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RAS Extension Pyro[®] Kit Handbook

For quantitative measurement of mutations in exons 3 and 4 of the human *KRAS* oncogene, and exons 2, 3, and 4 of the human *NRAS* oncogene using PyroMark Q24, PyroMark Q24 Advanced, and PyroMark Q48 Autoprep

Table of Contents

Kit Contents	3
Controls	4
Shipping and Storage	5
Intended Use	6
Safety Information	7
Quality Control	8
Introduction	9
Principle and procedure	9
Equipment and Reagents to be Supplied by User	11
Important Notes	12
Description of protocols	13
Protocol: PCR Using the PyroMark PCR Kit	14
Protocol: Assay and Run Setup	18
Procedure	18
Troubleshooting Guide	23
Contact Information	24
Ordering Information	25
Document Revision History	27

Kit Contents

RAS Extension Pyro Kit	(24)
Catalog no.	1088848
Number of reactions	24
Seq Primer KRAS 59/61	2 × 24 µL
Seq Primer KRAS 117	2 × 24 µL
Seq Primer KRAS 146	2 × 24 µL
Seq Primer NRAS 12/13	2 × 24 µL
Seq Primer NRAS 59	2 × 24 µL
Seq Primer NRAS 61	2 × 24 µL
Seq Primer NRAS 117	2 × 24 µL
Seq Primer NRAS 146	2 × 24 µL
PCR Primer KRAS 59/61	24 µL
PCR Primer KRAS 117	24 µL
PCR Primer KRAS 146	24 µL
PCR Primer NRAS 12/13	24 µL
PCR Primer NRAS 59	24 µL
PCR Primer NRAS 61	24 µL
PCR Primer NRAS 117	24 µL
PCR Primer NRAS 146	24 µL
PyroMark PCR Master Mix, 2x	4 × 850 µL
CoralLoad® Concentrate, 10x	1.2 mL
H ₂ O	6 × 1.9 mL
Unmethylated Control DNA, 10 ng/µL	3 × 100 µL

Controls

Unmethylated Control DNA is included in the kit as a positive control for PCR and sequencing reactions. This control DNA has a wild-type genotype in the regions sequenced using this kit and is required for adequate result interpretation. Include a sample containing Unmethylated Control DNA for each assay in every Pyrosequencing® run. In addition, include a negative control (no template DNA) in every PCR setup for at least one assay.

Shipping and Storage

The RAS Extension Pyro Kit is shipped on dry ice. PyroMark PCR Master Mix, CoralLoad Concentrate, Unmethylated Control DNA, and all primers should be stored at -30°C to -15°C upon arrival.

The RAS Extension Pyro Kit is stable until the kit expiration date when stored under these conditions.

Intended Use

The RAS Extension Pyro Kit is intended for molecular biology applications. This product is 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 components.

CAUTION



Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Safety Data Sheets (SDSs) or OSHA,* ACGIH,[†] or COSHH[‡] documents. Venting for fumes and disposal of waste must be in accordance with all national, state, and local health and safety regulations and laws.

* OSHA: Occupational Safety and Health Administration (United States of America).

[†] ACGIH: American Conference of Government Industrial Hygienists (United States of America).

[‡] COSHH: Control of Substances Hazardous to Health (United Kingdom).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the RAS Extension Pyro Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Principle and procedure

The RAS Extension Pyro Kit is used for quantitative measurement of mutations in exons 3 and 4 of the human *KRAS* gene, and exons 2, 3, and 4 of the human *NRAS* gene. The kit consists of eight assays (Figure 1).

The eight regions are amplified separately by PCR and sequenced across the defined region. Mutations in the covered region lead to distinct patterns in the Pyrogram[®] trace that are distinguishable from traces obtained from wild-type samples. The assays for *KRAS* codons 117 and 146, and *NRAS* codons 12/13, 59, 61, 117, and 146, are sequenced in the forward direction. The assay for *KRAS* codons 59/61 is sequenced in the reverse direction. The product consists of a PCR primer mix and a sequencing primer for each assay. The primers are delivered in solution, with each vial containing 24 µL of primer or primer mix.

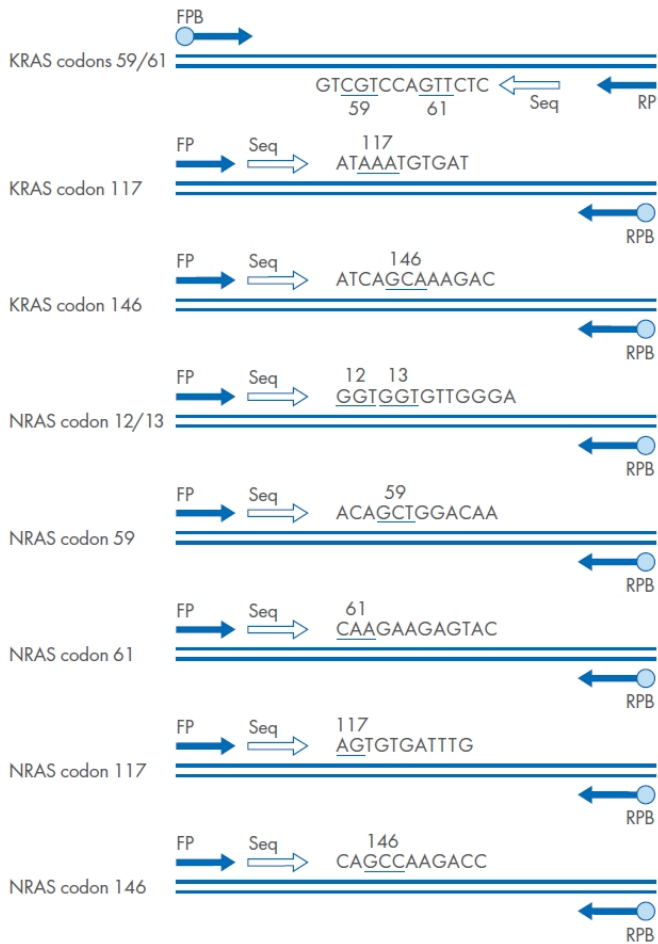


Figure 1. Illustration of the RAS Extension assay. The indicated sequence is the analyzed sequence for a wild-type sample. **FP**, forward PCR primers; **RP**, reverse PCR primers; **Seq**, sequencing primers. B behind the Primers indicates biotinylation.

Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- DNA preparation reagents
- For further information on required equipment and reagents, refer to the user manual of the instrument being used:
 - PyroMark Q24: See Section 5.3, "Sample Preparation," in the *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240).
 - PyroMark Q24 Advanced: See Section 5.3, "Sample and Reagent Preparation," in the *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341).
 - PyroMark Q48 Autoprep: See the "Preparing Templates and Reagents" section in the *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971).

Important Notes

- Create an Assay Setup as described below. This must only be done once, before running the RAS Extension Pyro assays for the first time (see “Protocol: Assay and Run Setup”, page 18).
- Ensure that the reactions are thoroughly mixed and prepared and incubated at the recommended temperatures.
- The Sequencing Primer stock solution has a concentration of 10 μM .
 - **PyroMark Q24:** Mix 0.8 μL of undiluted Sequencing Primer stock solution (10 μM) with 24.2 μL Annealing Buffer to prepare 25 μL of a Sequencing Primer solution at a final concentration of 0.32 μM .
 - **PyroMark Q24 Advanced:** Mix 0.75 μL of undiluted Sequencing Primer stock solution (10 μM) with 19.25 μL Advanced Annealing Buffer to prepare 20 μL of a Sequencing Primer solution at a final concentration of 0.375 μM .
 - **PyroMark Q48 Autoprep:** Dilute the Sequencing Primer stock solution (10 μM) to a final concentration of 4 μM by mixing 0.8 μL Sequencing Primer stock solution with 1.2 μL Advanced Annealing Buffer for one reaction.
 - For manual sequencing primer loading, pipet 2 μL of diluted Sequencing Primer (4 μM) into each well.

Important: Wait until the software prompts you to add the Sequencing Primer (4 μM). Do not pipet the Sequencing Primer (4 μM) into the wells in the beginning of the run. For more information, see *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971).
 - For automatic sequence primer loading, prepare higher volumes of diluted Sequencing Primer (4 μM), as required.

Description of protocols

The first step is to amplify the target DNA by PCR, as described in “Protocol: PCR Using the PyroMark PCR Kit”, page 14. The RAS Pyro Assay and Run should be set up while the PCR is running, following the instructions in “Protocol: Assay and Run Setup,” page 18. After amplification, follow the protocols according to the respective instrument user manual to prepare the sequencing templates for Pyrosequencing analysis:

- *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240): See Section 5.3, “Sample preparation,” continuing from Section 5.3.3 “Immobilizing the PCR products to beads,” and Section 5.4 “Preparation of PyroMark Gold Q24 Reagents.”
- *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341): See Section 5.3, “Sample and reagent preparation,” continuing from Section 5.3.3, “Immobilizing the PCR products to beads.”
- *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971): See “Preparing templates and reagents,” continuing from Section 6.1.5, “Absorber strip”.

Finally, perform the Pyrosequencing run and analyze the data according to the relevant instrument user manual:

- *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240): See Section 5.5, “Processing a run on the PyroMark Q24 Instrument.”
- *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341): See Section 5.4, “Processing a run on the PyroMark Q24 Advanced.”
- *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971): See “Starting a run” and Section 7, “PyroMark Q48 Autoprep Software”.

Protocol: PCR Using the PyroMark PCR Kit

This protocol describes the setup and cycling conditions for the amplification of DNA using the PyroMark PCR Master Mix (included in kit). The eight PCR products are subsequently used for detection of mutations in exons 3 and 4 of the human *KRAS* gene, and exons 2, 3, and 4 of the human *NRAS* gene by Pyrosequencing analysis.

Important points before starting

- See the *PyroMark PCR Kit Handbook* (www.qiagen.com/HB-3794) for more detailed information.
- HotStarTaq® DNA Polymerase requires an activation step of 15 min at 95°C (step 5 of the protocol).
- Set up all reaction mixtures in an area separate from areas used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- Before opening the tubes containing PCR primers, spin briefly to collect contents at the bottom of the tubes.
- Adjust the concentration of the control and sample DNA, if necessary, to 0.4–2 ng/μL.

Procedure

1. Thaw the PyroMark PCR Master Mix, CoralLoad Concentrate, and primer solutions.

Important: Mix the solutions before use to avoid localized concentrations of salt.

2. Prepare a reaction mix for each PCR primer set according to Table 1.

Table 1. Preparation of reaction mix for each PCR primer mix

Component of reaction mix	Volume (µL) per reaction
PyroMark PCR Master Mix, 2	12.5
CoralLoad Concentrate, 10x	2.5
PCR Primer KRAS 59/61 or PCR Primer KRAS 117 or PCR Primer KRAS 146 or PCR Primer NRAS 12/13 or PCR Primer NRAS 59 or PCR Primer NRAS 61 or PCR Primer NRAS 117 or PCR Primer NRAS 146	1
RNase-free water	4
Total volume	20

3. Gently pipet the reaction mixture up and down to mix thoroughly, and dispense 20 µL into each PCR tube. It is not necessary to keep PCR tubes on ice, as HotStarTaq DNA Polymerase is inactive at room temperature.
4. Add 5 µL template DNA (2–10 ng of genomic DNA) to the individual PCR tubes, and mix thoroughly.

Note: Include a negative control sample (without template DNA) in every PCR setup for at least one assay. In addition, include a sample containing Unmethylated Control DNA for each assay in every Pyrosequencing run (see “Controls,” page 4).

5. Program the thermal cycler according to Table 2.

Table 2. Optimized cycling protocol for PyroMark PCR Master Mix

	Time	Temperature (°C)	Notes
Initial PCR activation step	15 min	95	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling			
Denaturation	20 s	95	
Annealing	30 s	53	
Extension	20 s	72	
Number of cycles			42
Final extension	5 min	72	
Hold	∞	4	

6. Place the PCR tubes in the thermal cycler and start the cycling program.

Note: After amplification, samples can be stored overnight at 2–8°C. For longer storage store at –30°C to –15°C.

7. Use 10 µL of PCR product for subsequent Pyrosequencing analysis. We recommend checking the PCR product before Pyrosequencing analysis, for example, by rapid analysis on the QIAxcel® system or by agarose gel analysis. See the *PyroMark PCR Kit Handbook* (www.qiagen.com/HB-3794) for details. The amplicon length for each assay of the RAS Extension Pyro Kit are listed in Table 3 below.

Table 3. Amplicon length of all RAS Extension assays in base pairs

Assay	Amplicon length (bp)
<i>KRAS</i> 59/61	125
<i>KRAS</i> 117	77
<i>KRAS</i> 146	114
<i>NRAS</i> 12/13	106
<i>NRAS</i> 59	67
<i>NRAS</i> 61	114
<i>NRAS</i> 117	92
<i>NRAS</i> 146	75

8. Proceed to “Protocol: Assay and Run Setup,” next page.

Protocol: Assay and Run Setup

These protocols are for setting up the assay parameters and creating a Run Setup for mutation analysis in *KRAS* and *NRAS*.

Use default settings in the software for all assay setups if not otherwise stated.

Procedure

1. Set up the RAS extension assays by selecting **New AQ** Assay in the PyroMark Q24, PyroMark Q24 Advanced, or PyroMark Q48 Autoprep Software.
2. Table 4 shows the “Sequence to Analyze” for eight *KRAS* and *NRAS* assays. Type the assay-specific sequence in the “Sequence to Analyze” field of the software.

Note: The “Sequence to Analyze” can also be modified after the run (if not locked) to analyze for mutations at different positions in the *KRAS* and *NRAS* gene. To reanalyze and target additional mutations, go to **Analysis Setup** and change the default “Sequence to Analyze” to one of the additional “Sequence to Analyze” listed in Table 5 and Table 6. Click **Apply**, and then click **To All** when the Apply Analysis Setup window appears.

Note: We strongly recommend reanalyzing all samples with no mutation detected using the standard “Sequence to Analyze”, as well as samples that received a “Check” or “Failed” quality assessment or show peaks that do not match the height of the histogram bars. “Check” and “Failed” quality assessments may indicate a mutation that is not addressed by the standard “Sequence to Analyze”, resulting in peak height deviations.

3. Manually enter the assay-specific “Dispensation Order” from Table 4.

Note: Do not use the **Generate Dispensation Order** button. Both “Sequence to Analyze” and “Dispensation Order” must be entered in manually.

Click the **Analysis Parameters** tab and ensure that the **A peak reduction factor** is set to 0.86 for analysis of *NRAS* codon 61.

- Click the toolbar, and save the assay as *KRAS 59/61*, *KRAS 117*, *KRAS 146*, *NRAS 12/13*, *NRAS 59*, *NRAS 61*, *NRAS 117*, or *NRAS 146*.

Table 4. Assay setup: “Sequence to analyze” and “Dispensation order” for the eight RAS Extension Pyro assays

Assay	Sequence to analyze	Dispensation order
KRAS 59/61	CTCDTGACCTGCTGT	GCTCAGTCAGACTAGCATGA
KRAS 117	ATAAHTGTGA	GATGACTCGTG
KRAS 146	ATCAVCAAAGA	GATCAGCTGAGC
NRAS 12/13	GNTGNTGTTGGGAAAAGC	TACGACTCAGCATCGTAGAG
NRAS 59	ACAGNTGGAC	TGACTAGCATGA
NRAS 61	CNAGAAGAGTA	TCGTATCGAGAG
NRAS 117	ABTGTGATTT	GACGTGTGA
NRAS 146	CANCCAAGACCA	GCAGTCAGAC

Table 5. Common mutations in the human *KRAS* gene detected by the RAS Extension Pyro Kit with respective “Sequence to analyze”

Nucleic acid substitution	Amino acid substitution	Cosmic ID (V70)	Sequence to analyze
KRAS Codon 59 (GCA)			
175G>A	A59T	546	CTCTTGACCTGNTGT
176C>G	A59G	28518	CTCTTGACCTNCTGT
KRAS Codon 61 (CAA)			
183A>C*	Q61H	554	CTCDTGACCTGCTGT
182A>T*	Q61L	553	CTCTHGACCTGCTGT

Table 5. Common mutations in the human KRAS gene detected by the RAS Extension Pyro Kit with respective “Sequence to analyze” (continued)

Nucleic acid substitution	Amino acid substitution	Cosmic ID (V70)	Sequence to analyze
182A>G*	Q61R	552	CTCTHGACCTGCTGT
183A>T*	Q61H	555	CTCDTGACCTGCTGT
181C>G*	Q61E	550	CTCTTSACCTGCTGT
KRAS Codon 117 (AAA)			
351A>C*	K117N	19940	ATAAHTGTGA
351A>T*	K117N	28519	ATAAHTGTGA
KRAS Codon 146 (GCA)			
436G>A*	A146T	19404	ATCAVCAAAGA
436G>C	A146P	19905	ATCAVCAAAGA
437C>T*	A146V	19900	ATCAGBAAAGA

* For these mutations, the ability to detect different mutation frequencies on the PyroMark Q48 Autoprep was validated.

Table 6. Common mutations in the human NRAS gene detected by the RAS Extension Pyro Kit with respective “Sequence to analyze”

Nucleic acid substitution	Amino acid substitution	Cosmic ID (V70)	Sequence to analyze
NRAS Codon 12 (GGT)			
34G>A*	G12S	563	NGTNGTGTGGGAAAAGC
34G>T	G12C	562	NGTNGTGTGGGAAAAGC
34G>C	G12R	561	NGTNGTGTGGGAAAAGC
35G>A*	G12D	564	GNTGNTGTGGGAAAAGC
35G>T	G12V	566	GNTGNTGTGGGAAAAGC

Table 6. Common mutations in the human NRAS gene detected by the RAS Extension Pyro Kit with respective “Sequence to analyze” (continued)

Nucleic acid substitution	Amino acid substitution	Cosmic ID (V70)	Sequence to analyze
35G>C	G12A	565	GNTGNTGTTGGGAAAAGC
NRAS Codon 13 (GGC)			
37G>A	G13S	571	NGTNGTGTGGGAAAAGC
37G>T	G13C	570	NGTNGTGTGGGAAAAGC
37G>C	G13R	569	NGTNGTGTGGGAAAAGC
38G>A*	G13D	573	GNTGNTGTTGGGAAAAGC
38G>T	G13V	574	GNTGNTGTTGGGAAAAGC
38G>C	G13A	575	GNTGNTGTTGGGAAAAGC
NRAS Codon 59 (GCT)			
175G>A	A59T	578	ACAVCTGGAC
176C>G	A59G	–	ACAGNTGGAC
NRAS Codon 61 (CAA)			
181C>A*	Q61K	580	VAAGAAGAGTA
182A>G*	Q61R	584	CNAGAAGAGTA
182A>T*	Q61L	583	CNAGAAGAGTA
183A>T	Q61H	585	CANGAAGAGTA
183A>C	Q61H	586	CANGAAGAGTA
183A>G	Q61Q	587	CANGAAGAGTA
NRAS Codon 117 (AAG)			
351G>C*	K117N	–	ABTGTGATTT
351G>T*	K117N	–	ABTGTGATTT

Table 6. Common mutations in the human NRAS gene detected by the RAS Extension Pyro Kit with respective “Sequence to analyze” (continued)

Nucleic acid substitution	Amino acid substitution	Cosmic ID (V70)	Sequence to analyze
NRAS Codon 146 (GCC)			
436G>A	A146T	27174	CANCCAAGACCA
436G>C	A146P	–	CANCCAAGACCA
437C>T*	A146V	–	CAGBCAAGACCA

* For these mutations, the ability to detect different mutation frequencies on the PyroMark Q48 Autoprep was validated.

Run setup

1. Create a new run file by select **New Run**.
2. Setup the plate by adding the assays to selected wells.
3. Proceed with the Run Setup, preparation of samples and run according to the instructions in *PyroMark Q24 User Manual*, *PyroMark Q24 Advanced User Manual*, or *PyroMark Q48 Autoprep User Manual*.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx (for contact information, visit www.qiagen.com).

Refer to the user manual for the PyroMark instrument which is used.

- When using PyroMark Q24, see Section 7, “Troubleshooting Guide,” in the *PyroMark Q24 User Manual*.
- When using PyroMark Q24 Advanced, see Section 7, “Troubleshooting Guide,” in the *PyroMark Q24 Advanced User Manual*.
- When using PyroMark Q48 Autoprep, see Section 9, “Troubleshooting Guide,” in the *PyroMark Q48 Autoprep User Manual*.
- For an assay-specific Troubleshooting Guide, see “Troubleshooting Guide,” in the *RAS Extension Pyro Handbook* for PyroMark Q24 (www.qiagen.com).

Contact Information

For technical assistance and more information, please see our Technical Support Center at support.qiagen.com or contact one of the QIAGEN Technical Service Departments or local distributors (visit support.qiagen.com).

Ordering Information

Product	Contents	Cat. no.
Assays & Controls		
RAS Extension Pyro Kit	For 24 reactions: Forward primer, Reverse primer, and Sequencing primer for the mutation analysis of <i>KRAS</i> and <i>NRAS</i> using the PyroMark Q24, PyroMark Q24 Advanced, or PyroMark Q48 Autoprep	970590 1088848
PyroMark Control Oligo	For installation check of the system	979203
PyroMark Q24		
PyroMark Q24	Instrument, for laboratory use only	9001514
PyroMark Q24 Software	Analysis software, for laboratory use only	9019062
PyroMark Q24 Advanced		
PyroMark Q24 Advanced CpG Reagents (4 × 24)	For 4 × 24 samples for use on the PyroMark Q24 Advanced: Enzyme Mixture, Substrate Mixture, Buffers, and Nucleotides for CpG and long-read sequencing runs	970922
PyroMark Q24 Advanced	Instrument, software, and installation for advanced Pyrosequencing analysis of 24 samples in parallel	9002270
PyroMark Q48 Autoprep		
PyroMark Q48 Autoprep	Instrument, software, and pipette	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 Autoprep Starter Kit	PyroMark Q48 Magnetic Beads (300), PyroMark Q48 Advanced CpG Reagents (4 × 48), PyroMark Control Oligo, PyroMark Q48 Discs (50) and PyroMark Q48 Absorber Strips (100)	974230
PyroMark Q48 Advanced Reagents (4 × 48)	Reagents for 4 × 48 PyroMark Q48 Autoprep standard reactions	974002
PyroMark Q48 Advanced CpG Reagents (4 × 48)	Reagents for 4 × 48 PyroMark Q48 Autoprep CpG and long-read reactions	974022

Product	Contents	Cat. no.
PyroMark Q48 Magnetic Beads (300)	Magnetic streptavidin-coated Sepharose beads for running 300 PyroMark Q48 Autoprep reactions	974203

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 Support or your local distributor.

Document Revision History

Date	Description
04/2026	Initial release

Limited License Agreement for RAS Extension Pyro V2 Kit

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