September 2012

PyroMark Q24 Advanced Software User Guide

For use with the PyroMark Q24 Advanced System

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Contents

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Use PyroMark Q24 Advanced Software only with the PyroMark Q24 Advanced System.

Product Warranty and Satisfaction Guarantee

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Introduction

About this user guide

This user guide provides information about the functions and features of PyroMark Q24 Advanced Software. Please refer to the *PyroMark Q24 Advanced User Manual* for complete information about the proper care, maintenance, and use of the PyroMark Q24 Advanced Instrument and PyroMark Q24 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 Q24 Advanced Software in the following sections:

- Introduction
- Installation of the analysis software
- PyroMark Q24 Advanced Software
- Start the software
- Set up an AQ, SNP, or CpG assay
- Set up an SEQ assay
- Set up a run
- Process the run on the PyroMark Q24 Advanced Instrument
- Analyze the run
- View, print, and save analysis reports
- Manage instrument methods
- General hints and tips
- Troubleshooting guide

Installation of the Analysis Software

Minimum specifications

The computer used for data analysis should have the following as minimum specifications:

- Windows® 7 32 bit (English version) Operating System
- Pentium® IV processor, 3 GHz or higher
- 1 GB of RAM
- 100 MB free hard drive capacity
- Display resolution of 1280 x 800 pixels, True Color (32 bit)
- USB port
- CD-ROM
- Pointer device (mouse or similar)

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.

Install or upgrade PyroMark Q24 Advanced Software

To install or upgrade PyroMark Q24 Advanced Software:

1. **Ensure that the computer meets the minimum requirements (see Minimum specifications).**
2. **Close any programs running on the computer.**
3. **Insert the PyroMark Q24 Advanced Software CD into the CD-drive.**
4. **In the CD menu, click "Install PyroMark Q24 Advanced Software".**
5. **If the CD menu does not appear automatically, select "(My) Computer" in the Windows "Start" menu. Right-click the CD-drive with the software CD and select "Explore". Double-click the file "autorun.exe".**
6. **Follow the instructions that appear in the "Setup Wizard".**

Note: You may be prompted by the "Setup Wizard" to install the .NET Framework 4.0. If so, restart installation of the PyroMark Q24 Advanced Software after the .NET Framework installation has been completed (i.e., open the CD menu and click "Install PyroMark Q24 Advanced Software").

7. **When the software has been successfully installed, click "Exit Setup" in the CD menu.**
8. **Please use Windows Update (www.update.microsoft.com) to check for any critical updates to the .NET Framework 4.0.**

Note: If the computer is connected to a network, network policy settings may prevent you from completing this procedure. For more information, contact your system administrator.

Uninstall PyroMark Q24 Advanced Software

To uninstall PyroMark Q24 Advanced Software:

1. **Select "Control Panel" in the Windows "Start" menu.**
2. **Click "Add or Remove Programs" (XP) or "Uninstall a program" under the Programs category (Vista).**
3. **In the list of programs, select "PyroMark Q24".**
4. **Click "Remove" (XP) or "Uninstall" (Vista).**
5. **Repeat steps 2–4 for PyroMark Launcher.**

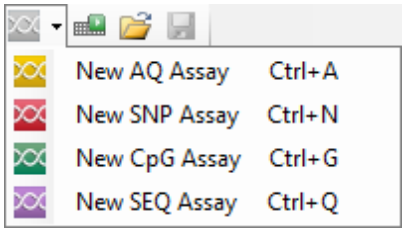
PyroMark Q24 Advanced Software

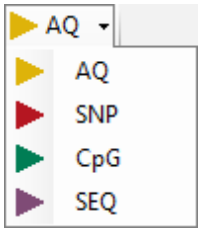
The PyroMark Q24 Advanced System is a complete solution comprising instrument, vacuum workstation, chemistry, and software.

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 at CpN sites
- 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





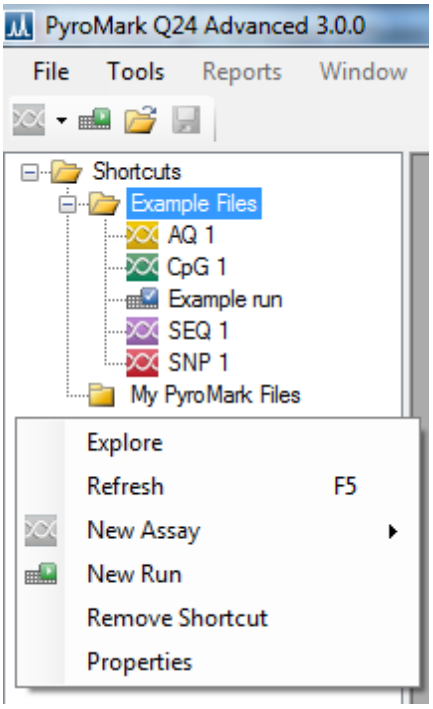
PyroMark Q24 Advanced Software has four analysis modes:

- AQ: A variety of quantification studies of mutations such as SNPs and InDels
- SNP: Genotype analysis of SNPs and InDels. This mode does not provide quantification of alleles as in AQ mode.
- CpG: Methylation analysis of single or multiple CpG sites. For methylation analysis at CpN sites, a CpG assay is used with CpN mode enabled during the assay setup.
- SEQ: Base-calling of unknown sequences

The four different types of analysis can be performed on the same PyroMark Q24 Plate. To toggle between the analysis modes in the “Analysis“ view, select “AQ”, “SNP”, “CpG”, or “SEQ” 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
- SEQ assay file
- A run file that has not been processed
- A 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, or by the pressing the "F5" key.
- 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 SEQ 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.

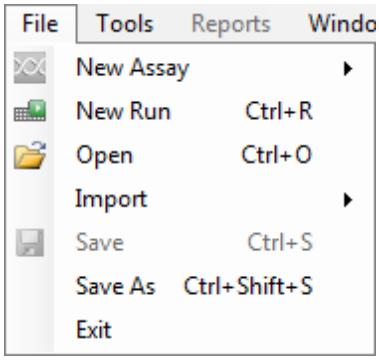
  Copy a processed run file and rerun it by right-clicking the run file and selecting "Copy and Rerun" from the context menu.

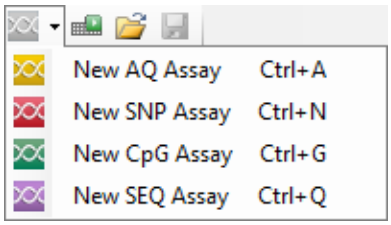
  Note: Only the run setup, not the run and analysis data, will be copied.
- Copy a file by right-clicking the folder containing the file and selecting "Explore" from the context menu. Windows Explorer opens. For more information, press the "F1" key to open the online help for Windows Explorer.

  Note: To avoid losing data, do not copy a file that is open in PyroMark Q24 Advanced 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 "SEQ").
- 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.

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 SEQ 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.

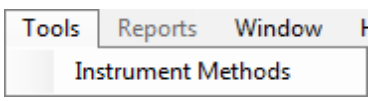
Select "Create New AQ/SNP/CpG Assay from Assay Design File" from the "Import" submenu to create a new AQ, SNP, or CpG assay based on an assay file (***.xml**) created with PyroMark Assay Design Software. The software will import the sequence to analyze and the names of the variable positions.

Select "Save" or click in the toolbar to save 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.

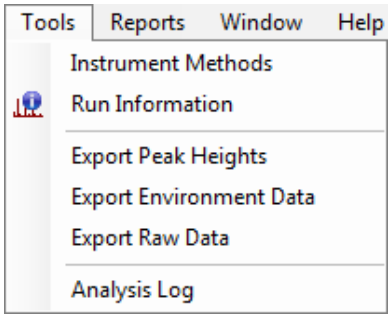
Select "Exit" to shut down the software.

Tools menu for unprocessed run files



Select "Instrument Methods" to view the settings for the instrument methods and, if necessary, import new instrument method files according to settings supplied by QIAGEN (see Manage Instrument Methods).

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.

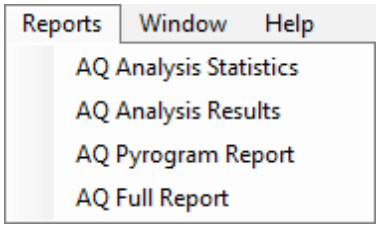
Select "Export Raw Data" to save the intensities and dispensation data as a text file.

Select "Export As FASTA" to save base-called sequences in FASTA format (SEQ 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 SEQ), 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.

Reports menu for:
AQ runs



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

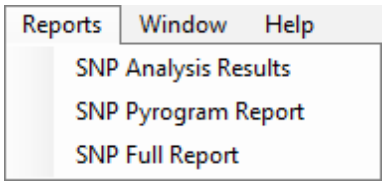
The "Analysis Results" report includes well information and analysis results for all or selected wells.

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

SNP runs

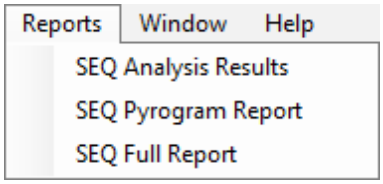
all or selected wells.



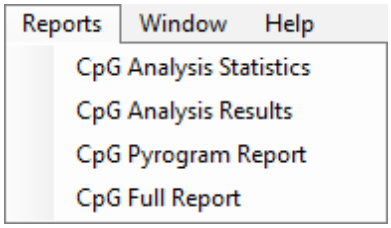
The report options are only available for processed runs. For more information on the reports, see View, Print, and Save Analysis Reports.

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.

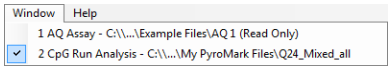
SEQ runs



CpG runs

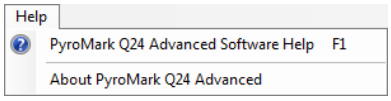


Window menu



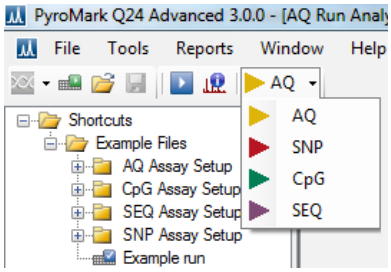
Toggle between open files in the software using the "Window" menu.

Help menu



Select "PyroMark Q24 Advanced Software Help" or press the "F1" key to open this user guide.

Analysis toolbar



Click to 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 "SEQ" in the toolbar to toggle between the analysis modes.

Workflow views

PyroMark Q24 Advanced Software is organized into views that reflect the Pyrosequencing workflow: Assay Setup, Run Setup, and Analysis. The active view is indicated in the status bar at the top of the window.

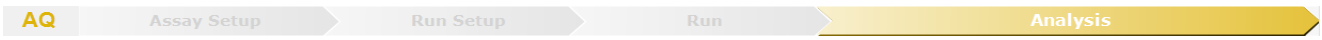
Assay Setup view

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 method, 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.

Analysis view

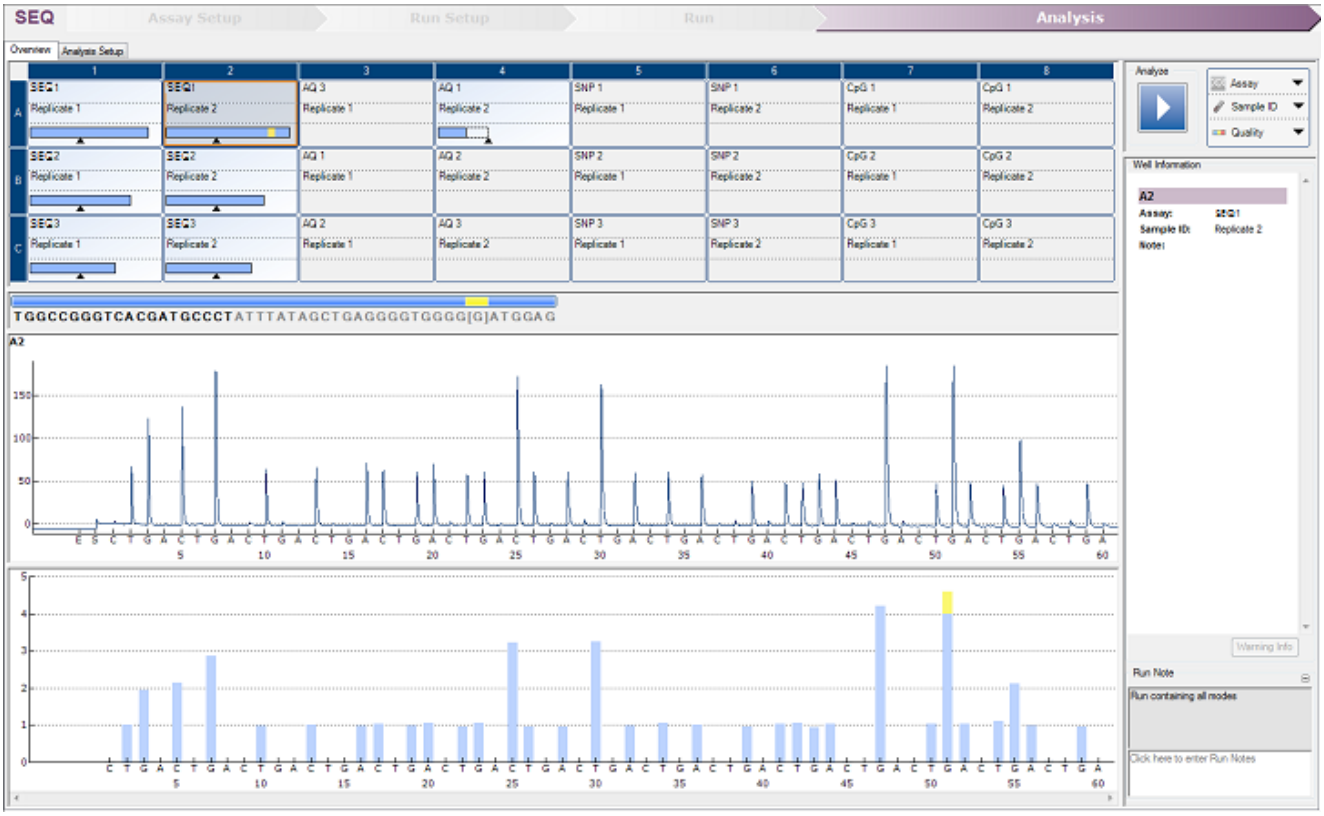


When a run is completed on the PyroMark Q24 Advanced, the "Analysis" view opens. Alternatively, this view becomes active when any processed run file is opened. The color of the workflow arrow "Analysis" in the status bar reflects the selected analysis mode. This view is used to manage the analysis of 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.

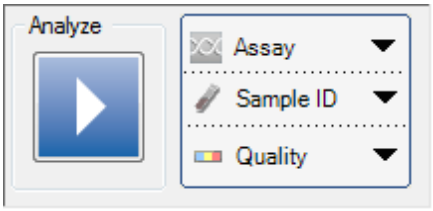
Overview tab

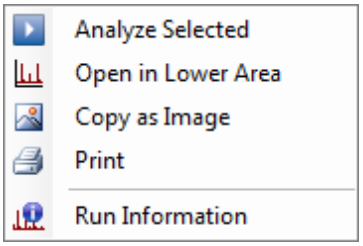
The "Overview" tab in the "Analysis" view displays the plate overview with well-specific information. Directly below, in the "Pyrogram" pane, is the Pyrogram of the well selected in the plate overview. The corresponding histogram is displayed beneath the Pyrogram. To the right is the "Well Information"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



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 SEQ 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).

Above the "Well Information" pane are dropdown menus to specify the type of information to be displayed for each well, and an easy access button to initiate analysis of selected wells.



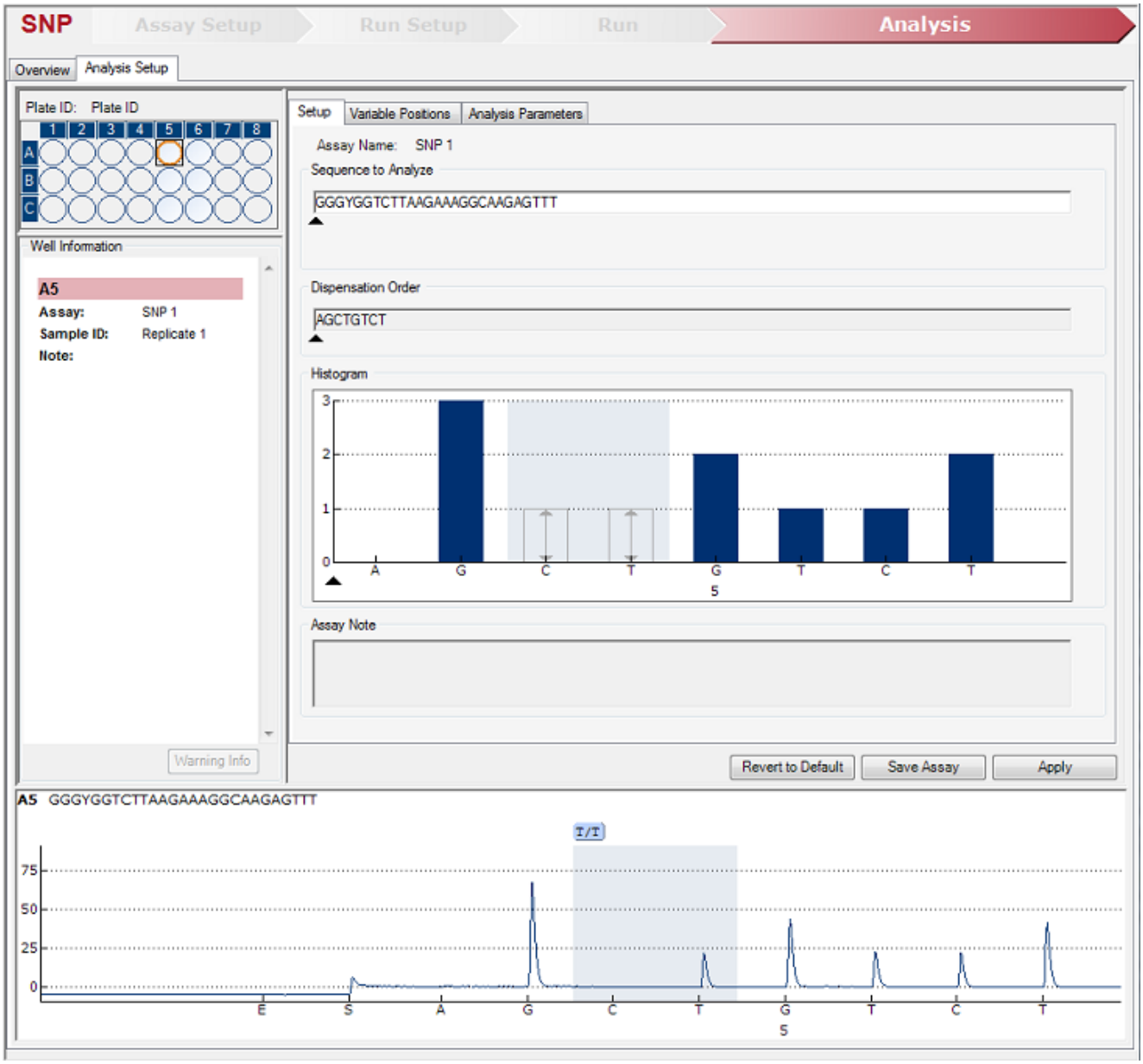


Right-clicking on the plate overview in this tab opens a context menu with the following options:

- Analyze Selected: initiates analysis of all selected wells
- Open in Lower Area: replaces the histogram with one or more Pyrograms of 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)

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.

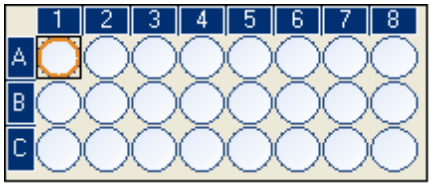
Details about the assay of a selected well are visible in the large main pane, including

assay name, sequence to analyze, nucleotide dispensation order, histogram, and notes entered during assay setup. In addition, three tabs display the analysis setup, variable positions analyzed, and the analysis parameters used.

Select wells

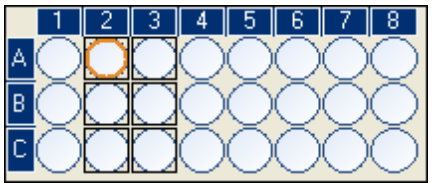
The following methods for selecting wells can be used in the plate overview of both the "Overview" and the "Analysis Setup" tabs. The color schematic for wells also applies to both tabs.

To select a single well, simply click on it.

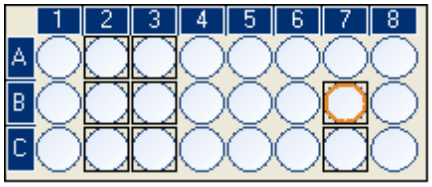


To select a rectangular group of wells, for example A2–A3, B2–B3, and C2–C3:

- Press and hold down the left mouse button while dragging the mouse pointer from well A2 to C3, or
- Select well A2 and press and hold down the "Shift" key while selecting well C3, or
- Select well A2 and press and hold down the "Shift" key while pressing the "Right Arrow" key twice and the "Down Arrow" key twice.



To add wells to the selection above, for example wells B7 and C7, press and hold down the "Ctrl" key while selecting the wells.



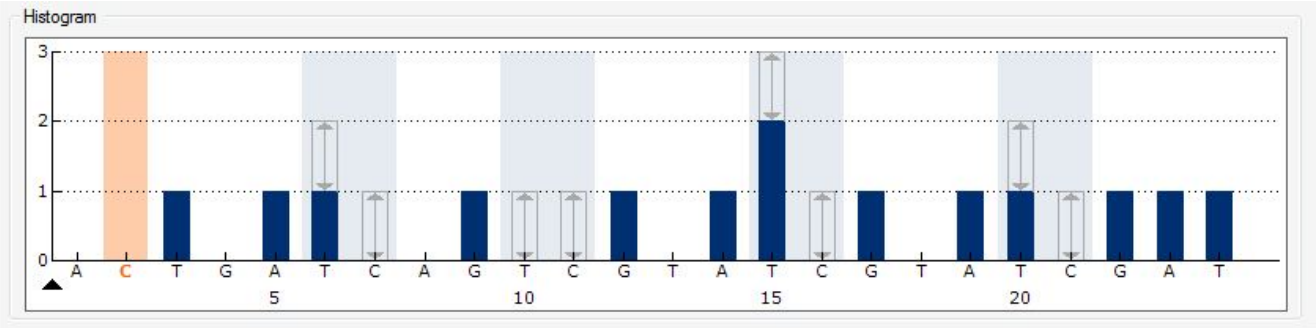
To deselect a well, press and hold down the "Ctrl" key while selecting the well.

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 or more variable positions) are highlighted with a blue-gray background color
- When showing reference peaks, blue diamonds are displayed above the reference peaks. A filled blue diamond indicates the reference peak is enabled, and a hollow blue diamond indicates the reference peak is disabled. Reference peaks may be disabled or disabled by left-clicking the peak.
- The bisulfite treatment control is highlighted with a orange background color, and the base is displayed in orange. 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

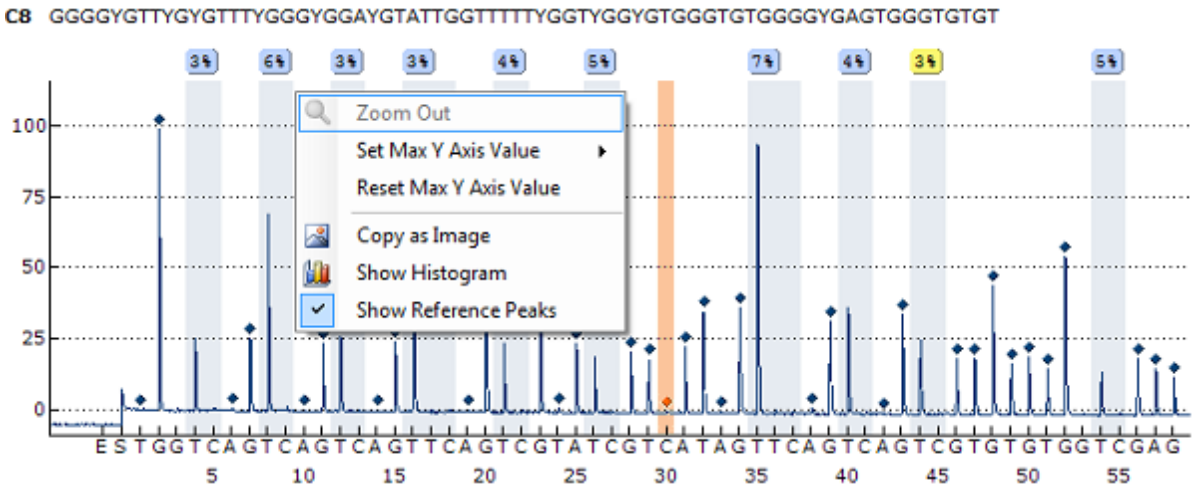
The histogram can be copied as an image to the clipboard by right-clicking the histogram and selecting "Copy as Image" from the context menu. The image can be

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



Pyrogram showing a CpG assay result.

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

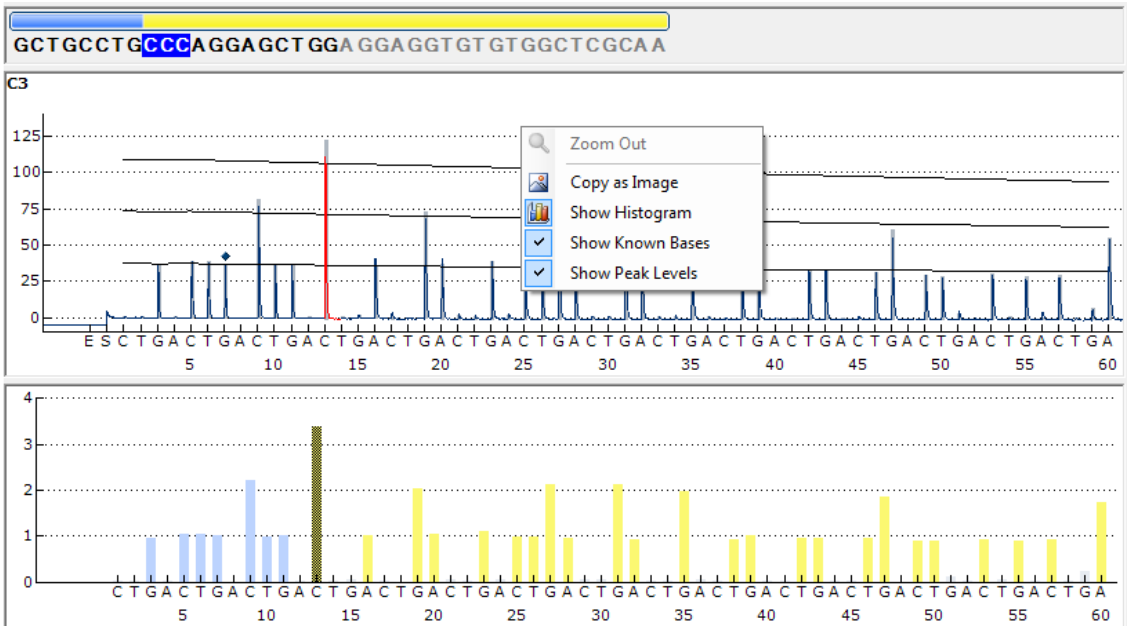
- The well name and the sequence to analyze are shown in the upper left corner.
- The analysis result (allele frequencies in AQ mode A: 4% T: 96%, genotype in SNP mode T/T, or methylation percentage 23% or average methylation percentage Average: 25% in CpG mode or CpN mode) is displayed above each variable 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%.

  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. A filled blue diamond indicates the reference peak is enabled, and a hollow blue diamond indicates the reference peak is disabled.
- Bisulfite treatment controls are highlighted with a light orange 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.
- The Pyrogram Y axis scale may be manually adjusted by right-clicking on it and selecting "Set Max Y Axis Value". The value is limited to three digits and must be confirmed by pressing the enter button or by clicking the mouse button.

SEQ 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 SEQ 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%).

The Pyrogram Y axis scale may also be manually adjusted by right-clicking on it and selecting "Set Max Y Axis Value". The value is limited to three digits and must be confirmed by pressing the enter button or by clicking the mouse button.

Export Pyrogram as an image

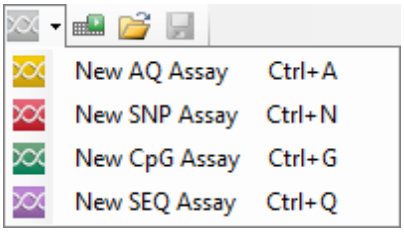
The Pyrogram can be copied as an image to the clipboard by right-clicking the Pyrogram area and selecting "Copy as Image" from the context menu. The image can be pasted into applications that support Enhanced Metafile (EMF) images.

Start the Software

In the Windows "Start" menu, select "(All) Programs/PyroMark/PyroMark Q24 Advanced". The *PyroMark Q24 Advanced Software User Guide* (this publication) can be accessed at any time by pressing the "F1" key when in the software.

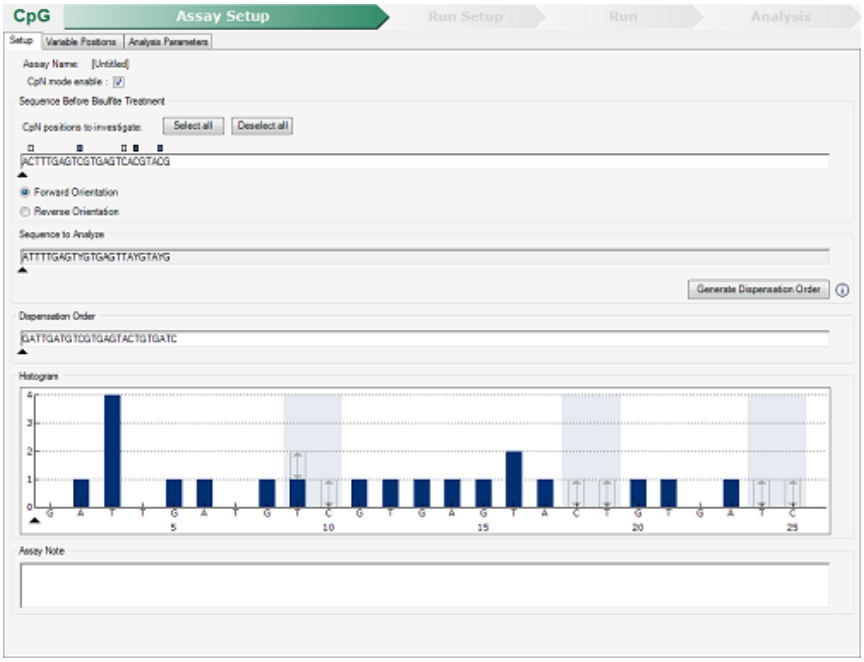
Set Up an AQ, SNP, or CpG Assay

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. For analyzing methylation at CpN sites, create a "New CpG Assay" and then enable the CpN mode by checking the box next to "CpN mode enabled".**

   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. Note: If creating a CpG assay, we recommend entering the "Sequence Before Bisulfite Treatment" (see Enter the sequence before bisulfite treatment). This enables the software to automatically generate the sequence to analyze and select the most appropriate bisulfite treatment control.**

   Note: When CpN mode is enabled, each CpN position in the sequence before bisulfite treatment is indicated by a small check box above the position. The user should select which position(s) to analyze by individually clicking the appropriate check boxes, or by clicking "Select all" or "Deselect all". Selected boxes are indicated by blue color and deselected boxes are indicated by white color. All of the selected CpN positions will be considered during the analysis as long as they are not deselected in the "Variable Positions" tab.

3. **Click the "Generate Dispensation Order" button (see Generate the dispensation order).**
4. **If creating a CpG assay, check that the software has selected a bisulfite treatment control. If no bisulfite treatment control has been automatically selected, add one manually, preferably at the beginning of the sequence (see Add or remove bisulfite treatment controls).**
5. **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.

6. **Optional: Set up the variable positions in the "Variable Positions" tab.**
7. **Before running your samples, validate your assay using a reference DNA sample (see Appendix B of the *PyroMark Q24 Advanced User Manual*).**
8. **Optional: If applicable, during the assay validation, edit the analysis parameters.**
9. **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 Q24 Advanced Instrument cannot be unlocked (i.e., it will not be possible to edit the analysis parameters or results after the assay has been processed).**
10. **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

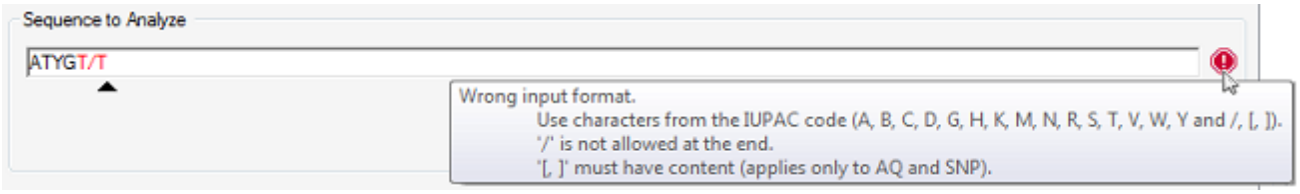
Type or paste the sequence to analyze into the "Sequence to Analyze" text box. If creating a CpG assay, we recommend entering the sequence before bisulfite treatment, if it is known (see **Enter the sequence before bisulfite treatment)**.

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 to Analyze" 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 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.

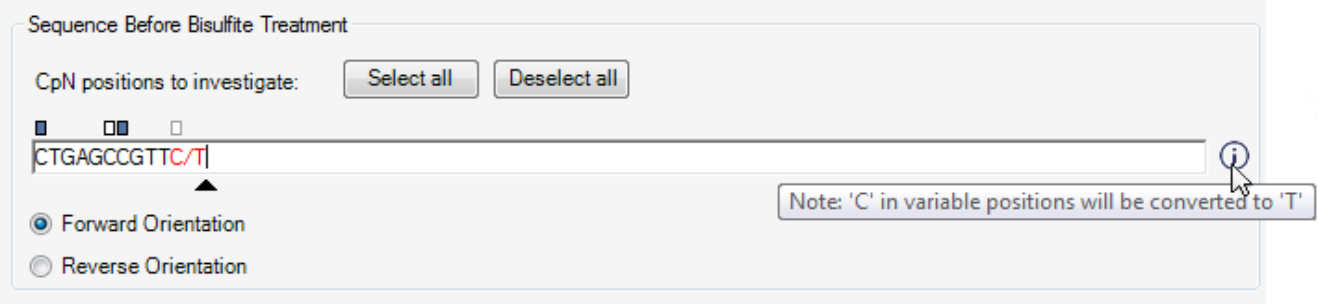
Enter the sequence before bisulfite treatment

If creating a CpG assay, we recommend entering the sequence before bisulfite treatment into the "Sequence Before Bisulfite Treatment" text box. This enables the software to automatically generate the sequence to analyze and select the most appropriate bisulfite treatment control.

The following rules apply when entering the DNA "Sequence Before Bisulfite Treatment" in the software:

- The allowed characters for sequence input are A, C, G, and T as well as IUPAC codes
- Variable positions such as SNPs can be entered using either IUPAC codes or a forward slash (/) as a separator between the two potential bases (e.g., C/G)
- The sequence should not include more than 400 characters or 100 variable positions
- InDels are not supported

If the software detects inconsistencies in the sequence before bisulfite treatment, the user is informed by an information symbol (ⓘ)next to the "Sequence Before Bisulfite Treatment" text box. The warning text is shown in a tooltip when moving the mouse over the symbol.



If the sequence before bisulfite treatment is edited such that it becomes invalid, the check boxes along with the "Select all" and "Deselect all" buttons will be grayed out, indicating that they are disabled and no longer selectable. The check boxes and buttons become enabled again once the errors in the "Sequence Before Bisulfite Treatment" are corrected.

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 sites, CpN sites, and SNPs can be included in a forward assay:

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

The following CpG sites, CpN sites, and SNPs can be included in a reverse assay:

- CpG site: CG/A
- CpN site: AG/A, CG/A, GG/A, and TG/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

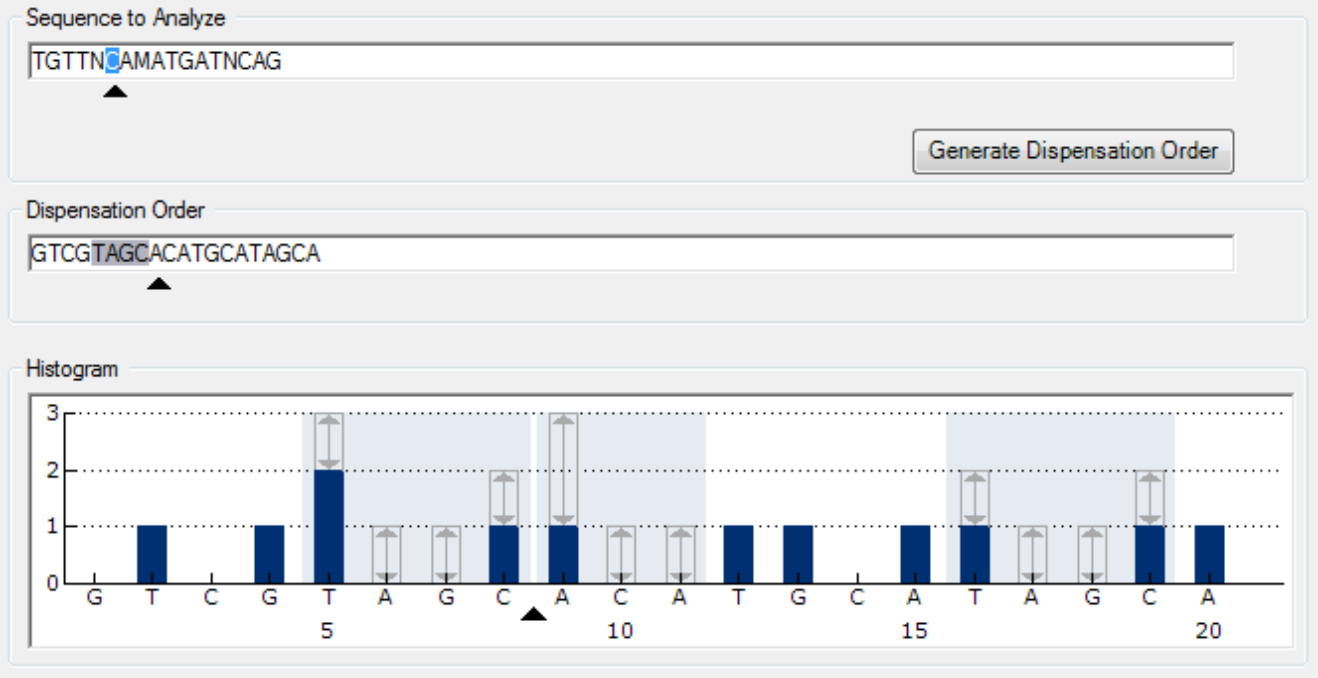
A dispensation order for the entered sequence to analyze is generated by the software by clicking the "Generate Dispensation Order" button. The generated dispensation order includes blank dispensations that are used as negative controls.

When creating CpG assays, the dispensation order should also include bisulfite treatment controls. If the user enters the sequence before bisulfite treatment, the sequence to analyze will be automatically generated, and one bisulfite control will be automatically selected, if possible. If the user directly enters the sequence to analyze, the bisulfite control will need 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.

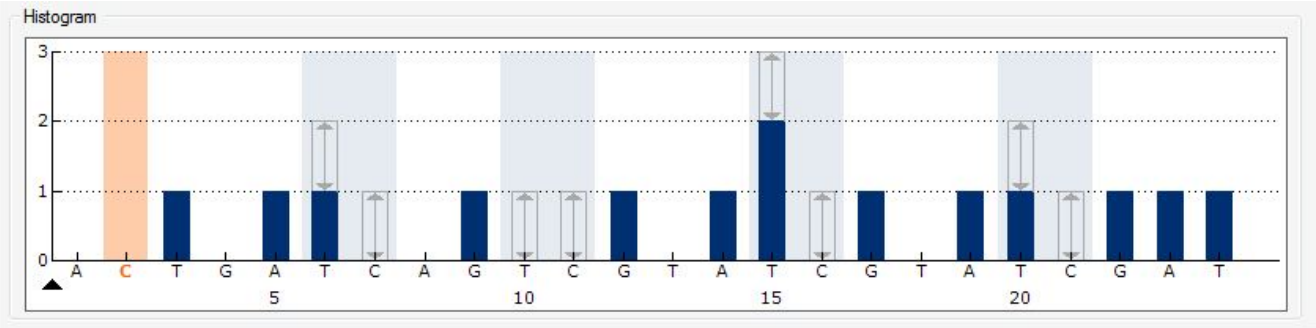
Note: If it is not possible for the sequence to come in phase before 32 alleles are dispensed, the dispensation order will not be completed. For example, the sequence ACTCDDDDG will have the dispensation order ACTC, since the four D polymorphisms will generate an out-of-phase stretch over too many alleles.

Dispensation warnings

If the dispensation order contains a warning, this is displayed by an information symbol ⓘ 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 an 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.

We recommend directly entering the sequence before bisulfite treatment, because this enables the software to generate the sequence to analyze and automatically select one appropriate bisulfite treatment control. If the user directly enters the sequence to analyze, or if the software does not find a suitable bisulfite control, one bisulfite control will need to be added manually, by the user, after the dispensation order has been generated.

A bisulfite treatment control can be added manually by adding a C before or after a T in a forward assay, or by adding a G before or after an A in a reverse assay, in the dispensation order. This is only possible if it's known that in the sequence before bisulfite treatment, the suggested bisulfite treatment controls are Cs converted to Ts (read as Gs and As in a reverse assay).

Only one bisulfite control may be included. If additional controls are manually added in the dispensation order, they will be displayed as red Cs in the histogram.

Note: For CpG assays with CpN mode enabled, if the sequence before bisulfite treatment is entered, the software will use an unselected CpN site as a bisulfite treatment control.

Set up the variable positions

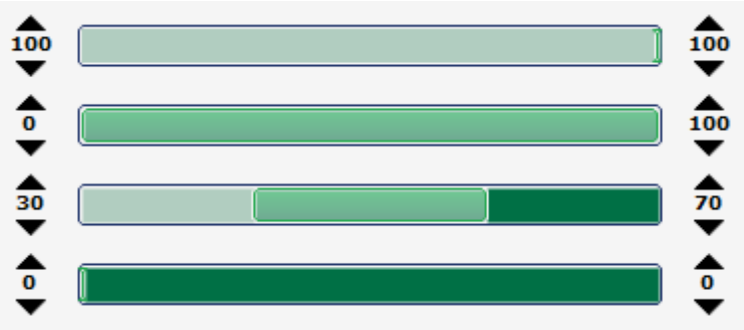
The variable positions can be set up in the "Variable Positions" tab. The available parameters are listed below.

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, CpG, or CpN 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 or CpN methylation. Setting this parameter for all the CpG or CpN sites allows easy identification of sites (in the analysis results) that are outside the user-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 Q24 Advanced 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 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 20.

	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 10. **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.

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, we recommend that the reference peaks 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.

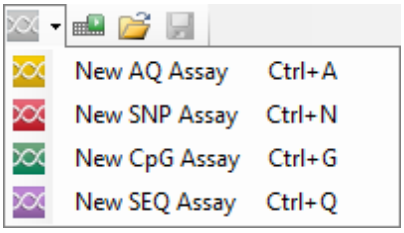
Adjust heights of histogram bars

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".**

Set Up an SEQ Assay

Workflow to set up an SEQ Assay



1. **Click in the toolbar and select "New SEQ 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 SEQ 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.

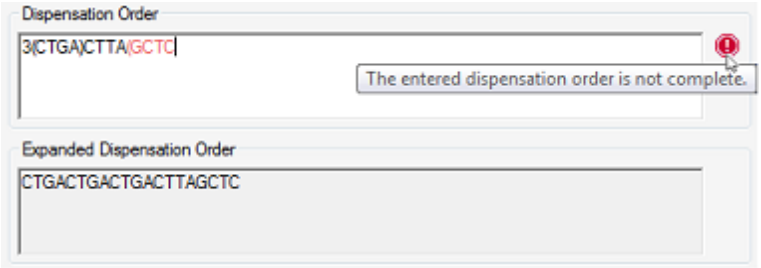
4. **Before running your samples, validate your assay using a reference DNA sample (see Appendix B of the *PyroMark Q24 Advanced User Manual*).**
5. **Optional: If applicable, during the assay validation, edit the analysis parameters.**
6. **Optional: Lock the assay for editing by clicking the "Lock Assay" button at the bottom of the assay setup window. A locked assay ( ) that has been run on the PyroMark Q24 Advanced 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 parentheses, 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 Q24 Advanced 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 4. **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 2. **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.

Known bases

If there are any known bases in the dispensation order, we recommend 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 Methods" drop-down list, select the instrument methods file that corresponds to the PyroMark Q24 Cartridge to be used. To import new instrument methods files, see Manage instrument methods.**
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 Q24 Advanced 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 methods	Select an instrument methods file according to the reagents and cartridge that will be used for the run (see Manage instrument methods). **Note**: We recommend that only instrument method settings supplied by QIAGEN are used.
Plate ID	**Optional**: Enter ID of the PyroMark Q24 Plate. **Note**: If you position the mouse pointer over a run file in the shortcut browser, a tooltip displays the entered plate ID.
Bar code	**Optional**: Enter a bar code number for the plate or, if you have a bar code reader connected to your computer, place the mouse cursor in the "Bar code" text box and scan the bar code.
Reagent lot number	**Optional**: Enter the lot number for the PyroMark Gold Q24 Reagents to be used. The lot number can be found on the product label. **Note**: We recommend entering the reagent ID so that any unexpected problems with the reagents can be traced.
Estimated run time	The estimated run time. Calculated by the software if instrument methods 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

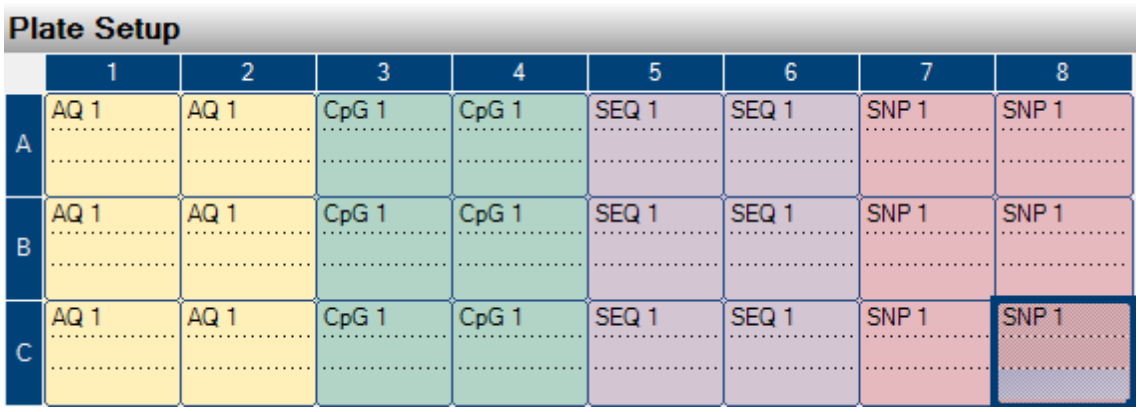
To add assays to wells, you can either:

- 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 "Ctrl" 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: It is not possible to add assays created with PyroMark Q24 Software or PyroMark Q96 Software.

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



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 SEQ assays are purple. Different shades of each color indicate wells with different assays of the same type.

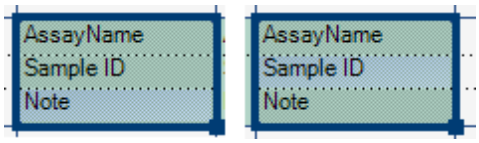
Copy or delete contents from cells

- 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"

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.



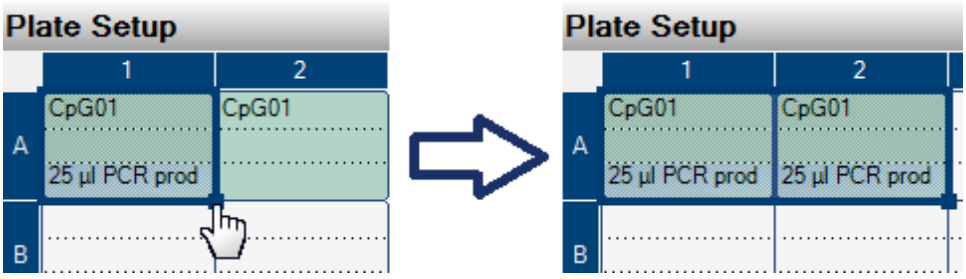
A selected cell is highlighted with a blue background color.

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.

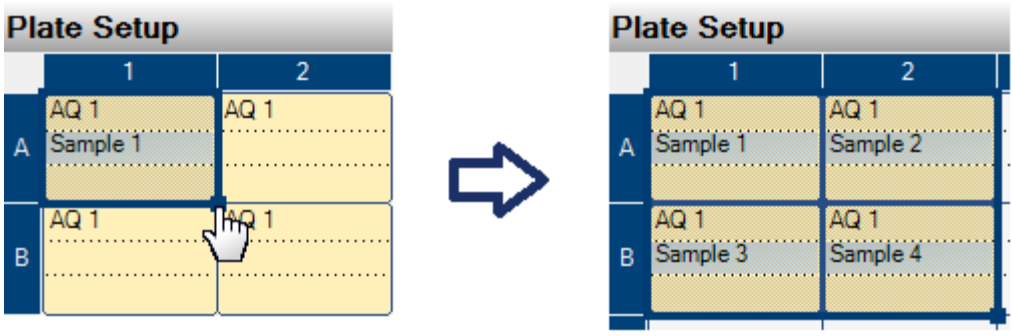
   First, release the left mouse button, then the "Ctrl" key. When the left mouse button is released, the sample ID of the first selected cell is incremented and pasted into the selected cells.

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

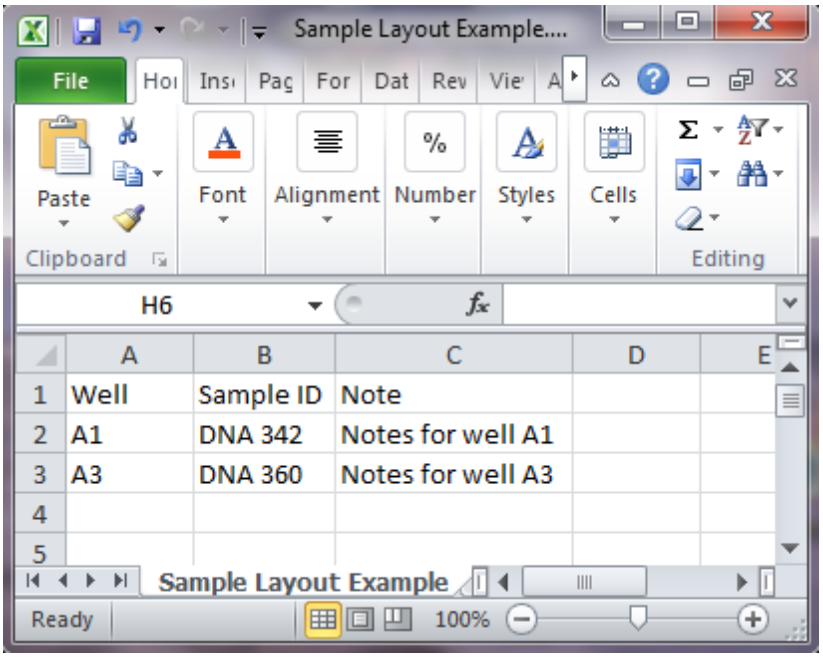
By using the "Import/Insert Sample Layout File" or "Paste Sample Layout" feature, you can easily use the same layout in several runs and reuse information available in existing documentation.

Using the import/insert sample layout file feature

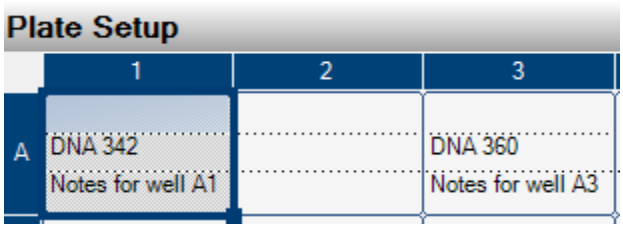
You can, for example, generate layout files from your Laboratory Information Management Systems (LIMS). Sample and note layout files can also be created in Microsoft Excel, Notepad, and similar applications. The layout file must have two or three columns: "Well", "Sample ID", and "Note" (optional). Each 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.

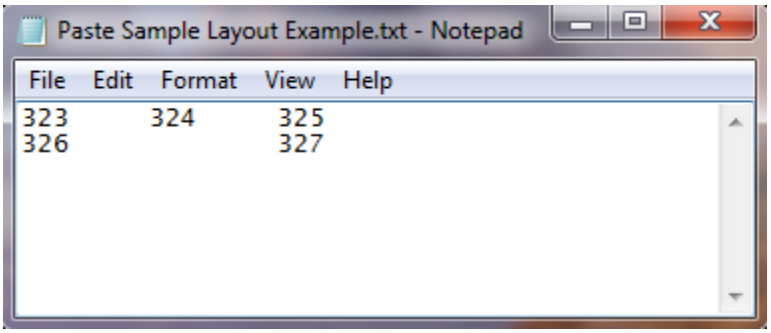
Using the paste sample layout feature

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.

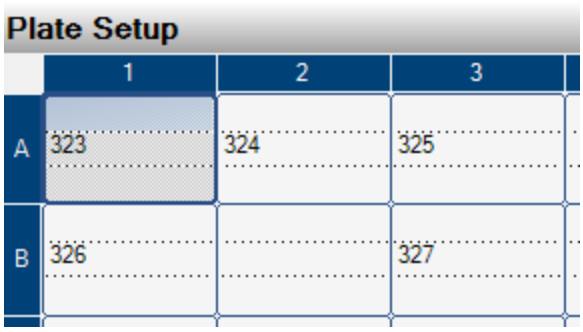
To paste a sample layout into an existing run file:

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 SEQ)
- 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

When a run is set up and ready to run on the PyroMark Q24 Instrument, perform the following steps:

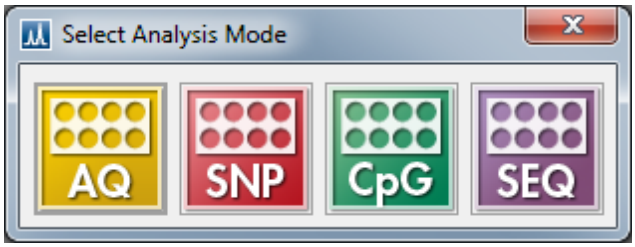
1. **Prepare your samples.**
2. **Fill the PyroMark Q24 Cartridge with the required volumes of reagents.**
3. **Load the reagent cartridge and PyroMark Q24 Plate into the instrument.**
4. **Insert the USB stick containing the run file into the USB port at the front of the instrument.**
5. **Select the run file and start the run.**
6. **When the run has been completed and data transferred to the USB stick, remove the USB stick.**
7. **Unload the plate and the reagent cartridge.**

For more information, refer to the *PyroMark Q24 Advanced User Manual*.

Analyze a Run

Workflow to analyze a run

1. **Move the processed run file from the USB stick to a computer running PyroMark Q24 Advanced Software; insert the USB stick into the computer's USB port and move the run file to the desired location using Windows Explorer.**
2. **Open the run file in PyroMark Q24 Advanced Software either by selecting "Open" in the "File" menu or double-clicking the file ( ) in the shortcut browser. If several assay types are included, select the analysis mode in the dialog box that opens. The analysis modes displayed in the dialog box are limited to the assay types on the plate.**



Note: To update the contents of a folder in the shortcut browser, right-click it and select "Refresh" from the context menu, or press the "F5" key.

Note: It is also possible to open the run file by double-clicking it in Windows Explorer.

3. **All wells on the plate with an Assay Setup corresponding to the selected analysis mode will be automatically analyzed.**
4. **View the analysis results.**
5. **Optional: If applicable, modify how the analysis is performed (see Edit analysis parameters).**
6. **Optional: Enter an analysis note in the "Note" text box in the "Overview" tab.**

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

7. **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 selected wells

Analysis of selected wells can be initiated by clicking the "Analyze Selected Wells" button in the "Overview" tab:

Analyze the selected wells (see Select wells).

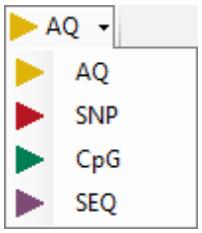
Note: It is also possible to right-click the selection and select "Analyze Selected" from the context menu.

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 Q24 Advanced Software has four analysis modes: AQ, SNP, CpG, and SEQ. To toggle between the modes, select "AQ", "SNP", "CpG", or "SEQ" 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. **After selecting the analysis mode, all wells with an Assay Setup corresponding to the analysis mode will be automatically analyzed, and the run will be automatically saved in the background after the analysis is complete.**

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

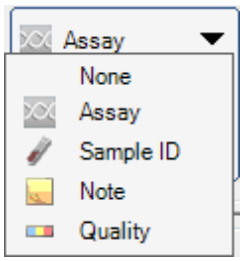
View the analysis results

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:

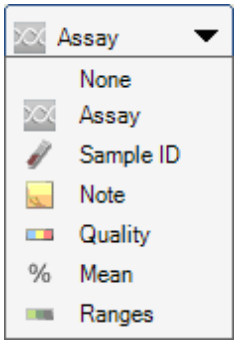
AQ, SNP, and SEQ assays



- 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 (or CpN sites if CpN mode was used) in the well.



Select to show the methylation bar. The methylation bar shows the methylation level for each CpG site (or CpN site if CpN mode was used) in the well. See Methylation colors.

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 SEQ 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.
- SEQ 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.

Clicking the "Warning Info" button (Warning Info) provides a short description and recommendation for each shown warning.

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, we recommend 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)
- The peaks in the compensated Pyrogram are colored according to their quality assessments (SEQ 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).

Homopolymers and uncertain bases

For sequences containing homopolymers, quantification of the base at the end of the homopolymer may be uncertain and should be checked by the user. Uncertain bases are indicated in these ways:

- By yellow in the quality bar of the plate overview
- By yellow in the quality bar of above the base-called sequence
- By "[ ]" surrounding the uncertain base in the base-called sequence.
- By a yellow bar above the blue bar in the histogram

See Edit base-called sequences to add or remove the uncertain base. If the uncertain base is added to the base-called sequence, the "[ ]" disappear, and the added base is shown in lowercase. If the uncertain base is deleted, the "[ ]" and the base are both deleted. If an analysis containing uncertain bases is exported to a FASTA-formatted report, uncertain bases with an intensity of 1.5 or higher will be included, while those with an intensity of less than 1.5 will not be included.

Methylation levels

In the CpG mode, a methylation bar in the "Overview" tab shows the methylation level for each CpG site (or CpN site if CpN mode was used) 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 SEQ assay) are displayed in the "Pyrogram" pane.

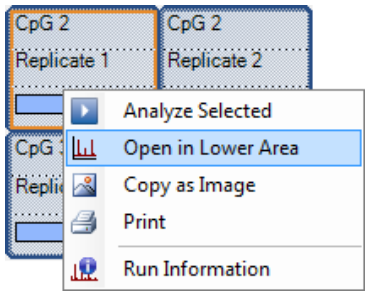
When a base is selected in the base-called sequence, the corresponding peak is highlighted in both Pyrogram areas, and vice versa.

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 or more additional Pyrograms of selected wells in the lower area of the "Pyrogram" pane:

1. **Highlight the wells (see Select wells) you wish to open in the lower area.**
2. **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 are linked, meaning that if the Y axis value for one of the Pyrograms is set manually, the entered value is used for both Pyrograms. The Pyrograms in the lower area are displayed one at a time.
3. **Select the well in the plate overview that should appear as the fixed Pyrogram in the upper area of the "Pyrogram" pane.**
4. **Use the scroll bar in the lower area of the "Pyrogram" pane to change the Pyrogram displayed.**



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 Q24 Advanced 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 SEQ 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 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, which is enabled if 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 SEQ 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:

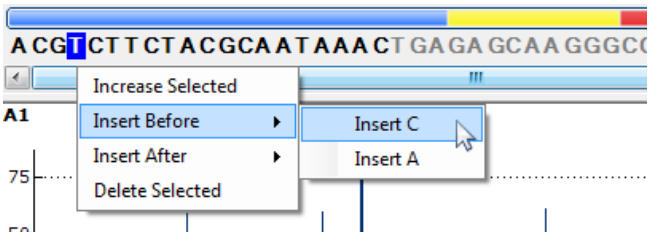
1. **Select a well that is using the modified assay and click "Save Assay". The "Save Assay As" dialog box opens.**
2. **Save the changes to the original file or save the modified assay as a new file:**

   Select destination (folder) from the "Save in" drop-down list.

   Enter filename in the "File name" text box and click "Save".

Edit 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. It is also possible to select a segment of base-called sequence by using the "Shift" and arrow keys.



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

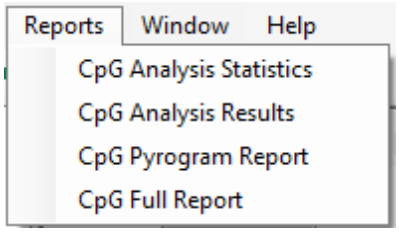
Note: If editing a base-called sequence, note that the quality assessments are still based on the original sequence (the sequence called by the software).

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

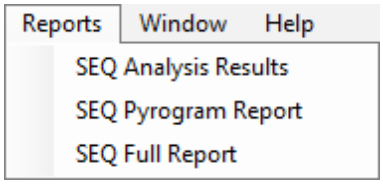
View, Print, and Save Analysis Reports

PyroMark Q24 Advanced Software offers the following analysis reports for processed runs.

Reports for AQ and CpG runs



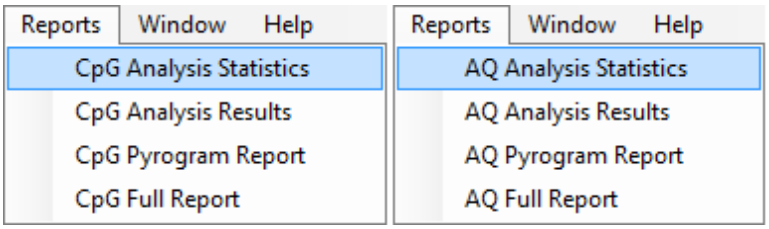
Reports for SNP and SEQ runs



- 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)



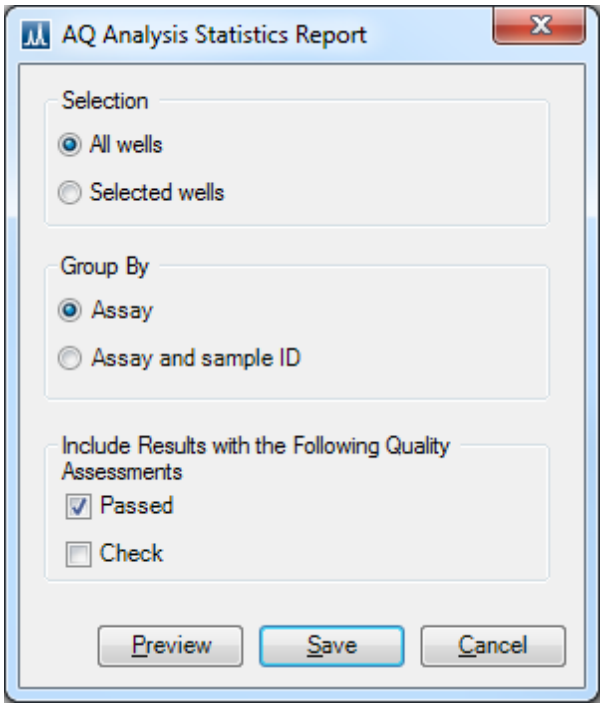
The Analysis Statistics report includes the following information for variable positions in all or selected wells (see Select wells):

- 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:

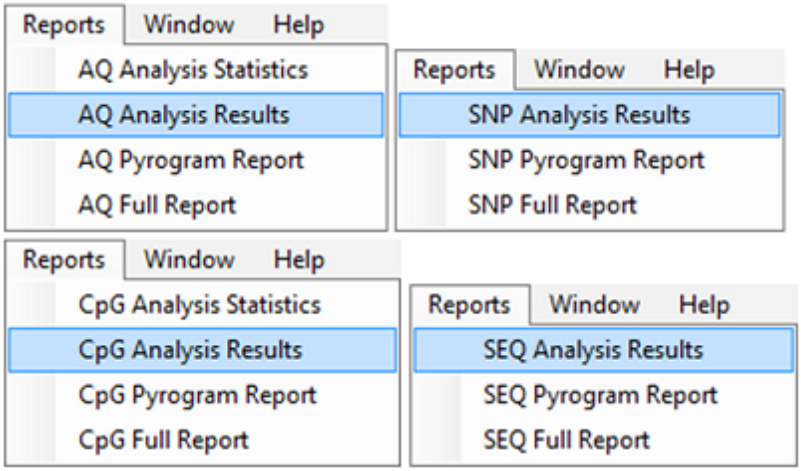
All wells/Selected wells	The wells to be included in the report.
Assay/Assay and sample ID	The analysis results statistics can be grouped according to: ▪ Assay Wells with the same assay will be grouped.

- 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):

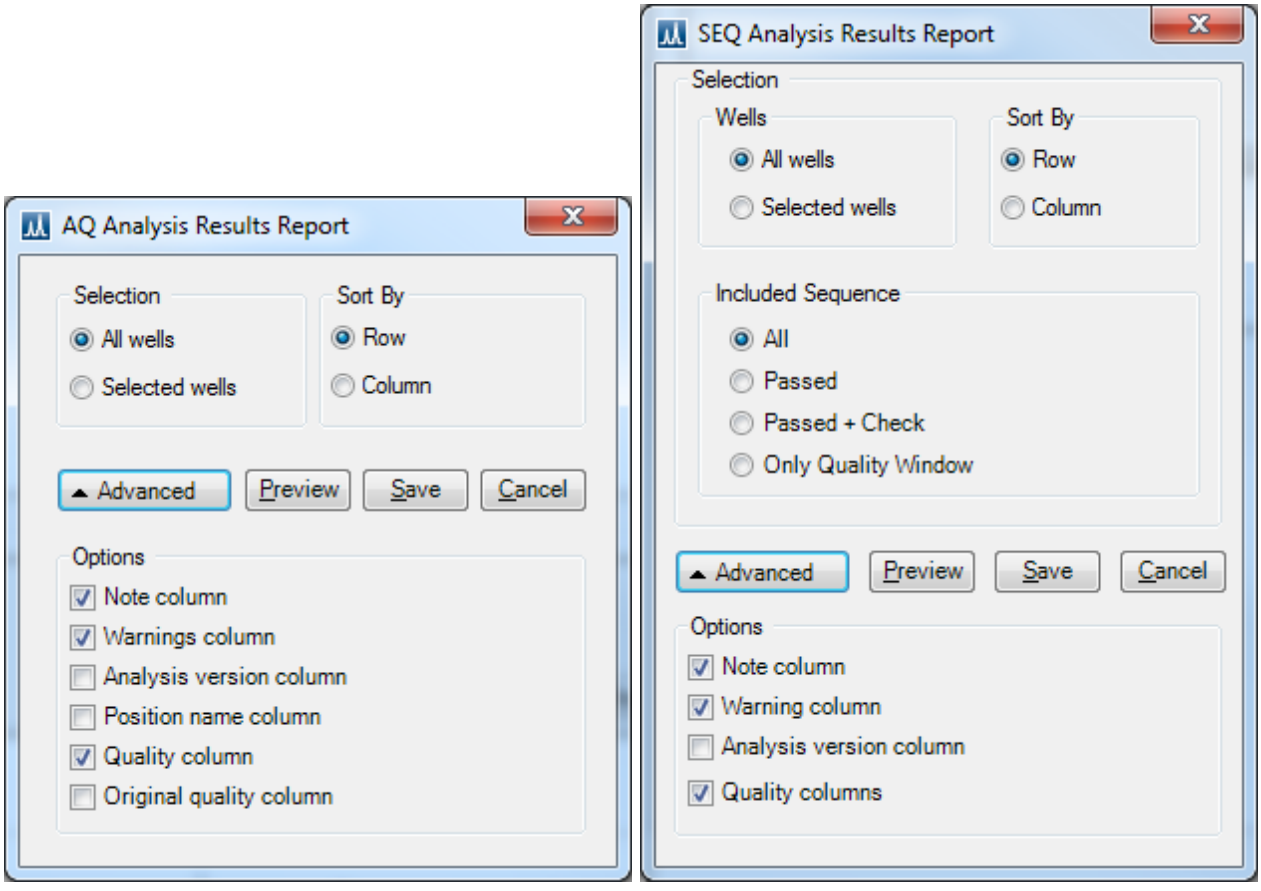
- Well information (well name, assay name, and sample ID)
- The allele frequencies (AQ report), genotypes (SNP report), methylation percentages (CpG report), or base-called sequences (SEQ report) and the quality assessments
- The mean methylation percentage and the standard deviation of all passed CpG sites (or CpN sites if CpN mode was used) 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 (SEQ 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



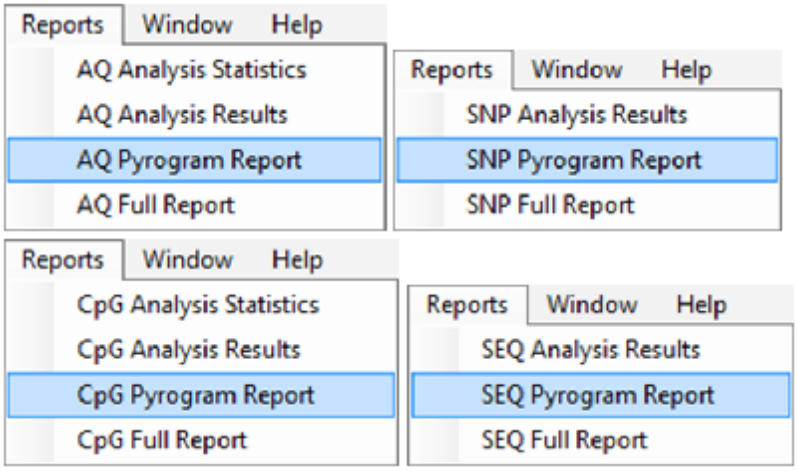
In the "Analysis Results Report" dialog box, there are the following options:

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 SEQ 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 SEQ 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 SEQ report.

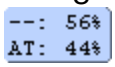
To view the report before saving or printing it, click "Preview".

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 SEQ 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 and methylation averages (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. 

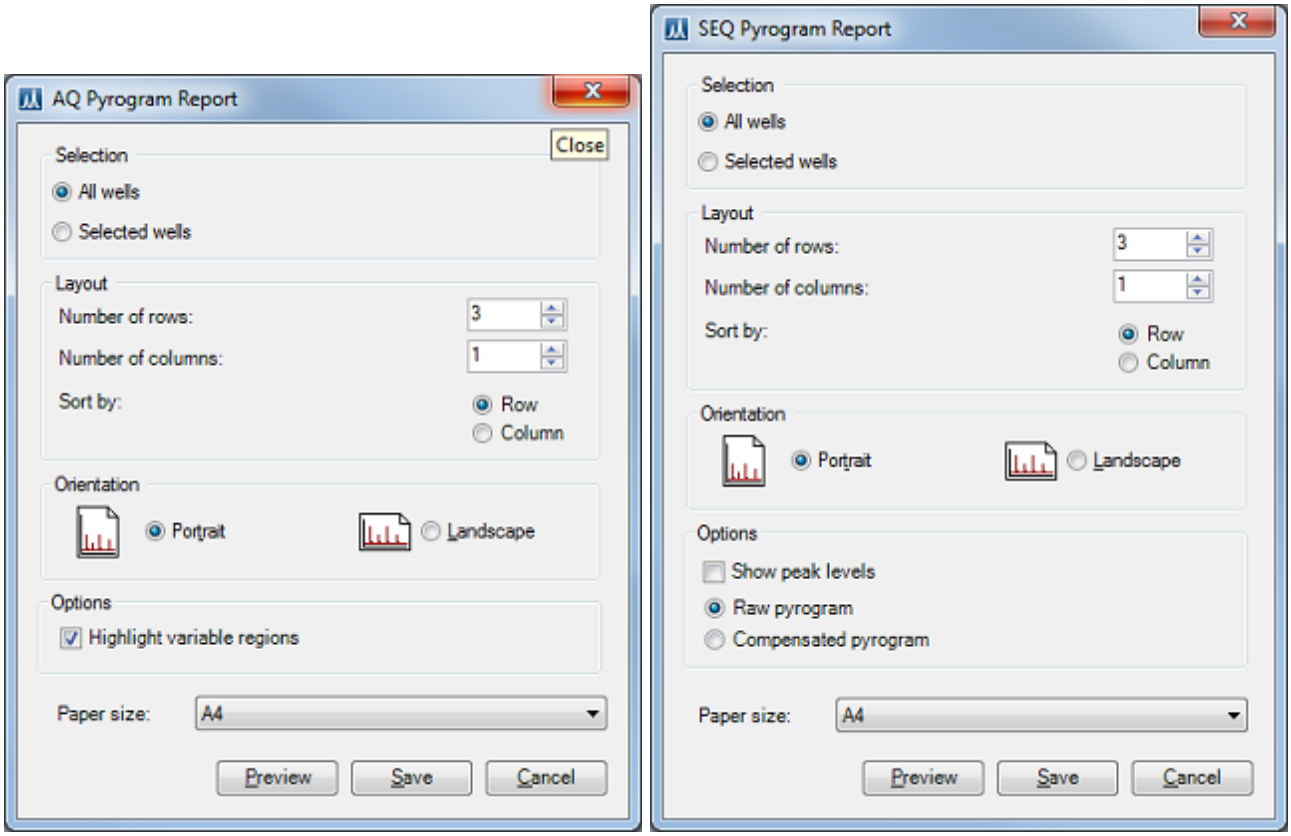
(in white) indicates that the software does not support the analysis, e.g., analysis of SNP in the CpG mode. 

(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 orange background color (CpG report only)

The following information and colors are displayed and used in the SEQ 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. Compensated Pyrograms are only shown for analyzed wells.

Report options



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

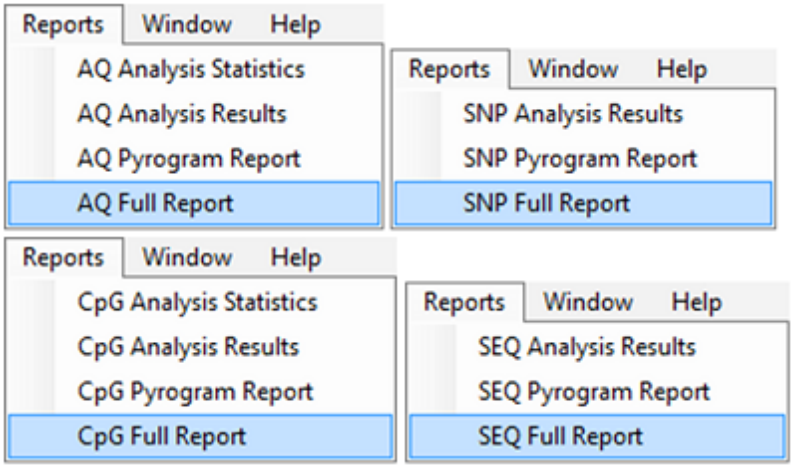
All wells/Selected wells	The wells to be included in the report.
Number of rows/columns	The number of columns and rows of a Pyrogram on each sheet.
Sort by row/column	The sorting order of the wells.
Portrait/Landscape	The paper orientation.
Highlight variable regions	If this option is checked, the variable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

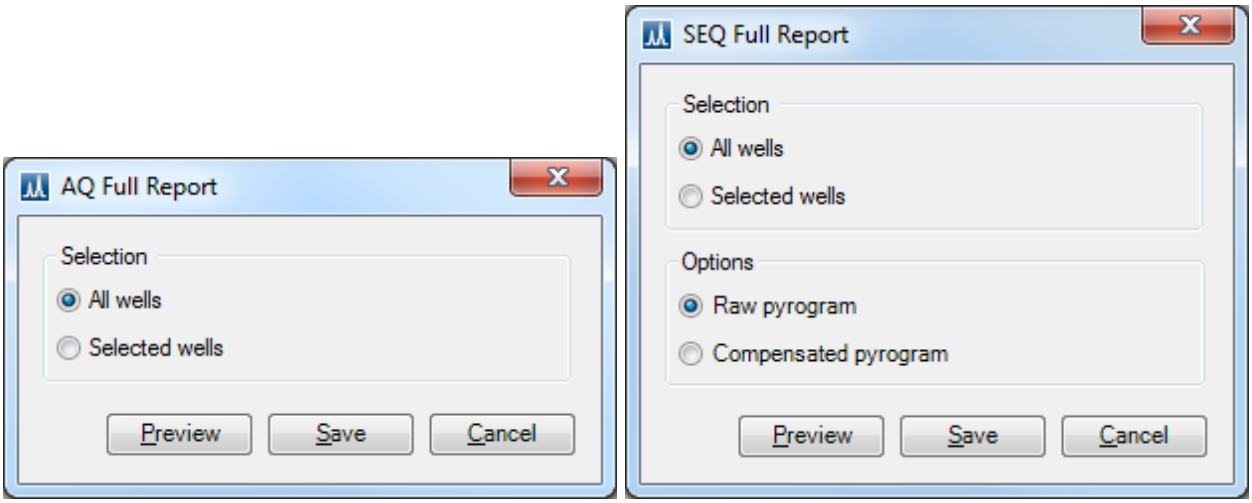


The full report includes the following information for all or selected wells (see Select wells):

- Run parameters (run name, run date and time, instrument method, 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 (SEQ 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 SEQ 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.

Supported files

PyroMark Q24 Advanced Software supports the following file types:

AQ assay	***.paq**
SNP assay	***.psnp**
CpG assay	***.pcpg**
SEQ assay	***.pseq**
Plate setup	***.pset**
Processed run	***.prun**

Files can be opened by either selecting “Open” in the Files menu or clicking  .

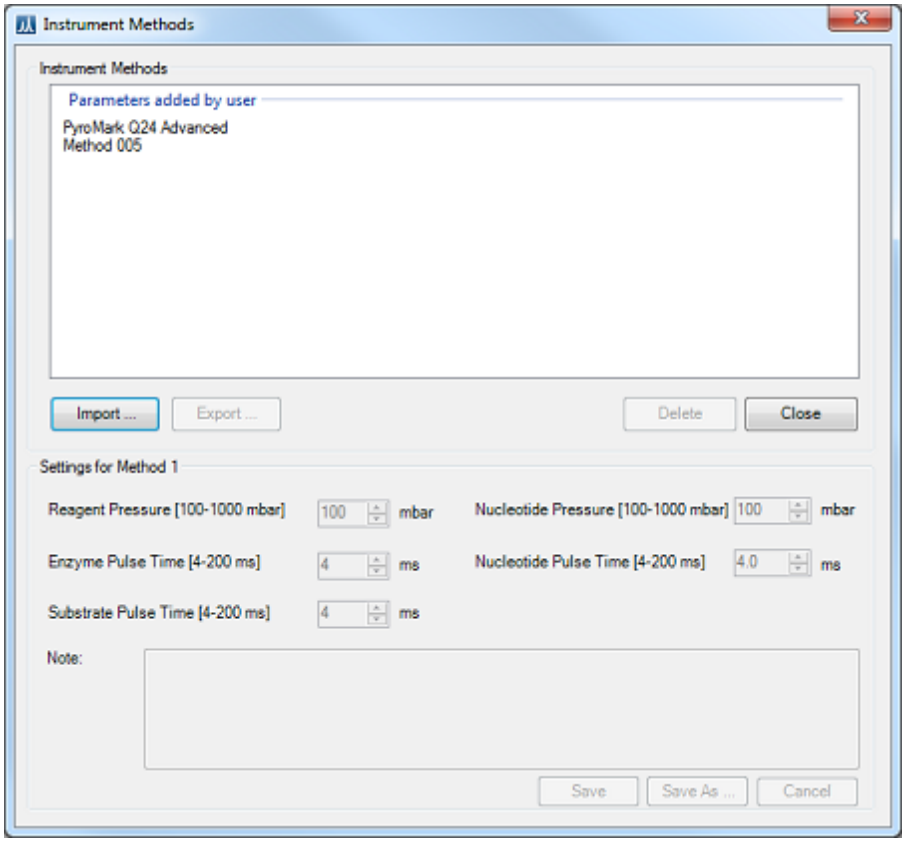
Note: Files created in PyroMark Q24 2.0 Software or in PyroMark Q96 2.5 Software are not supported.

Manage Instrument Methods

Instrument methods should be selected according to the reagent cartridge that will be used for the run. The code number printed on the PyroMark Q24 Cartridge corresponds to specific instrument parameter settings provided at http://www.qiagen.com/support/pyromarkparameters.aspx.

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

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



Workflow

1. **Go to http://www.qiagen.com/support/pyromarkparameters.aspx to download instrument methods files for the PyroMark Q24 Advanced Instrument.**
2. **Locate the instrument method file that corresponds to the method number printed on the label of the cartridge to be used.**
3. **Save the instrument method file to your computer.**
4. **Open the "Instrument Method" dialog box from the "Tools" menu.**
5. **Click the "Import" button and locate the downloaded instrument method file.**
6. **The downloaded instrument method file will now appear in the "Instrument Methods" dialog box under "Methods provided by user" and can be selected in the dropdown list when creating a new Run Setup.**

Instrument methods

In the "Instrument methods dialog box, the following parameters are shown.

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 Q24 Advanced 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 SEQ), 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 Q24 Advanced 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) Failed to load file.	The instrument methods file is probably corrupted. Import a new instrument methods file (see Manage Instrument Methods).
f) Failed to load instrument methods file. Instrument type is not supported.	The instrument methods file is from another instrument type (e.g., PyroMark Q24). Use only instrument methods files expressly for PyroMark Q24 Advanced.
g) Failed to save file. Access denied.	The file being saved is open in another application. Save the file with a different name.
h) 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.
i) 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".

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

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 Q24 Advanced Software

The following is a list of use, warning, and error messages that may appear in the PyroMark Q24 Advanced 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 Q24 Advanced instrument, refer to the *PyroMark Q24 Advanced User Manual.*

Message text	Comments and suggestions
General setup messages	
a) The dispensation order is very long	The dispensation order is very long (more than 200). Shorten the dispensation order to less than 200.
b) The InDel position contains a homopolymer.	The dispensations used to analyze the InDel will give a high signal. Quantification may be uncertain. Check results carefully.
c) Wrong input format.	One of the characters used is not recognized by the software.Use characters from the IUPAC code (A, B, C, D, G, H, K, M, N, R, S, T, V, W, Y and /, [,])
d) Sequence not in phase at the end of dispensations.	One allele is sequenced ahead of the other(s) and the two (or more) alleles are not in phase with the last dispensation. Add more sequence information, i.e., lengthen the sequence to analyze.
e) The sequence contains less reference peaks than required.	For reliable results, the software needs at least 5 dispensations with known results (reference peaks). Do not remove reference peaks.
f) The generated dispensation order contains less reference peaks than required.	For reliable results the software needs at least 5 dispensations with known results (reference peaks). Add more sequence information, i.e., lengthen the sequence to analyze.
General analysis messages	
a) Failed due to possible dispensation error at dispensation:	Desired nucleotide not properly dispensed. Clean cartridge properly (refer to *PyroMark Q24 Advanced User Manual*) and rerun. Discard cartridge if problem persists. If problem occurs in

Message text	Comments and suggestions
	many wells, perform an XY calibration.
b) Failed due to very wide peaks.	Wide peaks indicate bad chemistry. Check instrument method. Check reagent storage conditions. Check shelf life of reagents. Verify that PyroMark Q24 Advanced Reagents were used. Redesign the assay (sometimes peak width is assay-dependent).
c) Missing peaks between dispensations:	The software has detected that all four nucleotides have been dispensed, but no signal was detected. Wash the cartridge carefully, in case there was a dispensation error. Change cartridge. Perform an XY calibration of the dispensation unit.
d) Failed due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g. a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
e) Failed due to baseline drift.	Too high increase or decrease in baseline. Turn on instrument 30 minutes before starting a run. Verify that the environmental temperature did not change (e.g., air-conditioner cycling on or off) and remains under 32°C during the run.
f) Failed reference sequence pattern (overall).	Peak heights deviate from expected levels. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/ contamination. Check sequence to analyze. Check for unknown SNPs.
g) Failed due to high peak height deviation at dispensation:	Peak height deviates from expected level at indicated dispensation.Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
h) Failed due to high peak height deviation in surrounding sequence.	Peak heights near the variable position deviate from expected levels. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/contamination. Check sequence to

Message text	Comments and suggestions
	analyze. Check for unknown SNPs.
i) Failed due to low peak height.	Signal intensity too low. Use more PCR product. Check sample preparation procedure (i.e., ensure that the beads are all transferred to the Q24 plate). Check PCR product immobilization procedure (e.g., mixer speed, that the beads sediment quickly). Check PCR yield. Verify that the enzyme and substrate mixtures are not expired, are stored correctly, and are completely dissolved.
j) Failed due to high noise level.	The noise of the baseline generated by the instrument is too high. Check instrument lid. Contact QIAGEN Technical Services.
k) Failed due to high peak height deviation at more than 5 dispensations.	Peak heights deviate from expected levels at more than 5 dispensations. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/contamination. Check sequence to analyze. Check for unknown SNPs.
l) Uncertain due to high peak height deviation at more than 5 dispensations.	Peak heights deviate from expected levels at more than 5 dispensations. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/contamination. Check sequence to analyze. Check for unknown SNPs.
m) Uncertain due to possible dispensation error at dispensation:	Desired nucleotide not properly dispensed. Clean cartridge properly (refer to *PyroMark Q24 Advanced User Manual*) and rerun. Discard cartridge if problem persists. If problem occurs in many wells, perform an XY calibration.
n) Uncertain due to initial baseline drift.	Too high increase/decrease of initial baseline. Turn on instrument 30 minutes before starting a run. Verify that the environmental temperature did not change (e.g., air-conditioner cycling on or off) and remains under 32°C during the run.
o) Uncertain due to wide peaks.	Wide peaks indicate bad chemistry. Check instrument method. Check reagent storage conditions. Check shelf life of reagents. Verify that

Message text	Comments and suggestions
	PyroMark Q24 Advanced Reagents were used. Redesign the assay (sometimes peak width is assay-dependent).
p) Missing peaks between dispensations:	The software has detected that all four nucleotides have been dispensed, but no base was detected. Wash the cartridge carefully, in case there was a dispensation error. Change cartridge. Perform an XY calibration of the dispensation unit.
q) Uncertain due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
r) Uncertain due to baseline drift.	Too high increase or decrease in baseline. Turn on instrument 30 minutes before starting a run. Verify that the environmental temperature did not change (e.g., air-conditioner cycling on or off) and remains under 32°C during the run.
s) Uncertain reference sequence pattern (overall).	Peak heights deviate from expected levels. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/ contamination. Check sequence to analyze. Check for unknown SNPs.
t) Uncertain due to high peak height deviation at dispensation:	Peak height deviates from expected level at indicated dispensation. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
u) Uncertain due to high peak height deviation in surrounding sequence.	Peak heights near the variable position deviate from expected levels. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/contamination. Check sequence to analyze. Check for unknown SNPs.
v) Uncertain due to low peak height.	Signal intensity too low. Use more PCR product. Check sample preparation procedure (i.e., ensure that the beads are all transferred to the Q24 plate).

Message text	Comments and suggestions
	Check PCR product immobilization procedure (e.g., mixer speed, that the beads sediment quickly). Check PCR yield. Verify that the enzyme and substrate mixtures are not expired, are stored correctly, and are completely dissolved.
w) Uncertain due to high noise level.	The noise of the baseline generated by the instrument is too high. Check instrument lid. Contact QIAGEN Technical Services.
Assay setup messages in AQ mode	
a) Deselected by user.	Variable position was deselected for analysis by the user.
b) Sequence uncertain due to lack of terminal sequence information.	The last dispensation is the terminal base of the sequence to analyze. Add at least one more base at the end of the sequence to analyze, so that one or more additional base dispensations is generated. The added base must be different from the base currently at the end of the sequence to analyze.
c) Last variable position not analyzable due to lack of terminal sequence information.	The last dispensation is included in terminal variable position. Add at least one more base at the end of the sequence to analyze, so that one or more additional base dispensations is generated. The added base must be different from the base currently at the end of the sequence to analyze.
d) Sequence not in phase at the end of dispensations.	One allele is sequenced ahead of the other(s) and the two (or more) alleles are not in phase with the last dispensation. Add more sequence information, i.e., lengthen the sequence to analyze.
e) Variable positions with common dispensations cannot be analyzed.	Two or more variable sites share dispensations and can therefore not be distinguished. If possible, use recommended dispensation order.
Analysis messages in AQ mode	
a) Failed due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g. a certain sum of the peak heights. Check annealing procedure. Check instrument

Message text	Comments and suggestions
	method. Check sequence to analyze. Check for unknown SNPs.
b) Missing peaks in variable site.	The software has detected that all nucleotides in the variable site have been dispensed, but no signal was detected. Check sequence to analyze. Wash the cartridge carefully, in case there was a dispensation error. Change cartridge.
c) Not analyzable due to lack of data.	No peaks detected. Check PCR yield. Check sample preparation. Check sequencing primer. Check cartridge. Check reagents. Check correct usage of buffers.
d) Uncertain due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g. a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
Assay setup messages in CpG mode	
a) Cannot resolve sequence direction.	A forward CpG assay cannot contain a C after bisufite treatment. A reverse CpG assay cannot contain a G after bisulfite treatment.
b) Deselected by user.	Variable position was deselected for analysis by the user.
c) Sequence uncertain due to lack of terminal sequence information.	The last dispensation is the terminal base of the sequence to analyze. Add at least one more base at the end of the sequence to analyze, so that one or more additional base dispensations is generated. The added base must be different from the base currently at the end of the sequence to analyze.
d) Last variable position not analyzable due to lack of terminal sequence information.	The last dispensation is included in terminal variable position. Add at least one more base at the end of the sequence to analyze, so that one or more additional base dispensations is generated. The added base must be different from the base currently at the end of the sequence to analyze.

Message text	Comments and suggestions
e) Sequence not in phase at the end of dispensations.	One allele is sequenced ahead of the other(s) and the two (or more) alleles are not in phase with the last dispensation. Add more sequence information, i.e., lengthen the sequence to analyze.
f) Analysis not supported.	This dispensation order will support the analysis of SNPs in a different application mode.
g) Variable positions with common dispensations cannot be analyzed.	Two or more variable sites share dispensations and can therefore not be distinguished. If possible, use recommended dispensation order.
h) The position is not valid as bisulfite treatment control.	According to the sequence before bisulfite treatment this is not a possible bisulfite treatment control position.
Analysis messages in CpG mode	
a) Failed bisulfite conversion at dispensation:	Failed bisulfite treatment controls are hints for incomplete bisulfite conversion. Review the bisulfite conversion process. Check annealing process. Check nucleotide degradation. Normal Pyrosequencing specific background may trigger this warning, although bisufite treatment was complete.
b) Failed due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
c) Failed due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
d) Missing peaks in variable site.	The software has detected that all nucleotides in the variable site have been dispensed, but no signal was detected. Check sequence to analyze. Wash the cartridge carefully, in case there was a dispensation

Message text	Comments and suggestions
	error. Change cartridge.
e) Not analyzable due to lack of data.	No peaks detected. Check PCR yield. Check sample preparation. Check sequencing primer. Check cartridge. Check reagents. Check correct usage of buffers.
f) Uncertain bisulfite conversion at dispensation:	Uncertain bisulfite treatment controls are hints for incomplete bisulfite conversion. Review the bisulfite conversion process. Check annealing process. Check nucleotide degradation. Normal Pyrosequencing specific background may trigger this warning, although bisufite treatment was complete.
g) Uncertain due to high peak height deviation in variable position	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
h) Uncertain due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.

Assay setup messages in SNP mode

a) The InDel position contains a homopolymer.	The dispensations used to analyze the InDel will give a high signal. Quantification may be uncertain. Check results carefully.
b) Genotyping may be uncertain: the variable position contains a homopolymer.	The dispensations used to analyze the genotype will give a high signal. Genotyping may be uncertain. Check results carefully.
c) Sequence uncertain due to lack of terminal sequence information.	The last dispensation is the terminal base of the sequence to analyze.Add at least one more base at the end of the sequence to analyze, so that one or more additional base dispensations is generated.

Message text	Comments and suggestions
	The added base must be different from the base currently at the end of the sequence to analyze.
d) Last variable position not analyzable due to lack of terminal sequence information.	The last dispensation is included in terminal variable position.Add at least one more base at the end of the sequence to analyze, so that one or more additional base dispensations is generated. The added base must be different from the base currently at the end of the sequence to analyze.
e) Sequence not in phase at the end of dispensations.	One allele is sequenced ahead of the other(s) and the two (or more) alleles are not in phase with the last dispensation.Add more sequence information, i. e., lengthen the sequence to analyze.

Analysis messages in SNP mode

a) Failed genotype determination.	The genotype determination is uncertain, because the pattern of peaks is too similar to another genotype. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs. Check PCR performace (i. e., ensure enough starting material with sufficient quality).
b) Failed due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
c) Failed due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g., a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
d) Missing peaks in variable site.	The software has detected that all nucleotides in the variable site have been dispensed, but no signal was detected. Check sequence to analyze. Wash the cartridge carefully, in case there was a dispensation

Message text	Comments and suggestions
	error. Change cartridge.
e) Not analyzable due to lack of data.	No peaks detected. Check PCR yield. Check sample preparation. Check sequencing primer. Check cartridge. Check reagents. Check correct usage of buffers.
f) Failed genotype determination.	The genotype determination is uncertain, because the pattern of peaks is too similar to another genotype. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs. Check PCR performace (i. e., ensure enough starting material with sufficient quality).
g) Uncertain due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g. a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
h) Uncertain due to high peak height deviation in variable position.	Peak heights deviate from expected levels. Even at a variable position, the software expects a certain pattern, e.g. a certain sum of the peak heights. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
Messages in SEQ mode	
a) Basecalling not consistent with entered known bases	The known bases entered by the user do not match the result. Remove/change entered known bases.
b) Possible dispensation error at dispensation:	Desired nucleotide not properly dispensed. Clean cartridge properly (refer to *PyroMark Q24 Advanced User Manual*) and rerun. Discard cartridge if problem persists. If problem occurs in many wells, perform an XY calibration.
c) Baseline drift	Too high increase or decrease in baseline. Turn on instrument 30 minutes before starting a run. Verify that the environmental temperature did not change

Message text	Comments and suggestions
	(e.g., air-conditioner cycling on or off) and remains under 32°C during the run.
d) Very low peak height from dispensation:	Signal intensity too low starting from given dispensation. Use more PCR product. Check sample preparation procedure (i.e., ensure that the beads are all transferred to the Q24 plate). Check PCR product immobilization procedure (e.g., mixer speed, that the beads sediment quickly). Check PCR yield. Verify that the enzyme and substrate mixtures are not expired, are stored correctly, and are completely dissolved.
e) High pre-sequencing signal	When substrate mixture is added to the reaction, a light signal is generated.Check sample preparation procedure.
f) Not analyzable due to lack of data	No peaks detected. Check PCR yield. Check sample preparation. Check sequencing primer. Check cartridge. Check reagents. Check correct usage of buffers.
g) Peak height deviates from the expected peak level arround dispensation:	Peak heights deviate from expected levels around the indicated dispensation. Check annealing procedure. Check instrument method. Check for unknown SNPs.
h) Peak height deviates from the expected peak levels (overall).	Peaks deviates from expected level. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/ contamination. Check for unknown SNPs.
i) High noise level.	The noise of the baseline generated by the instrument is too high. Check instrument lid. Contact QIAGEN Technical Services.
j) Missing peaks in cycle starting at dispensation: 1	The software has detected that all four nucleotides have been dispensed, but no base was detected. If possible, enter known bases. Wash the cartridge carefully, in case there was a dispensation error. Change cartridge. Perform an XY calibration of the dispensation unit.

Message text	Comments and suggestions
k) Missing peaks in cycle starting at dispensation:	The software has detected that all four nucleotides have been dispensed, but no base was detected. If possible, enter known bases. Wash the cartridge carefully, in case there was a dispensation error. Change cartridge. Perform an XY calibration of the dispensation unit.
l) Peak height deviates from the expected peak level at dispensation:	Peak height deviates from expected level at indicated dispensation. Check annealing procedure. Check instrument method. Check sequence to analyze. Check for unknown SNPs.
m) Baseline drift	Too high increase or decrease in baseline. Turn on instrument 30 minutes before starting a run. Verify that the environmental temperature did not change (e.g., air-conditioner cycling on or off) and remains under 32°C during the run.
n) Low peak height from dispensation:	Signal intensity too low starting from given dispensation. Use more PCR product. Check sample preparation procedure (i.e., ensure that the beads are all transferred to the Q24 plate). Check PCR product immobilization procedure (e.g., mixer speed, that the beads sediment quickly). Check PCR yield. Verify that the enzyme and substrate mixtures are not expired, are stored correctly, and are completely dissolved.
o) Peak height deviates from the expected peak level arround dispensation:	Peak heights deviate from expected levels around the indicated dispensation. Check annealing procedure. Check instrument method. Check for unknown SNPs.
p) Peak height deviates from the expected peak levels (overall).	Peaks deviate from expected level. Check annealing procedure. Check instrument method. Ensure there is no nucleotide degradation/contamination. Check for unknown SNPs.
q) Very high noise level.	The noise of the baseline generated by the instrument is too high. Check instrument lid. Contact QIAGEN Technical Services.

Message text	Comments and suggestions
r) Wide peaks from dispensation: 1.	Wide peaks indicate bad chemistry. Check instrument method. Check reagent storage conditions. Check shelf life of reagents. Verify that PyroMark Q24 Advanced Reagents were used. Redesign the assay (sometimes peak width is assay-dependent).
s) Wide peaks from dispensation:	A certain peak width is needed for correct determination of peak heights. Peaks become wider over time, which is normal. Check results carefully.
t) Wide peaks from dispensation: 1.	Wide peaks indicate bad chemistry. Check instrument method. Check reagent storage conditions. Check shelf life of reagents. Verify that PyroMark Q24 Advanced Reagents were used. Redesign the assay (sometimes peak width is assay-dependent).
u) Wide peaks from dispensation:	A certain peak width is needed for correct determination of peak heights. Peaks become wider over time, which is normal. Check results carefully.

References

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