CE IVD

RAID-CRC Symptomatic qPCR Kit

Colorectal Cancer Symptomatic qPCR Kit

INSTRUCTIONS FOR USE

Reference number: REF

RAID-CRC Symptomatic qPCR Kit: CRC-01-2230-01

The **RAID-CRC Symptomatic qPCR kit** is an *in vitro* diagnostic device intended exclusively for use by trained laboratory professionals (professional user).

Intended purpose

The **RAID-CRC Symptomatic qPCR kit is intended for the detection of advanced colorectal neoplasia**, including precancerous lesions and colorectal cancer, in symptomatic individuals who have obtained a positive result in the faecal immunochemical test (FIT).

The clinical performance of RAID-CRC Symptomatic has been evaluated in a population of symptomatic individuals referred for diagnostic colonoscopy (Malagón *et al.*, 2019). Clinical sensitivity and specificity were 94% and 85% for colorectal cancer, and 80% and 90% for advanced colorectal neoplasia when using a FIT cut-off of 50 ng of haemoglobin/g of faeces (Malagón *et al.*, 2019). The predictive values were 51% (PPV) and 99% (NPV) for colorectal cancer, and 70% (PPV) and 94% (NPV) for advanced neoplasia.

The RAID-CRC Symptomatic assay detects specific bacterial markers in DNA extracted from patient stool samples using quantitative polymerase chain reaction (qPCR). The test analyses a panel of **four faecal bacterial markers** indicative of both favourable and unfavourable intestinal health conditions: *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Peptostreptococcus stomatis* and Eubacteria.

The kit **enables amplification and quantification of the gene fragments characteristic** of the microorganisms listed above. Results are provided both qualitatively (Positive/Not compatible) and quantitatively (Cq values and genomic copies/µL). Result generation is performed through the GoodGut-Test™ platform (https://goodgut-test.eu), which has been verified and validated for use with this kit as an *in vitro* diagnostic accessory.

This product is not automated and is intended for use solely by professional laboratory personnel.

Principles of the test

The **RAID-CRC Symptomatic qPCR kit** has been optimised through the analysis of a **multiplex qPCR reaction** using specific primers and fluorescent probes. The result is an **easy-to-use** tool with **high sensitivity**, **specificity**, **reproducibility**, and a **broad dynamic range**.

The assay is based on the 5'-exonuclease activity of the DNA polymerase enzyme. During DNA amplification, the enzyme cleaves probes hybridised to the complementary DNA sequence, separating the quencher from the reporter. This reaction generates an increase in fluorescence proportional to the amount of hydrolysed DNA sequence. Fluorescence is measured using validated real-time PCR thermocyclers (see Annex 2).

The RAID-CRC Symptomatic qPCR Kit requires performing one qPCR analysis per sample to obtain a diagnosis. A total of 230 reactions can be performed with each kit. The master mix is provided in a ready-to-use vial, in a 4X formulation that includes all components needed to perform the qPCR. The primers and probes are supplied lyophilised in separate vials, each sufficient for 230 complete reactions. RNase-free water is also provided in a vial, allowing for the 230 reactions. In addition, the kit includes a positive control provided in a separate tube, enabling verification of the correct performance of each qPCR analysis.

Note: *Throughout this document, the term* ***reagents*** *refers collectively to the 4X Master Mix, the oligonucleotides, the RNase-free water, and the positive control.*

Requirements for the RAID-CRC Symptomatic analysis

The **RAID-CRC Symptomatic qPCR kit** has been optimised for the quantification of the bacterial markers specified above from DNA extracted from stool samples. To ensure the validity of the analysis, the following requirements must be met:

Intended test population:

- **Symptomatic individuals** presenting symptoms compatible with colorectal cancer, such as rectal bleeding, changes in bowel habits, unexplained weight loss, iron-deficiency anaemia, and/or the presence of an abdominal mass.
- The test must be applied exclusively to individuals with a **positive FIT result**, using a **cut-off value of 50 ng of haemoglobin per mL** obtained with the **Eiken Chemical FIT collection tube,** equivalent to 10 µg of haemoglobin per g of faeces.

  Note*: If a different FIT cut-off is used, please contact the manufacturer (support@goodgut.eu).*

Exclusion criteria:

Stool samples must not be collected from individuals who:

- Are under 18 years of age.
- Are pregnant.
- Are undergoing or have received oral or intravenous antibiotic treatment during the month prior to sample collection.
- Have undergone a colonoscopy during the month before sample collection.
- Have previously undergone surgical resection of any part of the digestive tract.
- Have received chemotherapy and/or radiotherapy within the last 6 months.

Pre-analytical requirements:

- FIT determination must be performed using a dedicated analyser from **Eiken Chemical**, and FIT values must be expressed in **ng of haemoglobin per mL**.
- Stool samples **must be processed within 48 hours of collection**.
- During this period, sample collection must be performed using the **Eiken Chemical FIT collection tube**.
- If collection with the FIT tube cannot be performed within the first 48 hours, the sample must be frozen until collection can take place.
- Once collected in the FIT tube, the sample must be stored at 2–8 °C for a minimum of 48 hours before FIT determination and subsequent DNA extraction.
- DNA extraction must be performed within 18 days of sample collection using the FIT tube. If this is not possible, the FIT tube must be stored at –20 °C until the day of analysis, after having remained at least 48 hours at 2–8 °C.
- Because the stool sample is diluted in the FIT tube solution, a preliminary preparation step must be performed before DNA extraction:
  1. Homogenise the FIT tube by gentle manual inversion.
  2. Transfer the contents of the FIT tube into a 1.5-mL microtube (typically between 1.0 and 1.5 mL).
  3. Centrifuge the 1.5-mL tubes for 10 minutes at 4,000 × g.
  4. Remove the supernatant, leaving 100–200 µL of the initial volume.
  5. Homogenise the pellet by pipetting.
  6. Transfer the pellet into the bead-containing tubes provided in the DNeasy PowerSoil Pro Kit and proceed according to the manufacturer’s instructions.

***Note**: Instead of adding 250 mg of soil in step 1 of the PowerSoil protocol, insert the resuspended volume from the FIT tube.*

- After DNA extraction, the DNA may be used immediately for qPCR or stored at –20 °C until analysis.

Compliance with these requirements is essential to ensure that the results obtained fall within the reference ranges established for colorectal cancer detection using the RAID-CRC Symptomatic qPCR kit.

Kit contents

The **RAID-CRC Symptomatic qPCR kit** contains 1 vial of 4X Master Mix, 1 vial of RNase-free water, 12 vials of oligonucleotides (including forward primer, reverse primer and probe), 1 vial of positive control, and one Quick Start Protocol leaflet.

***Table 1.** Components included in the RAID-CRC Symptomatic qPCR kit and reagent information.*

RAID-CRC Symptomatic Kit (230 reactions | 10 µL/reaction)	Concentration	Vial colour	Quantity
Multiplex Master Mix	4X	Clear tube with red cap	1 vial (625 µL)
Primer SYM_f1	2.5 µM	Amber glass	1 vial (80 µL*)
Primer SYM_r1	2.5 µM	Amber glass	1 vial (80 µL*)
Probe SYM_FAM (containing fluorophore FAM and quencher BHQ1)	2.5 µM	Amber glass	1 vial (120 µL*)
Primer SYM_f2	5.0 µM	Amber glass	1 vial (100 µL*)
Primer SYM_r2	5.0 µM	Amber glass	1 vial (100 µL*)
Probe SYM_HEX (containing fluorophore HEX and quencher BHQ1)	5.0 µM	Amber glass	1 vial (120 µL*)
Primer SYM_f3	5.0 µM	Amber glass	1 vial (100 µL*)
Primer SYM_r3	5.0 µM	Amber glass	1 vial (100 µL*)
Probe SYM_CY5 (containing fluorophore CY5 and quencher BHQ2)	5.0 µM	Amber glass	1 vial (120 µL*)
Primer SYM_f4	5.0 µM	Amber glass	1 vial (100 µL*)
Primer SYM_r4	5.0 µM	Amber glass	1 vial (100 µL*)
Probe SYM_ROX (containing fluorophore ROX and quencher BHQ2)	5.0 µM	Amber glass	1 vial (120 µL*)
Positive control SYM	10^5-10^7 copies/µL**	Clear	1 vial (185 µL)
RNase-free water	NA	Clear	1 vial (1.9 mL)
Quick Start Protocol	NA	NA	1 leaflet

NA: Not applicable. * Volume indicated for resuspending the lyophilised oligonucleotide with Tris-HCl pH 8.0 to obtain a final concentration of 2.5 µM for marker 1, and 5.0 µM for markers 2, 3, and 4.

Reagents, materials and equipment not provided in the kit

To perform the RAID-CRC Symptomatic qPCR analysis correctly, the following reagents, materials, and equipment are required but **not included** in the kit:

- Eiken Chemical FIT Collection Tube
- DNA extraction kit (see Annex 1 for compatibility verification)
- Thermocycler (see Annex 2 for compatibility verification)
- Tris-HCl pH 8.0 buffer (for oligonucleotide resuspension)
- Microcentrifuge tubes
- PCR or qPCR tube strips and optical strip caps (8-tube strips)
- Filtered pipette tips

- Biosafety cabinet
- Mechanical disruptor or vortex with tube adaptor
- Microcentrifuge for 1.5-mL tubes
- Strip-tube centrifuge
- Micropipettes (0.5–10 µL, 10–100 µL, and 100–1000 µL)
- Powder-free disposable gloves

Shipping and storage conditions

The **RAID-CRC Symptomatic qPCR kit is shipped refrigerated** at temperatures between **2 °C and 8 °C**. Upon receipt, the Master Mix and positive control must be stored between **–30 °C and –15 °C** in a stable-temperature freezer and protected from light. Lyophilised primers and probes may be stored at **room temperature** until they are resuspended in Tris-HCl buffer (pH 8.0). **Once resuspended**, they must be stored between **–30 °C and –15 °C** in a stable-temperature freezer and protected from light.

In-use stability

Shelf-life after opening: once opened, reagents **remain stable until the expiration date indicated on the label**, provided they are stored between **–30 °C and –15 °C**. Outside this temperature range, the product may remain for a **maximum of 24 hours between 2 °C and 8 °C** without affecting its specifications.

Freeze–thaw cycles: reagents may undergo up to **10 freeze–thaw cycles**. If more thawing cycles are expected, it is recommended to prepare additional aliquots of the reagents.

Safety information

- This product is intended **exclusively for professional use.**
- **Do not use** the kit after the expiration date indicated.
- **A unidirectional workflow** must be established, beginning in the extraction area and proceeding to the amplification and detection areas. Samples, reagents, and equipment must **not be returned** to the previous area once each step is completed.
- Prepare the Master Mix in a **DNA-free biosafety cabinet**. **Avoid loading samples and positive controls into the qPCR plate inside the same cabinet.**
- **Good Laboratory Practices (GLP)** must be strictly followed:
  - Wear protective clothing, disposable gloves, safety goggles, and a mask.
  - Do not eat, drink, or smoke in the work area.
  - Wash hands after completing the analysis.
- Consumables and reagents used for qPCR must be disposed of in **biohazard waste containers**.
- **Regular decontamination of equipment**, particularly micropipettes and work surfaces, is recommended.

Caution: *The 4X Master Mix contains* ***1,2,4-triazole****, a hazardous substance. It may impair fertility or cause harm to the unborn child. It may also pose risks to breastfed children. Special instructions must be obtained before use. Do not handle the kit until all safety instructions have been read and understood. Good Laboratory Practices described in this section must be followed, and personnel must be informed of the risks associated with handling the product.* ***In the event of actual or suspected exposure: consult a physician****.*

Caution: *Do* ***NOT*** *add bleach or acidic solutions directly to sample preparation waste, as toxic irritating vapours may be produced.*

Information on interfering substances

Refer to the section **Requirements for the RAID-CRC Symptomatic Analysis** (page 1).

Quality control

In accordance with the **GoodGut Quality Management System** (ISO 13485 certified), each lot of the **RAID-CRC Symptomatic qPCR kit** is tested under predefined specifications to ensure **activity**, **efficiency**, and **sensitivity**

The Certificate of Analysis can be found in the professional area of the GoodGut website: https://professionalarea.goodgut.eu/.

Limitations of use

The reagents in this kit are designed to function exclusively with this qPCR kit. **Use with other assays is not recommended.**

The reagents are only compatible with the instruments specified in **Annex 2**.

To date, no additional components have been identified that could influence the measurements.

Accessories of the RAID-CRC Symptomatic qPCR Kit

To obtain the diagnostic result, use the **GoodGut-Test™** web platform (https://goodgut-test.eu). Access to the platform is provided separately upon purchase of the RAID-CRC Symptomatic qPCR kit.

A **user manual** is supplied together with a **DEMO** explaining the operation of the platform, intended for use by professional laboratory personnel. If you have not received it, please contact support@goodgut.eu.

The **recommended computer configuration** for using the GoodGut-Test™ web platform is shown in Table 2.

Table 2. *Recommended computer configuration for the GoodGut-Test™ web platform.*

	For WINDOWS	For MAC
Scale	125%	125%
Screen resolution	1920 x 1080	1920 x 1080
Screen resolution	Landscape	Landscape

An Internet connection is required to use the GoodGut-Test™ web platform. The platform is compatible with Google Chrome, Microsoft Edge, and Mozilla Firefox.

Reference measurement procedure

To ensure correct performance of the RAID-CRC Symptomatic qPCR kit, a **positive control** of known concentration must be included in every multiplex qPCR run (see *RAID-CRC Symptomatic qPCR Kit Protocol*, page 8). A **No Template Control (NTC)** must also be included to verify the absence of reaction contamination.

Positive control
As the assay is based on a multiplex qPCR that simultaneously analyses 4 biomarkers, the positive control consists of a mixture of specific target sequences corresponding to each assay. For each lot of positive control, a **tolerance range** has been established based on the analysis of three independent duplicate runs selected at random.

After running and interpreting the assay, **the Ct value of the positive control must fall within the established range for the corresponding lot**. If the Ct value falls outside the accepted range, the results cannot be considered reliable.

The **GoodGut-Test™** web platform automatically indicates whether the positive controls are accepted or rejected. In case of rejection, the sample analysis must be repeated

The **tolerance ranges for positive control** are available in the technical specifications of the RAID-CRC Symptomatic qPCR kit, supplied with each lot at the time of purchase and accessible in the GoodGut professional area: https://professionalarea.goodgut.eu/.

No template control (NTC)

The NTC is used to confirm that the reaction mix is free from contamination. Each marker included in the RAID-CRC Symptomatic qPCR kit has a **predefined minimum Ct value**.

After running the assay, **the Ct value of the NTC must be above the predefined minimum threshold**. If the Ct value of the NTC is below the acceptable minimum, the results are not reliable. The **GoodGut-Test™** web platform indicates whether the NTCs are accepted or rejected. In the event of rejection, the analysis must be repeated.

The minimum accepted Ct values for NTCs are included in the technical specifications of the RAID-CRC Symptomatic qPCR kit, supplied with each lot and accessible in the GoodGut professional area: https://professionalarea.goodgut.eu/.

Reagent information

***Table 3**. Information on the reagents included in the RAID-CRC Symptomatic qPCR Kit.*

Component	Description
Multiplex Master Mix 4X	The DNA polymerase is a modified form of a recombinant 94 kDa DNA polymerase isolated from *Thermus aquaticus*. The enzyme is supplied in an inactive state with no enzymatic activity at room temperature and is activated by incubating for 1 minute at 95 °C. The master mix contains Tris-HCl, KCl, NH_4Cl, $MgCl_2$, and additives enabling rapid cycling, as well as ultrapure dATP, dCTP, dGTP, and dTTP.
RNase-free water	Distilled RNase-free water for molecular biology applications.
Primers (forward and reverse)	For each marker, two primers (forward and reverse) are provided, purified by desalting and preloaded in the corresponding vial.
Probes	For each marker, a probe purified by HPLC is provided, preloaded in the corresponding vial.
Positive Control SYM	The positive control contains a distinct mixture of qPCR amplification products. All controls undergo rigorous quality verification, including size confirmation by capillary electrophoresis and sequence identification by mass spectrometry.

RAID-CRC Symptomatic qPCR kit protocol

To obtain the RAID-CRC Symptomatic results, the following protocol must be followed.

The concentration of primers/probes, as well as the cycling parameters (temperature for hybridisation, number of cycles and incubation times), have been fully optimised to achieve optimal analytical performance and specificity.

Before beginning, primers and probes must be resuspended in the volume of Tris-HCl buffer pH 8.0 indicated in the protocol (see Kit Contents section).

***Note**: For optimal resuspension of primers and probes, after adding the Tris-HCl buffer, incubate the tubes at room temperature for 1 hour or overnight at 4 °C without changing the container. Once resuspended, they must be stored between –30 °C and –15 °C in a stable-temperature freezer and protected from light.*

qPCR protocol steps:

1. **Preparation of PCR tube strips:**
   Determine the number of tubes and caps required for the intended reactions, taking into account all samples and controls to be included in each qPCR run (materials not included in the kit). Each qPCR analysis must include one **positive control** and one **No Template Control** (**NTC**).

2. **Initial preparation of reagents:**
   Thaw the 4X Multiplex Master Mix, primers, probes, and positive control supplied in the qPCR kit.

3. **Multiplex qPCR Analysis :**
   Add the components listed in Table 4 into a microcentrifuge tube. It is recommended to prepare a reaction mix volume of n × 1.1, where n is the number of reactions, to minimise pipetting error. Avoid exposing fluorescently labelled probes to light.

   Note: *The number of reactions performed simultaneously must not exceed the maximum capacity of the thermocycler.*

Table 4. Reaction mix for RAID-CRC Symptomatic qPCR (per reaction).

Component	Final concentration	Volume/reaction
Multiplex Master Mix 4X	1X	2,50 µL
Primer SYM_f1	50 nM	0,20 µL
Primer SYM_r1	50 nM	0,20 µL
Probe SYM_FAM	60 nM	0,24 µL
Primer SYM_f2	150 nM	0,30 µL
Primer SYM_r2	150 nM	0,30 µL
Probe SYM_HEX	200 nM	0,40 µL
Primer SYM_f3	150 nM	0,30 µL
Primer SYM_r3	150 nM	0,30 µL
Probe SYM_CY5	200 nM	0,40 µL
Primer SYM_f4	150 nM	0,30 µL
Primer SYM_r4	150 nM	0,30 µL
Probe SYM_ROX	200 nM	0,40 µL
RNase-free water	-	1,86 µL

Mix the reaction vigorously and briefly centrifuge. Dispense **8 µL** of the reaction mix into qPCR tubes compatible with the thermocycler in use.

Add **2 µL of the DNA sample** to each tube containing the reaction mix. Also, add **2 µL of the specific positive control** and leave one tube containing only the reaction mix as the **NTC**.

Seal the tubes with optical caps, vortex for 5 seconds, and briefly spin down to ensure the reaction settles at the bottom of the tube without droplets or bubbles

4. **Loading the thermocycler:**
   Select the appropriate channels (targets/dyes) for fluorescence acquisition during the combined hybridisation/extension step: **FAM**, **HEX**, **CY5** and **ROX**.

Table 5. Thermocycler protocol for RAID-CRC Symptomatic multiplex qPCR.

Step		Time (min:s)	Temperature (ºC)
qPCR activation		01:00	95
40 cycles	Denaturation	00:15	95
	Annealing + Extension	00:30	60

5. **Starting the analysis (*run*):**
   The samples and controls must be **analysed within the same run**.

Analysis and interpretation of the results:

1. **Data processing:**
   Sample analysis is performed using the qPCR instrument software, following the manufacturer's instructions.

   ***Note**: Before beginning the analysis, predefined analysis parameters (baseline and threshold values) must be selected for each primer/probe system, as indicated in the Technical Specifications of the RAID-CRC Symptomatic kit. These are provided upon kit purchase and available at the GoodGut Professional Area (https://professionalarea.goodgut.eu/).*

2. **Uploading results to the platform:**
   To obtain the RAID-CRC Symptomatic diagnostic output, the results from qPCR analysis, including positive and negative controls, must be uploaded to the **GoodGut-Test™** web platform (https://goodgut-test.eu/) following the **User Manual**.
   Data must be uploaded using Excel templates specific to the multiplex assay, which must include:
   - Sample identifier
   - Detection channel (dye)
   - Raw Ct (Cq) value

   The Excel template file can be downloaded directly from the platform.

For any technical support request or feedback, please contact support@goodgut.eu

In the event of an incident—defined as any malfunction or problem occurring with this In Vitro Diagnostic Medical Device during use or thereafter, which may have serious consequences for health—please contact the manufacturing laboratory (GoodGut S.L.U.). E-mail: vigilance@goodgut.eu and/or the competent authority of the country where the user and/or patient is established.

Symbol description:

	Contains a substance hazardous to health. Refer to the safety information
	In vitro diagnostic medical device in accordance with Regulation (EU) 2017/746
	Keep away from moisture
	Temperature limits
	Read the instructions for use before using the product
	Number of reactions that can be performed with the kit
	Date of manufacture
	Reference number
	Lot number
	Date of expiration

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RAID-CRC Symptomatic qPCR Kit
Colorectal Cancer Detection qPCR Kit
Basic UDI-DI: 8437023437RAIDCRCKC

GOODGUT
Enhancing digestive health

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ANNEX 1: Compatibility of DNA extraction kits and automated equipment

The DNA extraction kits and automated extractors that may be used to obtain reliable diagnostic results with the RAID-CRC Symptomatic assay are listed below (not included in the kit).

DNeasy PowerSoil Pro DNA Extraction Kit from QIAGEN (manual extraction)

- Kit reference: 47014 (50 reactions); 47016 (250 reactions), QIAGEN
- Before initiating DNA extraction, the sample preparation process described in the ***Pre-analytical requirements*** section of ***Requirements for the RAID-CRC Symptomatic analysis*** must be performed. Once this preparation is complete, proceed with DNA extraction according to the manufacturer's instructions.

QIAcube from QIAGEN (automated extraction)

- Use the DNeasy PowerSoil Pro DNA Extraction Kit (QIAGEN) with the QIAcube Connect automated extractor (QIAGEN).
- Before initiating DNA extraction, the sample preparation process described in the ***Pre-analytical requirements*** section of ***Requirements for the RAID-CRC Symptomatic analysis*** must be performed. Once this preparation is complete, proceed with DNA extraction according to the manufacturer's instructions.

ANNEX 2: Compatibility of real-time PCR equipment

The RAID-CRC Symptomatic multiplex assay can be performed on all qPCR thermocyclers equipped with a **low-profile block**, as listed below.

AriaDx (Agilent Technologies)

- Sample analysis is performed using the software provided with the real-time PCR instrument, following the manufacturer's instructions.
- Before analysing the data, select the **predefined analysis settings** for each primer + probe set (e.g., reference settings and threshold values) according to the **Technical Specifications of the RAID-CRC Symtomatic qPCR Kit**.
- Use tube strips and optical caps recommended by the thermocycler manufacturer

CFX96 (BioRad)

- Sample analysis is performed using the software provided with the real-time PCR instrument, following the manufacturer's instructions.
- Specifications for analysing results with the CFX96 software:
    - Select **BR White** as the plate type.
    - Apply **fluorescence drift correction**.
- Before analysing the data, select the **predefined analysis settings** for each primer + probe system (e.g., reference configuration and threshold values) according to the *Technical Specifications of the RAID-CRC Symptomatic qPCR Kit*.
- Use tube strips and optical caps recommended by the thermocycler manufacturer.

Note: *The RAID-CRC Symptomatic Technical Specifications for each lot are provided separately upon purchase of the kit and are also available in the Professional Area of the GoodGut website: https://professionalarea.goodgut.eu/.*