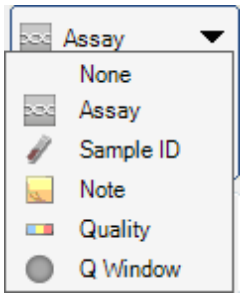
- Select to show the assay name.
- Select to show the sample ID.
- Select to show the well note.
- Select to show the quality bar. The quality bar shows the quality assessment of all variable positions in the well or of all the bases in the base-called sequence. See Quality colors.

CpG assays



- Select to show the mean methylation percentage of all CpG sites in the well.
- Select to show the methylation bar. The methylation bar shows the methylation level for each CpG site in the well. See Methylation colors.

SQA assays



Select to show the quality assessment at the end of the quality control window. The default number of bases included is 20.

Note: Wells with a high substrate peak will be marked with an information icon (ⓘ) in the plate overview. This will not affect the quality assessments.

Note: If analysis parameters, quality assessments, or base-called sequence for SQA results have been edited by the user, the well is marked with a warning icon (⚠).

Note: If an assay is locked, the well is marked with the 🔒 icon.

Print or export plate overview as an image

The plate overview can be printed or copied as an image (to the clipboard) by right-clicking the plate overview and selecting "Print" or "Copy as Image" from the context menu. The image can be pasted into applications that support Enhanced Metafile (EMF) images.

Analysis warnings

By selecting an analyzed (light blue) well, the analysis warnings (if any) are listed in the "Well Information" area. An analysis warning affects the quality assessment in the following way:

- AQ, SNP, and CpG assays: Affects the quality assessment for either all variable positions or a single position. If several warnings of the same kind were triggered, only the most serious ones are displayed in the "Well Information" area.
- SQA assays: Affects the quality assessment for either the whole sequence or from a specific dispensation and forward. All warnings triggered within the quality control window are displayed in the "Well Information" area.

For some of the warnings, the criteria for occurrence and the effect on the quality assessment can be modified by the user in the "Analysis Parameters" tab; see Edit analysis parameters.

Note: If a dispensation error occurs, it is recommended that the reagent cartridge is replaced.

Quality assessments

The quality assessments of the analysis results are displayed by:

- Quality bars () in the plate overview; see Get an overview of the results
- The background color of the analysis results (allele frequencies, the methylation percentages, or genotype in the Pyrogram, for example  , or the base-called sequence)
- Quality control windows () in the plate overview (SQA assays only)
- The peaks in the compensated Pyrogram are colored according to their quality assessments (SQA assays only)

Quality colors

- Blue: Passed
- Yellow: Check
- Red: Failed
- White: Not analyzed. Either analysis is not supported by the software (e.g., analyzing single nucleotide polymorphisms in CpG mode) or the variable position has been deselected by the user (AQ, SNP, and CpG assays only).

Methylation levels

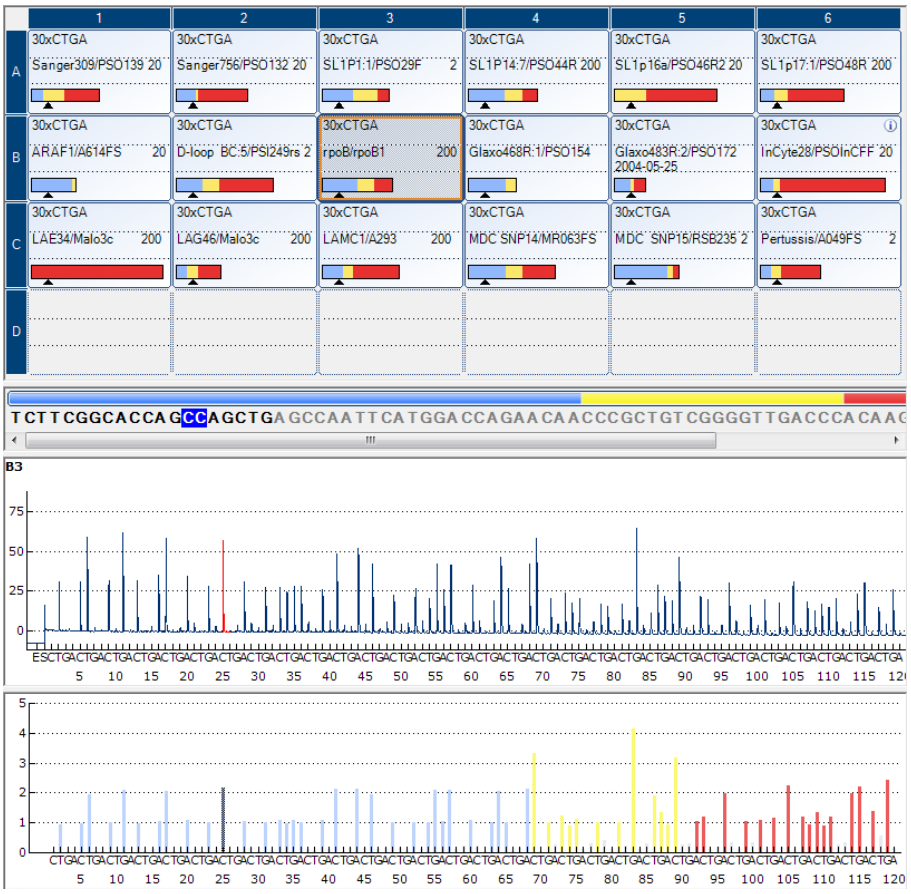
In the CpG mode, a methylation bar in the "Overview" tab shows the methylation level for each CpG site in the well (see Get an overview of the results).

Methylation colors

- Light green: Below the expected range
- Green: Within the expected range
- Dark green: Above the expected range

View and compare Pyrogram

By selecting an analyzed well in the "Overview" tab, the corresponding Pyrogram and theoretical histogram (if an AQ, SNP, or CpG assay) or compensated Pyrogram (if an SQA assay) are displayed in the "Pyrogram" pane.



When a base is selected in the base-called sequence, the corresponding peak is highlighted in both Pyrogram areas, and vice versa.

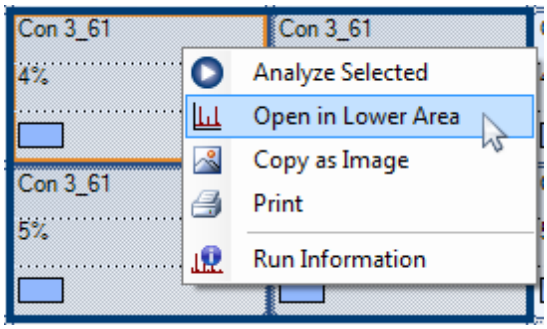
Simultaneously view Pyrograms of different wells

Pyrograms from 2 or more wells can be viewed simultaneously (e.g., if the user wishes to compare Pyrograms) by opening one ore more additional Pyrograms of selected wells in the lower area of the "Pyrogram" pane:

1. **In the "Overview" tab use the zoom tool to display the plate quadrant containing the wells you wish to compare.**
2. **Highlight the wells (see Select wells) you wish to open in the lower area.**
3. **Right-click the selection and select "Open in Lower Area" from the context menu.**

   Two or more Pyrograms are now displayed in the "Pyrogram" pane. In the upper area of the pane is the Pyrogram of the well in the plate overview displaying an orange outline. This Pyrogram is fixed. In the lower area are the one or more Pyrograms of the additional wells selected for the comparison. The Pyrograms in the lower area are displayed one at a time.

4. **Select the well in the plate overivew that should appear as the fixed Pyrogram in the upper area of the "Pyrogram" pane.**
5. **Use the scroll bar in the lower area of the "Pyrogram" pane to change the Pyrogram displayed.**



A Pyrogram with the same sequence to analyze can be zoomed simultaneously (i.e., linked zooming) by clicking in the top right corner of the upper area.

To close the Pyrogram list in the lower area, click **x** in the upper right corner of the lower area.

Zoom Pyrogram and view description of icons and colors

For information on icons and colors used in the Pyrogram area and how to zoom, see Pyrogram.

Edit analysis parameters

The default analysis settings have been set to give optimal analysis results for most assays. If changing these settings, ensure the changes are validated (see Appendix B of the *PyroMark Q96 ID User Manual*).

Note: When using QIAGEN kits, use the settings stated in the kit handbooks.

Note: It is not possible to edit the analysis parameters for a locked assay ( ).

1. **Select the well or wells (see Select wells) for which you wish to edit the analysis parameters.**

   Note: The changes will only be applied to wells that share the same assay and dispensation order as the displayed well. To edit the analysis parameters for all wells with the same assay and dispensation order, you only have to select one of the wells.

2. **Edit analysis parameters in the "Analysis Setup" tab:**

   To enable or disable variable positions and/or change expected methylation ranges (only CpG assays), see Set up the variable positions. To edit other analysis parameters for an AQ, SNP, or CpG assay, see Edit analysis parameters.

   To edit the analysis parameters for an SQA assay, see Edit analysis parameters.

   Note: It is not possible to change the assay name, dispensation order, or assay note.

3. **When finished, click "Apply". In the resulting message box, select if changes should be applied to all wells with the same assay name and dispensation order ("To all") or to only selected wells with the same assay name and dispensation order ("To selected").**

   Note: It is also possible to enable or disable reference peaks and/or bisulfite treatment controls (CpG assays only) in Pyrogram in the "Overview" tab (see Enable or disable reference peaks and bisulfite treatment controls). To apply changes made in Pyrogram, click the green button. This button is enabled when a change has been made.

4. **In the "Apply Analysis Setup" dialog box, apply the changes to all or the selected wells:**

   To apply the changes to all wells that share the same assay and dispensation order as the displayed well (i.e., all the white wells in the "Apply Analysis Setup" dialog box), click "To All".

   To apply the changes to the selected wells, (i.e., the white wells that are selected in the "Apply Analysis Setup" dialog box), click "To Selected".

   During the analysis, a progress dialog box is shown. The dialog box contains a progress bar, a stop button, and the name of the well that is being analyzed. The analysis can be stopped by clicking "Stop".

5. **To save the changes, click .**

   Note: If analysis parameters, quality assessments, or base-called sequence for SQA results have been edited by the user, the well is marked with a warning icon () in the "Overview" tab.

   Note: All changes are logged. To view the analysis log for a selected well, select "Analysis Log" from the "Tools" menu.

Use modified assay in other runs

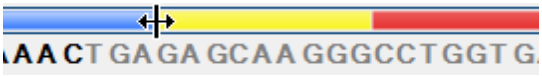
Changes made in the "Analysis Setup" tab will not be saved in the original assay file. To use the modified assay in other runs:

1. **Select a well that is using the modified assay and click "Save Assay". The "Save Assay As" dialog box opens.**
2. **Save the changes to the original file or save the modified assay as a new file:**
   Select destination (folder) from the "Save in" drop-down list.
   Enter filename in the "File name" text box and click "Save".

Edit quality assessments

To edit the quality assessment of an allele frequency, genotype, or methylation percentage, left-click the analysis result in the Pyrogram and select "Passed", "Check", or "Failed" from the context menu.

To edit the quality assessment of a base-called sequence, position the mouse pointer over the left or the right end of the "Passed", "Check", or "Failed" area, so that the pointer changes from a white arrow to ⇿, and move the mouse to the left or the right while holding down the left mouse button.



If a quality assessment has been edited by the user, a warning icon (⚠) is displayed in the plate overview in the "Overview" tab, a warning appears in the "Well Information" area, and if it is an AQ, CpG, or SNP assay, a border appears around the analysis result in the Pyrogram (e.g., 44%).

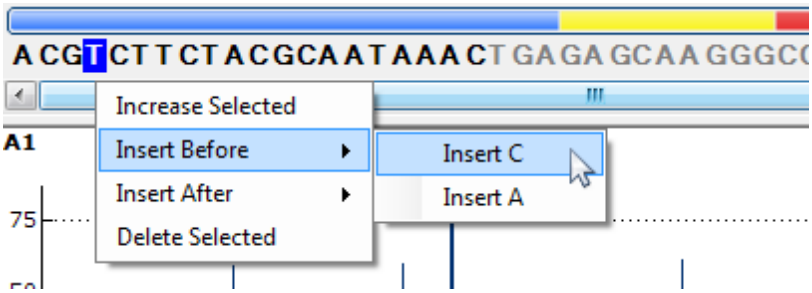
Note: All changes are logged. To view the analysis log for a selected well, select "Analysis Log" from the "Tools" menu.

Note: The quality assessments generated by the software are based on advanced analysis algorithms. It is not recommended to edit the quality assessments.

Note: It is not possible to edit the quality assessments for a locked assay (🔒).

Edit base-called sequences

To edit a base-called sequence, right-click it and select the desired option. It is also possible to copy segments or the complete base-called sequence. Click and drag the mouse across the segment to be copied (selected segment will be highlighted in blue) and press "C" while holding down the "Ctrl" key to copy the selection to the clipboard.



Note: All changes are logged. To view the analysis log for a selected well, select "Analysis Log" from the "Tools" menu.

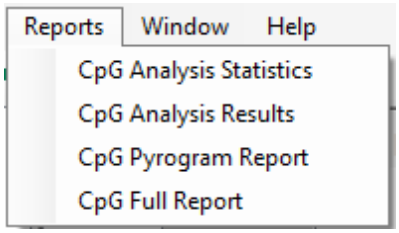
Note: If editing a base-called sequence, note that the quality assessments are still based on the original sequence (the sequence called by the software). See edit the quality assessments for more details.

Note: It is not possible to edit the base-called sequences for a locked assay ( ).

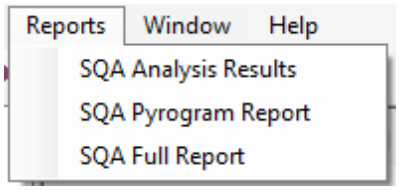
View, Print, and Save Analysis Reports

PyroMark Q96 Software offers the following analysis reports for processed runs.

Reports for AQ and CpG runs



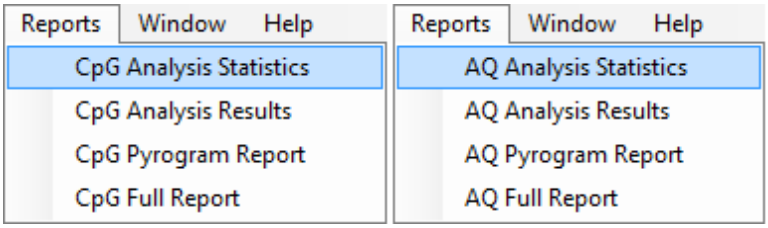
Reports for SNP and SQA runs



- Analysis Statistics Report. This includes analysis statistics for all or selected wells.
- Analysis Results Report. This includes well information and analysis results for all or selected wells.
- Pyrogram Report. This includes well information and Pyrogram for all or selected wells.
- Full Report. This includes run parameters, run log, well information, and analysis results (including Pyrogram) for all or selected wells.

Note: To view reports generated in PDF format, a PDF reader must be installed on the computer. Adobe Reader can be downloaded at www.adobe.com.

Analysis statistics report (only AQ and CpG modes)



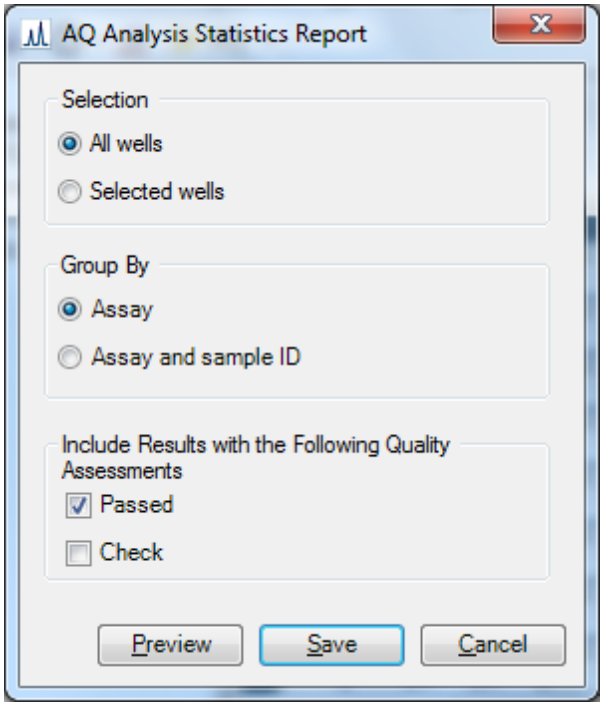
The Analysis Statistics report includes the following information for variable positions in all or selected wells (see Select wells):

- The mean allele frequencies (AQ report) or mean methylation percentage (CpG report)
- The highest and the lowest allele frequencies (AQ report) or methylation percentage (CpG report)
- The standard deviation
- The number of values and the wells used in each calculation

- If analysis parameters or quality assessments have been edited by the user, the affected wells are listed at the top of the report

The report can be saved as a text file (*.tsv or *.csv) or an HTML file (.html). The report can be imported into Microsoft Excel or other applications that can handle text files (*.tsv or *.csv) with data that is separated by semicolons (;) or tabs. This is useful when doing further calculations on the data.

Report options



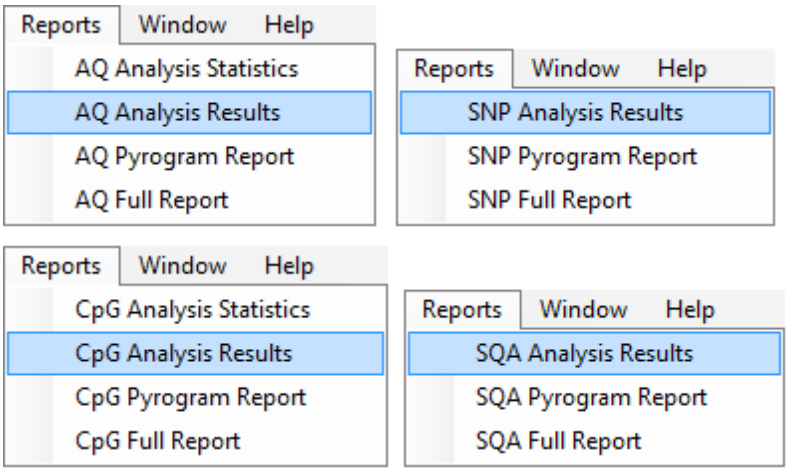
In the "Analysis Statistics Report" dialog box, there are the following options:

All wells/Selected wells	The wells to be included in the report.
Assay/Assay and sample ID	The analysis results statistics can be grouped according to: ▪ Assay Wells with the same assay will be grouped. ▪ Assay and sample ID Wells with the same assay and sample ID will be grouped. Can be useful when experiments with replicates are performed.

Passed/Check	The analysis results to be included. The calculations can be performed on results with passed and/or check quality assessment. **Note**: If all passed and check results are to be included in the report, you can exclude results within this group by turning off the "Analyze" option for these positions in the "Analysis Setup" tab (see Set up the variable positions).

To view the report before saving or printing it, click "Preview".

Analysis results report



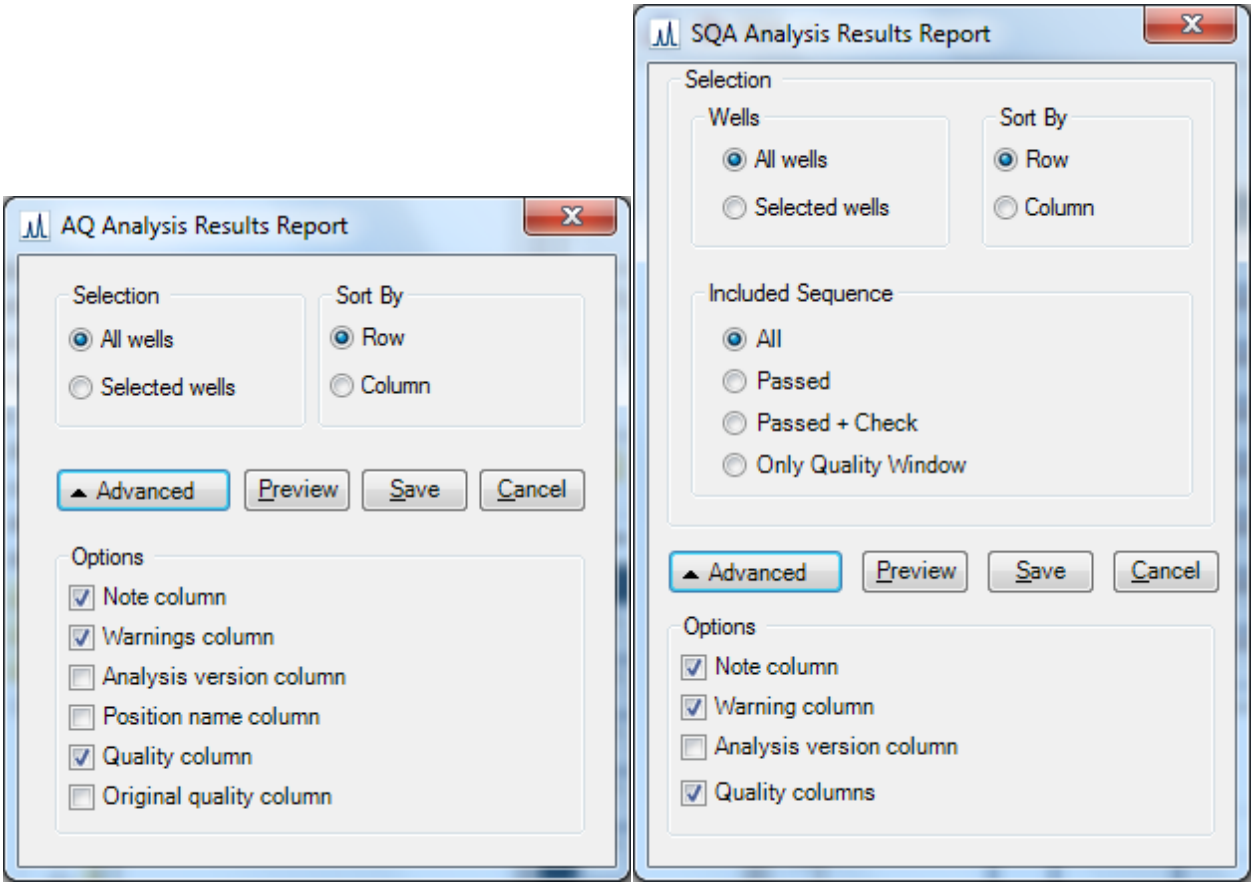
The Analysis Results report includes the following information for all or selected wells (see Select wells):

- Well information (well name, assay name, and sample ID)
- The allele frequencies (AQ report), genotypes (SNP report), methylation percentages (CpG report), or base-called sequences (SQA report) and the quality assessments
- The mean methylation percentage and the standard deviation of all passed CpG sites in a well (CpG report only)
- The highest and lowest methylation percentage in a well (CpG report only)
- Information on whether the analysis parameters, quality assessments, and analysis results (SQA report only) have been edited by the user or not

Optional: The analysis version, well notes, and analysis warnings. In the AQ and CpG reports, it is also possible to include the names and the original and/or the current quality assessments for the variable positions.

The report can be saved as a text file (*.tsv or *.csv) or an HTML file (.html). The report can be imported into Microsoft Excel or other applications that can handle text files (*.tsv or *.csv) with data that is separated by semicolons (;) or tabs. This is useful when doing further calculations on the data. The first line in the report states the name of the run. The following two or three lines contain the column headings. Each of the lines following the column headings contains detailed well information and statistics of a specified well.

Report options



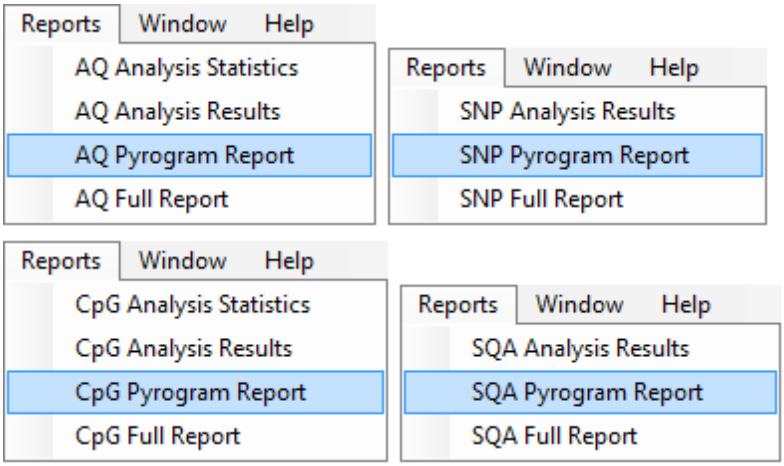
In the "Analysis Results Report" dialog box, there are the following options:

All wells/Selected wells	The wells to be included in the report.
Sort by row/column	The sorting order of the wells.

All/Passed/Passed + Check/Only Quality Window	The bases in the base-called sequences to be included in the report. This option is only available for the SQA report.
Note column	If this option is checked, a column with well notes is included.
Warnings column	If this option is checked, a column with analysis warnings is included.
Analysis version column	If this option is checked, a column with the analysis version is included.
Position name column	If this option is checked, a column with the names of the variable positions is included. This option is not available for the SQA report.
Quality column	If this option is checked, a column with the current quality assessments is included.
Original quality columns	If this option is checked, a column with the original quality assessments is included. This option is not available for the SQA report.

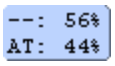
To view the report before saving or printing it, click "Preview".

Pyrogram report



The Pyrogram report includes well information (well name, assay name, sample ID, and well note) and Pyrograms for all or selected wells (see Select wells). If analysis parameters, quality assessments, or base-called sequence for SQA results have been edited by the user, this is stated in the report.

The following information, icons, and colors are displayed and used in the AQ, SNP, and CpG reports:

- The well name and the sequence to analyze
- The analysis result — allele frequencies (AQ report), genotypes (SNP report), or methylation percentages (CpG report) — is displayed above each variable position, for example 

(InDel) and 

. The background color shows the quality assessment of the analysis result; see Quality colors.

  Note: 

(in white) indicates that a site was deselected by the user. 

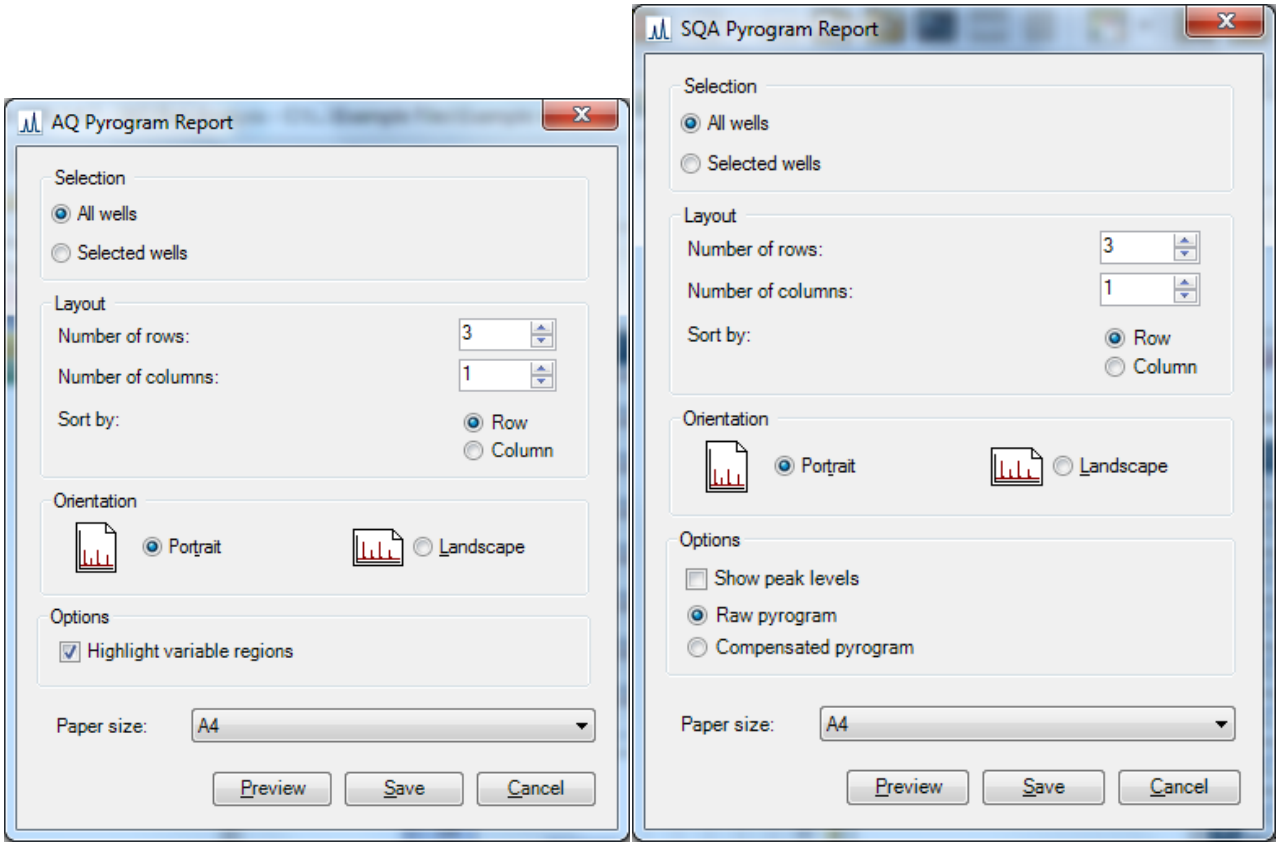
(in white) indicates that the software does not support the analysis, e.g., analysis of SNP in the CpG mode. 

(in red) indicates that the analysis was not possible due to lack of data.
- If desired, the variable positions are highlighted with a blue-gray background color
- Bisulfite treatment controls are highlighted with a light yellow background color (CpG report only)

The following information and colors are displayed and used in the SQA report:

- The well name
- The base-called sequence. The background color of a base in the sequence is according to its quality assessment; see Quality colors.
- If a compensated Pyrogram is included, the peaks are colored according to their quality assessments

Report options



In the "Pyrogram Report" dialog box, there are the following options:

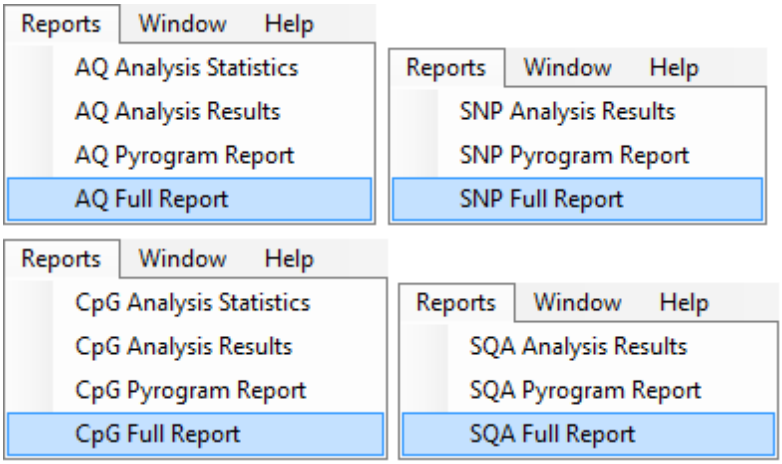
All wells/Selected wells	The wells to be included in the report.
Number of rows/ columns	The number of columns and rows of a Pyrogram on each sheet.
Sort by row/column	The sorting order of the wells.
Portrait/Landscape	The paper orientation.
Highlight variable regions	If this option is checked, the variable regions are highlighted with a blue-gray background color. This option is not available for the SQA report.

Show peak levels	If this option is checked, the calculated peak levels are shown in the Pyrogram. This option is only available for the SQA report.
Raw Pyrogram/ Compensated Pyrogram	The type of Pyrogram to be included in the report. This option is only available for the SQA report.
Paper size	The paper size (A4, A3, letter, or tabloid).

To view the report before saving or printing it, click "Preview".

Note: In order to view the report, a PDF reader must be installed on the computer. Adobe Reader can be downloaded at www.adobe.com.

Full report

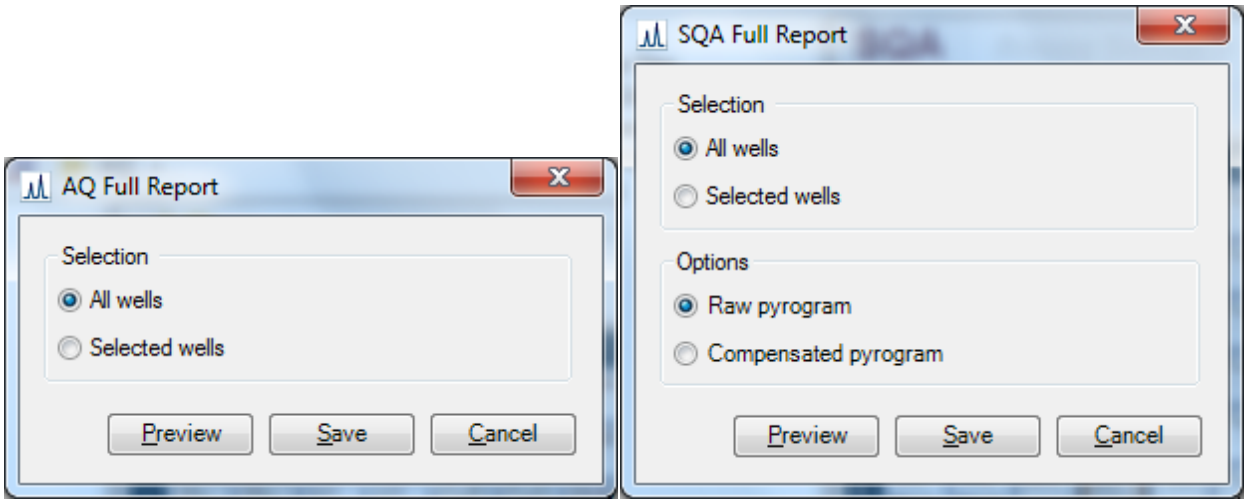


The full report includes the following information for all or selected wells (see Select wells):

- Run parameters (run name, run date and time, instrument parameters, instrument name, serial number, operator, plate ID, barcode, reagent ID, and run note) and a run log
- Well information (well name, assay name, sample ID, and well note), analysis version, AQ, SNP, or CpG assay, sequence to analyze
- Pyrogram. For information on icons and colors used in the Pyrogram area, see Pyrogram report.

- Allele frequencies (AQ report), genotypes (SNP report), methylation percentages (CpG report), or base-called sequences (SQA report), and the quality assessments
- Analysis warnings
- If analysis parameters or quality assessments have been edited by the user, the affected wells are listed

Report options



In the "Full Report" dialog box, there are the following options:

All wells/Selected wells	The wells to be included in the report.
Raw Pyrogram/ Compensated Pyrogram	The type of Pyrogram to be included in the report. This option is only available for the SQA report.

To view the report before saving or printing it, click "Preview".

Note: In order to view the report, a PDF reader must be installed on the computer. Adobe Reader can be downloaded at www.adobe.com.

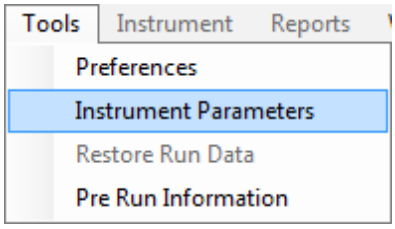
Open files created in other PyroMark software

PyroMark Q96 Software supports Run Setup, Assay Setup, and run files created in PyroMark CpG or Q-CpG Software. Files can be opened by either selecting "Open" in the Files menu or clicking  . Upon saving, the file is converted to the new format and can no longer be opened in previous software versions. To keep a file supported in previous software versions, save the file with the different name.

Unconverted Q-CpG assay and run files are designated with light-colored icons. See Shortcut browser for more information.

Note: Files created in PyroMark Q24 Software are not supported.

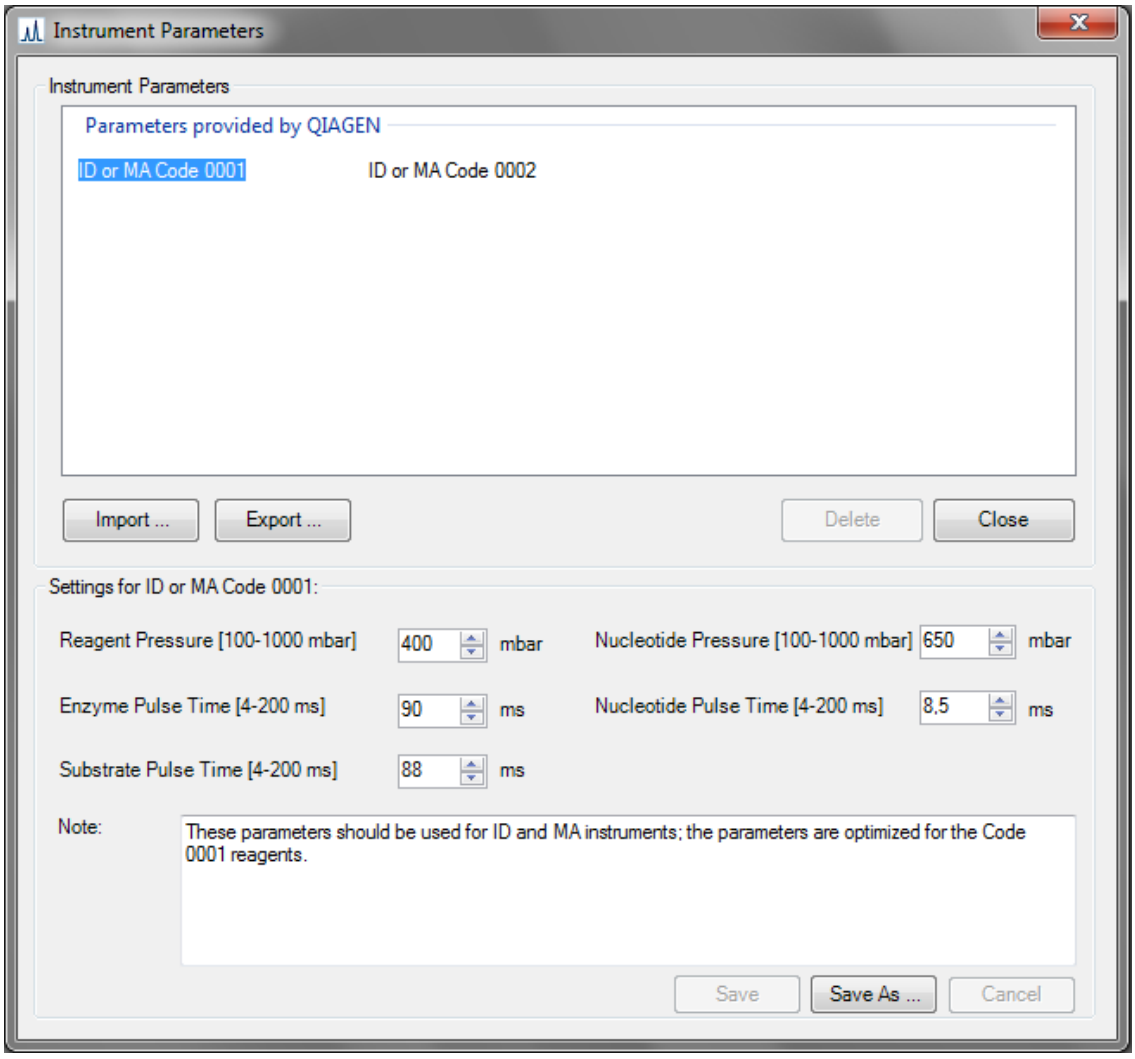
Manage Instrument Parameters



Instrument parameters should be selected according to the reagent cartridge that will be used for the run. The code number printed on the PyroMark Q96 Cartridge corresponds to specific instrument parameter settings provided at www.qiagen.com/Products/PyroMarkQ96ID.aspx.

Note: We recommend that only instrument parameters supplied by QIAGEN are used.

Important: In order to access instrument parameter settings online, you must register your instrument with a valid serial number (see back of instrument) at www.qiagen.com.



Workflow

1. **Select "Instrument Parameters" from the "Tools" menu. The Instrument parameters dialog box opens.**
2. **Right-click an existing instrument parameter file listed in the upper pane and select "Duplicate" from the context menu.**

   Available instrument parameter files are listed according to origin — files provided by QIAGEN with the software, and files created by the user.

   Note: An instrument parameter file can also be imported using the "Import" button (e.g., if the file is downloaded from www.qiagen.com). Existing instrument parameter files can also be exported and deleted.
3. **Enter a new file name (e.g., "Instrument parameters Code 0005").**

4. **Go to the Web page cited above and open the file "Managing instrument parameters for the PyroMark Q96 ID", found under the User Support tab. In the provided table, find the dispensing pressures and pulse time settings that correspond to the Code number printed on the PyroMark Q96 Cartridge to be used.**
5. **Enter the dispensing pressures and pulse times for Enzyme Mixture, Substrate Mixture, and nucleotides into the corresponding fields.**

   Optional: Enter a note regarding cartridge and reagents lots used.
6. **Save the instrument parameter file.**
7. **When creating a new Run Setup, select this new instrument parameter file from the dropdown menu that appears in the "Run Setup" view.**

Instrument parameters

In the "Instrument parameters" dialog box, the following parameters are available.

Reagent pressure	Pressure (millibar) for dispensation of the enzyme mix and substrate mix.
Enzyme pulse time	Dispensation time (milliseconds) for the enzyme mix.
Substrate pulse time	Dispensation time (milliseconds) for the substrate mix.
Nucleotide pressure	Pressure (millibar) for the dispensation of nucleotides.
Nucleotide pulse time	Dispensation time (milliseconds) for nucleotides.
Note	Note on the instrument method (optional).

General Hints and Tips

Validation of assays

Validate your assays using reference DNA samples; see Appendix B of the *PyroMark Q96 ID User Manual*.

Run log

A log is maintained for each run, detailing events and warnings that occur during a run. This log is available in the "Run Information" window, which can be accessed from the "Tools" menu.

Analysis log

All analyses performed are logged with analysis settings used, analysis mode (AQ, SNP, CpG, or SQA), analysis version, results (including analysis warnings), date and time of the analysis, and who performed the analysis. For information on who performed an analysis and who created an assay or run file to be correct, all users must log on to Windows using their own user accounts. For more information about user accounts and logging on and off, see Windows online help or contact your system administrator.

To view the analysis log for a selected well, select "Analysis Log" from the "Tools" menu.

Protection of files

To protect a file from being edited by another user, save the file in a folder that can only be accessed by you. Contact your system administrator for more information.

To protect a file from being accidentally overwritten by you or another user, set the "Read-only" attribute for the file using Windows Explorer:

1. **Close the file in the PyroMark Q96 Software.**
2. **Open Windows Explorer and locate the file.**
   This can be done by right-clicking the folder containing the file in the shortcut browser and selecting "Explore" from the context menu.
3. **In Windows Explorer, right-click the file and select "Properties" from the context menu.**

4. **When the "Properties" dialog box opens, turn on (☑) the "Read-only" attribute and click "OK".**

A backup should be performed frequently.

Protection of analysis results

It is not possible to edit the analysis parameters or results for a locked assay (🔒). To lock an assay, open the assay file and click the "Lock Assay" button at the bottom of the assay setup window. Lock the assay before adding it to the plate.

Troubleshooting Guide

Error or error message	Comments and suggestions
a) Red cross over wells in the "Overview" tab during analysis	The analysis of the well resulted in an error. Contact QIAGEN Technical Services.
b) "Exception" dialog box appears	Save the error report and send to QIAGEN Technical Services for information. Click "Continue" to proceed with analysis. If the dialog box remains, click "Quit" and restart the software.
c) Could not create assay from specified PyroMark Assay Design Software file	Ensure a valid assay file type (AQ, CpG, or SNP) is being imported.
d) Assay missing dispensation order. Cannot be added to well.	Add a dispensation order to the assay setup.
e) Connection test failed.	Check the cables and ensure that the instrument is turned on. Contact QIAGEN Technical Services for further assistance.
f) Failed to load file.	The instrument parameters file is probably corrupted. Create a new instrument parameters file (see Manage Instrument Parameters).
g) Failed to load instrument parameters file. Instrument type is not supported.	The instrument parameters file is from another instrument type (e.g., PyroMark Q24). Use only instrument parameters files expressly for PyroMark Q96 ID.
h) Failed to save file. Access denied.	The file being saved is open in another application. Save the file with a different name.
i) The assay setup for well A1 is locked and cannot be transferred to a new analysis mode.	Locked assays on a processed run cannot be unlocked. A new assay and run setup must be created.

Error or error message	Comments and suggestions
j) No well has a valid assay setup for the current analysis mode. Use "Analyze Selected Wells" or edit the sequence to analyze in the "Analysis Setup" tab.	Warning occurs in AQ, SNP, or CpG mode when "Analyze all" is clicked in an analysis mode that does not correspond to the assays in the plate. Specific wells can be analyzed in the current analysis mode by selecting the wells and clicking "Analyze Selected Wells". If the sequence to analyze has not been entered in the assay setup (e.g., in SQA assays) enter the sequence in the "Analysis Setup" tab.
k) No well has a valid assay setup for the current analysis mode. Use "Analyze Selected Wells".	Warning occurs in SQA mode when "Analyze all" is clicked and no wells contain SQA assays. Specific wells can be analyzed in the current analysis mode by selecting the wells and clicking "Analyze Selected Wells".

Error messages from PyroMark Launcher	Comments and suggestions
a) Unknown file type! File could not be opened.	The file type is not recognized and thus cannot be opened with available software.
b) PyroMark Q-CpG Software could not be located.	The pathway to PyroMark Q-CpG Software is not established. This error message can occur for other software applications as well.
c) Error Loading Q96 Software.	An error has occurred while loading the software, or the pathway to the software is not established. This error message can occur for other software applications as well.
d) Error Loading PyroMark Software.	An error has occurred while loading the software and the pathway to the software is not established.

For more information, see the Frequently Asked Questions page at the Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information in this user guide or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

Appendix: Messages of the PyroMark Q96 Software

The following is a list of use, warning, and error messages that may appear in the PyroMark Q96 Software during assay setup, assay run, and data analysis. Messages are organized by analysis mode, though general messages can occur in any mode. For troubleshooting specific to the PyroMark Q96 ID instrument, refer to the *PyroMark Q96 ID User Manual.*

Message text	Explanation
General setup messages	
a) The dispensation order is very long	The dispensation order is longer than 200 bases.
General analysis messages	
a) Not analyzable due to lack of data (overall)	Insufficient peaks in the Pyrogram to analyze the data. This warning overrides all other warnings.
b) High pre-sequencing signal	The substrate peak is high in comparison with the noise and single peak level.
c) Uncertain due to baseline drift	There is an abnormal baseline drift, which may have a negative impact on the result.
d) Failed due to possible dispensation error at dispensation	There is a possible error at the indicated dispensation(s). Affects the quality of all sites after the error.
e) No peaks detected between dispensations	No peaks are detected even though all nucleotides have been dispensed, which may indicate problems with the dispensation unit. Affects the quality color of all sites after the error.
f) Uncertain due to wide peaks	The mean peak width exceeds the check limit. Affects the quality of all sites in the sequence.
Assay setup messages in AQ mode	
a) Invalid sequence	There is an error in the entered sequence to analyze.

Message text	Explanation
b) Variable positions with common dispensations cannot be analyzed	Nucleotide dispensations for several polymorphisms are out-of-phase.
c) The generated dispensation order contains less reference peaks than required	The assay contains less than five constant peaks with an expected value greater than 1.
d) Sequence uncertain due to lack of terminal sequence information	The last nucleotide in the sequence to analyze has been dispensed, which means that the expected peak height of the last dispensation is unknown. **Note**: The sequence to analyze must be one nucleotide longer than the dispensation order.
e) Last variable position not analyzable due to lack of terminal sequence information	There is no sequence information entered after the last variable region, which means that the expected peak height of the variable position is unknown. **Note**: The sequence to analyze must be one nucleotide longer than the dispensation order.
f) Sequence not in phase at the end of dispensations	The sequence is not in phase after all nucleotides have been dispensed, which means that the expected peak height of the variable position is unknown.
g) Quantification may be uncertain: the variable position consists of more than 5 dispensations	Nucleotide dispensations occur out of phase at a polymorphism for more than 5 dispensations, which may have a negative impact on the result.
h) Quantification may be uncertain: the variable position requires more than 5 variable dispensations.	An InDel position requires more than the 5 dispensations to be analyzed, which may have a negative impact on the result.

Message text	Explanation
i) Quantification may be uncertain: the variable position contains a homopolymer.	A homopolymer of height =3 precedes or follows a single-base InDel. The warning indicates the site is very difficult to analyze.

Analysis messages in AQ mode

a) The sequence contains less reference peaks than required	The Pyrogram contains less than 5 constant peaks with an expected value greater than 1.
b) Not analyzable due to lack of data	Insufficient peaks in a site to analyze the data.
c) Deselected by user	The variable site has been deselected from analysis by the user.
d) The variable position contains a homopolymer	A homopolymer of height =3 precedes or follows a single-base InDel. The warning indicates the site is very difficult to analyze.
e) Uncertain due to low signal-to-noise ratio	The sum of the peaks in the variable region is not significantly different from the noise, which may have a negative impact on the result.
f) Failed due to low signal-to-noise ratio	The sum of the peaks in the variable region is not significantly different from the noise, which may have a negative impact on the result.
g) Uncertain due to low peak height	The single peak level at the indicated position is lower than the required peak height for "passed" quality (defined in the assay setup).
h) Failed due to low peak height	The single peak level at the indicated position is below the predefined "failed" quality value.
i) Uncertain due to high sum deviation in variable position	The sum of the peaks in a variable region differs from the expected single peak level at the polymorphic position, exceeding the check limit.
j) Failed due to high sum deviation in variable position	The sum of the peaks in a variable region differs from the expected single peak level at the polymorphic position, exceeding the fail limit.

Message text	Explanation
k) Uncertain due to high pattern deviation in variable position	The best match from possible frequency patterns deviates from the actual pattern, exceeding the check limit.
l) Failed due to high pattern deviation in variable position	The best match from possible frequency patterns deviates from the actual pattern, exceeding the fail limit.
m) Uncertain surrounding reference sequence pattern	The measured peak heights in the quality window deviate from the expected values, exceeding the check limit.
n) Failed surrounding reference sequence pattern	The measured peak heights in the quality window deviate from the expected values, exceeding the fail limit.
o) Uncertain due to high peak height deviation at dispensation	The measured peak height at the specified dispensation deviates from the expected value, exceeding the check limit.
p) Failed due to high peak height deviation at dispensation	The measured peak height at the specified dispensation deviates from the expected value, exceeding the fail limit.
q) Uncertain reference sequence pattern at more than 5 dispensations	The measured peak heights deviate from the expected values at more than 5 dispensations, exceeding the check limit.
r) Failed reference sequence pattern at more than 5 dispensations	The measured peak heights deviate from the expected values at more than 5 dispensations, exceeding the fail limit.
s) Analysis not supported	The variable site is not supported in AQ mode.

Assay setup messages in SNP mode

a) Sequence uncertain due to lack of terminal sequence information	The last nucleotide in the sequence to analyze has been dispensed, which means that the expected peak height of the last dispensation is unknown.
b) Invalid sequence	There is an error in the entered sequence to analyze.

Message text	Explanation
c) Last variable position not analyzable due to lack of terminal sequence information	There is no sequence information entered after the last variable region, which means that the expected peak height of the variable position is unknown.
d) Sequence not in phase at the end of dispensations	The sequence is not in phase after all nucleotides have been dispensed, which means that the expected peak height of the variable position is unknown.
e) Genotyping may be uncertain: the variable position requires more than 5 variable dispensations	The analyzed InDel region consists of more than 5 variable peaks. This may have a negative impact on the result because nucleotide dispensations occur out of phase at a polymorphism for more than 5 dispensations.
f) Genotyping may be uncertain: the variable position consists of more than 5 dispensations	Nucleotide dispensations occur out of phase at a polymorphism for more than 5 dispensations, which may have a negative impact on the result.
g) Genotyping may be uncertain: the variable position contains a homopolymer	A homopolymer of height =3 precedes or follows a single-base InDel. The warning indicates the site is very difficult to analyze.
h) Some genotypes will generate the same sequence pattern and will therefore not be distinguishable	More than one genotype have the same theoretical histogram and therefore cannot be distinguished.
i) The generated dispensation order contains less reference peaks than required	The assay contains less than five constant peaks with an expected value greater than 1.

Analysis messages in SNP mode

a) Failed genotype determination	The difference between the best and the second best genotype match is smaller than the fail limit.

Message text	Explanation
b) Uncertain genotype determination	The difference between the best and the second best genotype match is smaller than the check limit.
c) Analysis not supported	The variable site is not supported in SNP mode.
d) Deselected by user	The variable site has been deselected from analysis by the user.
e) The sequence contains less reference peaks than required	The Pyrogram contains less than 5 constant peaks with an expected value greater than 1.
f) Not analyzable due to lack of data	Insufficient peaks in a site to analyze the data.
g) Not analyzable due to lack of data (overall)	Insufficient peaks in the Pyrogram to analyze the data. This warning overrides all other warnings.
h) The variable position contains a homopolymer	A homopolymer of height =3 precedes or follows a single-base InDel. The warning indicates the site is very difficult to analyze.
i) Uncertain due to low signal-to-noise ratio	The sum of the peaks in the variable region is not significantly different from the noise, which may have a negative impact on the result.
j) Failed due to low signal-to-noise ratio	The sum of the peaks in the variable region is not significantly different from the noise, which may have a negative impact on the result.
k) Uncertain due to low peak height	The single peak level at the indicated position is below the required peak height for “passed” quality (defined in the assay setup).
l) Failed due to low peak height	The single peak level at the indicated position is below the predefined “failed” quality value.
m) Uncertain surrounding reference sequence pattern	The measured peak heights in the quality window deviate from the expected values, exceeding the check limit.

Message text	Explanation
n) Failed surrounding reference sequence pattern	The measured peak heights in the quality window deviate from the expected values, exceeding the fail limit.
o) Uncertain due to high sum deviation in variable position	The sum of the peaks in a variable region differs from the expected single peak level at the polymorphic position, exceeding the check limit.
p) Failed due to high sum deviation in variable position	The sum of the peaks in a variable region differs from the expected single peak level at the polymorphic position, exceeding the fail limit.
q) Uncertain due to high peak height deviation at dispensation	The measured peak height at the specified dispensation deviates from the expected value, exceeding the check limit.
r) Failed due to high peak height deviation at dispensation	The measured peak height at the specified dispensation deviates from the expected value, exceeding the fail limit.
s) Uncertain reference sequence pattern at more than 5 dispensations	The measured peak heights deviate from the expected values at more than 5 dispensations, exceeding the check limit.
t) Failed reference sequence pattern at more than 5 dispensations	The measured peak heights deviate from the expected values at more than 5 dispensations, exceeding the fail limit.
u) Uncertain due to wide peaks	The mean peak width exceeds the check limit. This affects the quality of all sites in the sequence.
v) Uncertain due to baseline drift	Baseline drift exceeds the check limit.
w) High pre-sequencing signal	The signal to noise value of the substrate peak is too high and the signal to single peak level exceeds the check limit.

Message text	Explanation
x) No peaks detected between dispensations	No peaks are detected after all nucleotides have been dispensed at a variable site. This affects the quality of all sites after the error.
y) Failed due to possible dispensation error at dispensation	A dispensation error has possibly occurred at the specified dispensation(s). This affects the quality of all sites after the error.
z) Some genotypes (G/T, G/T and G/G, T/T) generate the same sequence pattern and are not distinguishable	More than one genotype have the same theoretical histogram and therefore cannot be distinguished (only check quality is given). Note: the genotypes given in the warning message are specific to the affected assay.

Assay setup messages in CpG mode

a) Invalid sequence	There is an error in the entered sequence to analyze.
b) Cannot resolve sequence direction	The entered sequence to analyze includes nucleotides that are specific for either forward or reverse bisulfite-treated sequences.
c) A CpG site has to be biallelic	Occurs if the indicated CpG site includes an additional polymorphism; e.g., C/T/AG.
d) Variable positions with common dispensations cannot be analyzed	Nucleotide dispensations for several polymorphisms are out-of-phase. A variable region evaluated with nucleotide dispensations that apply to more than one polymorphism (common dispensation) cannot be analyzed. For example, the sequence to analyze A/GC/TCAC with the dispensation order CATCGTCA will not be analyzed, as the dispensations will be out-of-phase.
e) The generated dispensation order contains less reference peaks than required	The assay contains less than 5 constant peaks with an expected value greater than 1.

Message text	Explanation
f) Sequence uncertain due to lack of terminal sequence information	The last nucleotide in the sequence to analyze has been dispensed, which means that the expected peak height of the last dispensation is unknown.
g) Last variable position not analyzable due to lack of terminal sequence information	There is no sequence information entered after the last variable region, which means that the expected peak height of the variable position is unknown.
h) Sequence not in phase at the end of dispensations	The sequence is not in phase after all nucleotides have been dispensed, which means that the expected peak height of the variable position is unknown.
i) Quantification may be uncertain: the variable position consists of more than 5 dispensations	Nucleotide dispensations occur out of phase at a polymorphism for more than 5 dispensations, which may have a negative impact on the result.

Analysis messages in CpG mode

a) Analysis not supported	The variable site is not supported in CpG mode.
b) Deselected by user	The variable site has been deselected from analysis by the user.
c) Uncertain bisulfite conversion at dispensation	The peak for the bisulfite control at the indicated dispensation(s) is higher than the check limit. Affects the quality of all sites in the sequence.
d) Failed bisulfite conversion at dispensation	The peak for the bisulfite control at the indicated dispensation is higher than the fail limit. Affects the quality of all sites in the sequence.
e) The sequence contains less reference peaks than required	The Pyrogram contains less than 5 constant peaks with an expected value greater than 1.
f) Not analyzable due to lack of data	Insufficient peaks in a site to analyze the data.

Message text	Explanation
g) Uncertain due to low signal-to-noise ratio	The sum of the peaks in the variable region is not significantly different from the noise (below the check limit), which may have a negative impact on the result.
h) Failed due to low signal-to-noise ratio	The sum of the peaks in the variable region is not significantly different from the noise (below the fail limit), which may have a negative impact on the result.
i) Uncertain due to low peak height	The single peak level at the indicated position is below the required peak height for "passed" quality (defined in the assay setup).
j) Failed due to low peak height	The single peak level at the indicated position is below the predefined "failed" quality value.
k) Uncertain due to high sum deviation in variable position	The sum of the peaks in a variable region differs from the expected single peak level at the polymorphic position, and the difference exceeds the check limit.
l) Failed due to high sum deviation in variable position	The sum of the peaks in a variable region differs from the expected single peak level at the polymorphic position, and the difference exceeds the fail limit.
m) Uncertain due to high pattern deviation in variable position	The best match from possible frequency patterns deviates from the actual pattern, exceeding the check limit.
n) Failed due to high pattern deviation in variable position	The best match from possible frequency patterns deviates from the actual pattern, exceeding the fail limit.
o) Uncertain surrounding reference sequence pattern	The measured peak heights in the quality window deviate from the expected values, exceeding the check limit.
p) Failed surrounding reference sequence pattern	The measured peak heights in the quality window deviate from the expected values, exceeding the fail limit.

Message text	Explanation
q) Uncertain due to high peak height deviation at dispensation	The measured peak height at the specified dispensation deviates from the expected value, exceeding the check limit.
r) Failed due to high peak height deviation at dispensation	The measured peak height at the specified dispensation deviates from the expected value, exceeding the fail limit.
s) Uncertain reference sequence pattern at more than 5 dispensations	The measured peak heights deviate from the expected values at more than 5 dispensations, exceeding the check limit.
t) Failed reference sequence pattern at more than 5 dispensations	The measured peak heights deviate from the expected values at more than 5 dispensations, exceeding the fail limit.
Messages in SQA mode	
a) Base-calling not consistent with entered known bases	User-defined normalization peaks have been entered and the resulting base-calling is inconsistent with the information entered.
b) Low peak height	The single peak level at the beginning of the Pyrogram is lower than the required peak height for “passed” or “checked” quality (defined in the assay setup).
c) High homopolymer at dispensation	A homopolymer consisting of more than 5 nucleotides at the specified dispensation(s).
d) Large peak height variation	Large overall peak height variation. Gives uncertain/failed start quality if detected within the first 5 dispensations.
e) Large peak height variation around dispensation	Large peak height variation within single- or double-peak levels. Specifies the dispensations for which this warning is valid and gives uncertain/ failed quality to sequence from that dispensation on.

Message text	Explanation
f) Low signal-to-noise ratio (overall)	Low signal-to-noise ratio. Gives uncertain/failed start quality if detected within the first 5 dispensations.
g) Low signal-to-noise ratio from dispensation	Low signal-to-noise ratio. Gives uncertain/failed quality to sequence from that dispensation on.
h) Missing peaks in cycle starting at dispensation	No peaks are detected even though all nucleotides have been dispensed, which may indicate a problem with the dispensation unit. Gives uncertain quality starting at the last peak detected.
i) Peak height deviates from the expected peak level at dispensation	The measured peak height at the specified dispensation deviates excessively from the expected value. Affects the quality color of the indicated dispensation.
j) Risk for overlaid sequence from dispensation	A possible background sequence is detected in a certain area. Affects the quality color from the beginning of that area.
k) Spurious peak(s) at dispensation	Unclassified peaks over a certain value are detected. Affects the quality color starting at the specified dispensation.
l) Wide peaks from dispensation	Wide peaks detected. Gives uncertain quality starting at the dispensation where it was detected. If present within the first 5 dispensations results in an uncertain start quality.

References

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