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PyroMark[®] Q48 Advanced and PyroMark Q48 Advanced CpG Reagents Handbook

For performing PyroMark Q48 Autoprep
standard and long-read Pyrosequencing[®]
reactions

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Kit Contents

	PyroMark Q48 Advanced Reagents	PyroMark Q48 Advanced CpG Reagents
Catalog no.	974002	974022
Number of preps	(4 x 48)	(4 x 48)
Enzyme Mixture*	2 vials	2 vials
Substrate Mixture*	2 vials	2 vials
dATPaS	2 x 380 µl	2 x 1200 µl
dGTP	2 x 260µl	2 x 660 µl
dCTP	2 x 260µl	2 x 660 µl
dTTP	2 x 260µl	2 x 660 µl
PyroMark Advanced Annealing Buffer	2 x 10 ml	2 x 10 ml
PyroMark Binding Buffer	2 x 2 ml	2 x 2 ml
PyroMark Advanced Denaturation Solution	2 x 2 ml	2 x 2 ml

* Mixture supplied as a lyophilized preparation to be reconstituted in PyroMark Advanced Annealing Buffer before use.

Storage

Store PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents at 2–8°C upon receipt. Reconstituted Enzyme and Substrate Mixtures are stable for at least 5 days at 2–8°C. To minimize loss of activity, it is advisable to keep both the enzyme mixture and the substrate mixture in the vials supplied. Reconstituted Enzyme and Substrate Mixtures can be frozen and stored in their vials at –30°C to –15°C. Frozen reagents should not be subjected to more than 6 freeze–thaw cycles. The Substrate Mixture must be protected from exposure to light. All reagents are stable at the recommended storage conditions until the expiration date.

Important: Do not freeze the nucleotides.

Important: Do not dissolve the Enzyme and Substrate Mixtures in water.

Intended Use

PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents is tested against predetermined specifications to ensure consistent product quality.

Introduction

PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents are optimized for Pyrosequencing technology. The reagents are designed to generate a Pyrogram® with sharp and distinct peaks and low background, providing optimal conditions for mutation and SNP analyses. PyroMark Q48 Advanced CpG Reagents are recommended for assays with longer sequencing read lengths, such as with CpG methylation analysis or *de novo* sequencing.

PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents contain all enzymes, substrates, buffers, and nucleotides needed in the Pyrosequencing cascade (Figure 1). When nucleotides are incorporated into the analyzed DNA strand, pyrophosphate is released and converted to ATP. The generation of ATP drives a detectable light signal through a luciferase reaction and this is proportional to the number of nucleotides incorporated.

PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents are intended to be used together with the PyroMark Q48 Autoprep instrument and PyroMark Q48 Autoprep software).

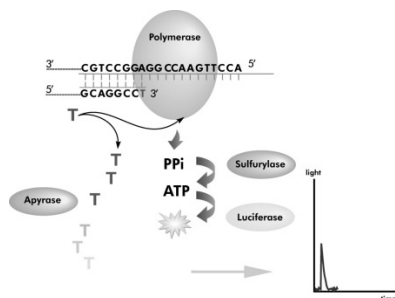


Figure 1. Schematic illustration of the Pyrosequencing cascade. As nucleotides are incorporated into the analyzed DNA strand, pyrophosphate is released and converted to ATP. Generated ATP drives the light reaction detected as a peak on the Pyrogram. Apyrase degrades any unincorporated nucleotides.

Enzyme mixture

The enzyme mixture contains all enzymes that are needed in the Pyrosequencing cascade. These are DNA polymerase for incorporation of nucleotides, ATP sulfurylase for conversion of pyrophosphate to ATP, and luciferase for generation of the light signal. Apyrase is included to degrade ATP and unincorporated nucleotides which switches off the light signal and regenerates the reaction solution. In addition, single-stranded binding protein (SSB) has been added to prevent formation of secondary structures in the DNA template and primers.

Substrate mixture

The substrate mixture consists of adenosine 5' phosphosulfate (APS) needed for generation of ATP, and luciferin, the substrate for luciferase. ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin. This conversion generates visible light in amounts that are proportional to the amount of ATP.

Nucleotides

Nucleotides included in the PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents are dissolved in a well-balanced buffer to prevent degradation of the nucleotides. It should be noted that deoxyadenosine alpha-thio triphosphate (dATP α S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since this deoxynucleotide is efficiently used by the DNA polymerase but not recognized by luciferase.

Buffers

Two buffers are included with the PyroMark Q48 Advanced Reagents and PyroMark Q48 Advanced CpG Reagents. PyroMark Binding Buffer enhances the immobilization of PCR products to the PyroMark Q48 Magnetic beads. Before the sequencing reaction, primers are diluted in PyroMark Advanced Annealing Buffer.

Equipment and Reagents to Be Supplied by User

The following equipment and reagents are required for template preparation.

- Pipets (adjustable)
- Sterile pipet tips (with filters for PCR setup)
- PyroMark Q48 Magnetic Beads (cat. no. 974203)
- PyroMark Q48 Autoprep instrument (cat. no. 9002470, including PyroMark Q48 Cartridges and PyroMark Q48 Autoprep software)
- PyroMark Q48 Discs (cat. no. 974901)
- PyroMark Q48 Absorber Strips (cat.no. 974912)
- Sequencing primer (final concentration 4 μM)
- High-purity water (Milli-Q 18.2 $\text{M}\Omega \times \text{cm}$ or equivalent)

Procedures

Notes before starting

Refer to the *PyroMark Q48 Autoprep User Manual* for detailed instrument operating procedures and important safety information.

Important: Do not freeze the nucleotides.

Important: Do not dissolve the Enzyme and Substrate Mixtures in water.

Dissolve the lyophilized Enzyme and Substrate Mixtures in 660 μ l each of PyroMark Advanced Annealing Buffer (included in the kit). Mix by swirling the vial gently. Do not vortex! In order to ensure that the mixture is fully dissolved, leave it at room temperature (15–25°C) for 5–10 min. Make sure that the solution is not turbid before filling the PyroMark Q48 Cartridge. If the reagents are not to be used immediately, place the reagent vials on ice or in a refrigerator.

Preparing templates and reagents

At the beginning of the run, the instrument will guide you through run preparation, including absorber strip insertion, injector loading, disc insertion and bead and template loading.

To start a sequencing run, select **Sequence** on the home screen and load the run either via USB stick or network connection (selectable on the left side of the screen). Start the run setup by clicking on its name and the arrow appearing behind the run name.

Samples to be analyzed using the PyroMark Q48 Autoprep should be prepared according to the instructions below. All reagents and solutions should be at room temperature (15–25°C) before starting. All steps are performed at room temperature unless otherwise stated.

DNA amplification

A biotinylated PCR product serves as the template for the Pyrosequencing reaction. Amplify the DNA to be analyzed by PCR with one of the primers biotinylated. To receive valid analysis data, see Appendix A – Assay design and validation in the *PyroMark Q48 Autoprep User Manual*.

Insertion of absorber strip

Insert the absorber strip into the absorber strip cavity by sliding it into place, ensuring that it sits level. Ensure that the ends meet on the left hand side of the absorber strip cavity (9 o'clock position).

Loading and priming of cartridges

Injector priming ensures the correct operation of the injectors by loading up the injector system with liquid and removing all the air. During injector priming some of the liquid in the reservoir will be used. Therefore, it is important that the minimum loading volume be adhered to, to ensure that adequate levels of reagent remain to achieve the sequencing run.

1. Open the injector cover and then open each individual cartridge lid.
2. Select injectors to prime by touching on the injector box. Selected injectors will display a droplet symbol.

All injectors requiring mandatory injector priming will be displayed with a red cross and drop symbol (✘💧). You cannot override these selected injectors.

Note: Any injectors not being used in the run will be left blank and cannot be selected for injector priming.

Injectors with a green tick (✔) indicate that they have been primed and do not require injector priming. However, the user may elect to perform another prime by touching on the injector box to display a drop symbol. Injectors with a question mark suggest

discretionary injector priming. These injectors were previously primed, but may require another prime as they have been idle for an extended period of time.

3. Select if this is the last run of the day. If there is more than one run per day, the user can enable the software by deselecting this option to calculate and add the extra volume required for the next run without requiring further injector priming.

Note: If the option “last run of the day” is selected, the software will calculate the minimum volumes required to achieve one run only. A mandatory clean will be required after the last run of the day.

4. Pipet each reagent into the designated injector, according to the volumes shown on the instrument touchscreen. Use the volumes displayed to ensure sufficient reagent is available for the initial injector prime as well as for dispensations during the run. Changing the response in steps 2 or 3 will change the required reagent volumes. Consider the following information when loading the injectors:

- **Important:** Ensure that the reservoir capacity of 3.8 ml is not exceeded.
- Information about the required sequencing primers can be brought up on screen by selecting the information button on the sequencing primer injectors.
- Ensure that the tip of the pipette does not touch the filter at the bottom.
- If loading volumes less than 100 μl , ensure that the solution is dispensed from the top of the reservoir neck.
- Slide the chamber lid toward the front of the instrument to make it easier to load into the reservoirs.
- When loading injectors try to avoid air bubbles in the liquid as they can influence single dispensations.
- In contrast to other PyroMark systems, PyroMark Wash buffer is not needed and is replaced by PyroMark Annealing buffer.

5. Once all the reservoirs have been filled with the required reagent, close and lock the cartridge lids using the lid locks.

Important: Remember to store the reagents at 4°C once all reservoirs are loaded to ensure long reagent shelf life.

6. Select the **Start Button** to begin injector priming and testing.

7. A confirmation message “Start prime and test?” will appear. To confirm select **Yes**.

All injectors selected for priming will undergo the prime protocol. The chamber will rise to the prime position and the injectors will be pressurized. If the instrument reports a problem with the pump reaching pressure please check that the cartridge lids are not open.

The chamber lid will move the injectors over the front half of the absorber strip and dispense the solution to prime the injectors. All injectors that have undergone a prime will be displayed with a question mark.

Accidentally moving the chamber lid during injector priming will pause injector priming and deliver a warning message indicating that the lid has moved. Select **Retry** to continue with the prime.

An **Abort** icon in the top right corner can be used to stop injector priming. A confirmation message, “Abort prime?” will appear. Select **Yes** to stop injector priming or **No** to continue. Following the abort, the injector status will be updated to denote the current state of each injector.

Following the prime, all required injectors will undergo a number of test shots. The test shots ensure that the injectors are operating correctly by observing the drop sensor data. Injectors that pass the test will be displayed with a green tick (✓).

Once injector priming has completed successfully, the software will progress to the next step with the chamber lid automatically opening.

Important: Navigating to **Home** following injector priming will require performing injector test shots in the following run.

Injectors that fail the test will have additional priming and testing to recheck. Further failure will mean the injectors are displayed with a red cross (✗).

If any of the injectors has failed the prime and test protocol, a **Failed Prime** warning will appear indicating which injectors have failed. Failure to pass injector priming could be due to insufficient reagent being loaded. The user has the option to **Retry** the priming and testing or **Skip** to the next part.

Note: If priming fails and more than one priming step must be performed, the volume in the failed injector is reduced and is not sufficient for dispensations during the run. If this occurs, the display will inform the user to load an additional 50 µl into the indicated injector to ensure that it contains the required volume (only the final volumes of the failed injectors are displayed).

If priming problems persist, refer to the *PyroMark Q48 Autoprep User Manual*.

Important: Using injectors that have failed priming and testing may result in poor data.

Inserting the PyroMark Q48 Disc into the instrument

The PyroMark Q48 Disc may be loaded with template and bead mix after insertion into the instrument. Alternatively, the disc can be loaded outside the instrument and inserted just before the run.

To insert a disc into the instrument, follow these steps:

1. Insert a PyroMark Q48 Disc into the instrument. Hold the disc using the disc grips, position the disc index hole (outer hole near well B1) over the disc index pin on the rotor hub, and insert.

The orientation of the disc grips should match up to those on the rotor. The disc detection system will recognize and warn the user if a disc is not properly inserted.

2. Lock the disc into position by screwing down the lock nut.

Important: The disc must be locked into position to avoid spilling the contents of the disc into the chamber.

Loading template and beads into the PyroMark Q48 Disc

The Pyrosequencing template (biotinylated PCR product) and PyroMark Q48 Magnetic Beads are loaded into a disc manually according to the following instructions.

Note: When running the P4 extra protocol, 5 µl PyroMark Binding Buffer must be manually pipetted into the disc wells along with the Pyrosequencing template and PyroMark Q48 Magnetic Beads.

Note: When running the Standard protocol, the PyroMark Binding Buffer is automatically injected into the sample wells by the instrument injector, so there is no need to manually pipet it into the wells.

Note: The two wells on the disc marked by the Ø symbol are waste wells; it is not necessary to load beads into these wells.

PyroMark Q48 Magnetic Beads

PyroMark Q48 Magnetic Beads clump very quickly after mixing due to their large size. Please follow these guidelines when pipetting the beads. Failure to do so will result in inconsistent bead amounts and poor sequencing results.

- Use the supplied electronic multi-dispense pipet when dispensing beads into a larger number of wells (>5)
- When using the multi-dispense pipet, never dispense more than 5 samples at a time, as the beads will clump inside the pipet

- Always use the reverse pipetting function to ensure sufficient volume for the last dispensation
- Ensure that the bead slurry is mixed before taking the next aliquot
- Always centrifuge any residual bead slurry from the top of the stock tube before returning to storage. The consistency of the slurry can change over time if residual solution is left at the top of the stock tube.

Note: See manual of the supplied multi-dispense pipet for detailed information on programming.

1. Vortex the bead slurry to homogeneity before dispensing.
2. Pipet 3 μ l PyroMark Q48 Magnetic Beads into the required wells of the PyroMark Q48 Disc immediately after resuspension.

Note: Position the pipet tip in the middle of the bead slurry to ensure consistency of each aliquot.

Note: Avoid excess liquid on the outside of the pipet tip. Excess liquid can be removed by gently touching the pipet tip against the top of the bead tube when removing the tip.

3. Use the image below as a visual reference example for required bead amount (image was taken after templates were also loaded into the wells). Well 1 contains too few beads; well 2 contains the correct amount of beads; well 3 contains too many beads.



Note: It is not necessary to equally distribute the beads within the well. At the beginning of the sequencing run the disc will be shaken, resulting in an equal distribution of beads.

4. Repeat the resuspension step between each pipetting step.
5. After dispensing beads into all required wells, discard the remaining beads in the pipet tip using the purge function.

6. Store the beads immediately after use according to the manufacturer's guidelines to ensure a longer shelf life.

Pyrosequencing template

1. Pipet 10 μ l biotinylated PCR product into the correct wells of the PyroMark Q48 Disc. The software indicates the well position for each sample. Alternatively, use the Pre-run printout from the PyroMark Q48 Autoprep Software as a sample position guide.
Note: If using less than 10 μ l of PCR product, add high-purity water to the sample to give a final volume of 10 μ l.
Samples with no names will be displayed as **No sample ID**.
Note: When running the P4 extra protocol, the user will be prompted by the instrument to additionally pipet 5 μ l PyroMark Binding Buffer into each well.
2. Ensure that the template thoroughly covers the beads inside the well.
3. Ensure that the disc is correctly inserted into the instrument and locked in place.

Starting a run

1. After the template has been loaded, begin the run by selecting the **Start** button.
2. A confirmation message will display "Close injector cover. Start run?" To start the run close the injector cover and select **Yes**.
To go back and make any adjustments or to confirm any setting, select **No**.
If the injector cover is not closed at the beginning of the run, the following warning will appear: "Injector cover check. Higher than expected signal detected. Check the injector cover is closed and select retry."

Ordering Information

Product	Contents	Cat. no.
Instrument and disposables		
PyroMark Q48 Autoprep	Instrument, software and pipet for advanced Pyrosequencing of 48 samples in parallel	9002470
PyroMark Q48 Discs (50)	50 discs for running PyroMark Q48 Autoprep reactions	974901
PyroMark Q48 Absorber Strips (100)	100 absorber strips for running PyroMark Q48 Autoprep reactions	974912
PyroMark Q48 Cartridge Set	Set of 3 cartridges for PyroMark Q48 Autoprep	9024321
PyroMark Q48 Nucleotide Cartridge	Nucleotide replacement cartridge for PyroMark Q48 Autoprep	9024322
PyroMark Q48 Reagent Cartridge	Reagent replacement cartridge for PyroMark Q48 Autoprep	9024323
PyroMark Q48 Primer Cartridge	Primer replacement cartridge for PyroMark Q48 Autoprep	9024324
Software and licenses		
PyroMark Q48 Software License (1)	1 additional license for PyroMark Q48 Software. Only valid together with PyroMark Q48 Autoprep Software	9024325
PyroMark Q48 Software License (5)	5 additional licenses for PyroMark Q48 Software. Only valid together with PyroMark Q48 Autoprep Software	9024326

Product	Contents	Cat. no.
PyroMark Assay Design SW 2.0	Software for convenient design of PCR and sequencing primers, optimized for Pyrosequencing analysis	9019077
PyroMark IdentiFire SW 1.0	Software for comparing and matching Pyrosequencing data to a local database	9019087
Reagents and consumables		
PyroMark Q48 Autoprep Starter Kit	PyroMark Q48 Magnetic Beads (300), PyroMark Q48 Advanced CpG Reagents (4 x 48), PyroMark Control Oligo, PyroMark Q48 Discs (50) and PyroMark Q48 Absorber Strips (100)	974230
PyroMark Q48 Advanced Reagents (4 x 48)	Reagents for 4 x 48 PyroMark Q48 Autoprep standard reactions	974002
PyroMark Q48 Advanced CpG Reagents (4 x 48)	Reagents for 4 x 48 PyroMark Q48 Autoprep CpG and long-read reactions	974022
PyroMark Q48 Magnetic Beads (300)	Magnetic streptavidin-coated Sepharose® beads for running 300 PyroMark Q48 Autoprep reactions	974203
PyroMark Control Oligo	For installation check of system	979203
PyroMark PCR Kit (200)*	PCR Master Mix for PCR reactions optimized for Pyrosequencing analysis	978703
PyroMark OneStep RT-PCR Kit (50)*	OneStep RT-PCR Enzyme Mix for RT-PCR reactions optimized for Pyrosequencing analysis	978801

* Larger kit sizes available. † 96-well format available.

Product	Contents	Cat. no.
EpiTect® Fast DNA Bisulfite Kit (50)*†	For 50 preps: Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59824
EpiTect Fast FFPE Bisulfite Kit (50) *†	For 50 preps: Deparaffinization Solution, Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59844
EpiTect Fast LyseAll Bisulfite Kit (50) *†	For 50 preps: Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59864

* Larger kit sizes available. † 96-well format available.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Trademarks: QIAGEN[®], EpiTect[®], Pyrogram[®], PyroMark[®], Pyrosequencing[®], Sample to Insight[®] (QIAGEN Group); Sepharose[®] (GE Healthcare).

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the PyroMark Q48 Advanced Reagents or PyroMark Q48 Advanced CpG Reagents to the following terms:

1. The PyroMark Q48 Advanced Reagents or PyroMark Q48 Advanced CpG Reagents may be used solely in accordance with the *PyroMark Q48 Advanced Reagents or PyroMark Q48 Advanced CpG Reagents Handbook* and for use with components contained in the Reagents only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of these Reagents with any components not included within these Reagents except as described in the *PyroMark Q48 Advanced Reagents or PyroMark Q48 Advanced CpG Reagents Handbook* and additional protocols available at www.qiagen.com.
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