CE

IVD

RAID-CRC SCREEN qPCR KIT

Colorectal Cancer Screening qPCR Kit

Ref number: REF

RAID-CRC SCREEN qPCR Kit: CRC-01-1250-01

The RAID-CRC Screen qPCR Kit is an *in vitro* diagnostic device for professional laboratory use (professional user).

Intended Purpose

The RAID-CRC Screen qPCR Kit is intended for the screening of advanced colorectal neoplasia in asymptomatic individuals equal to or older than 50 years who obtain a positive result in the faecal immunochemical test (FIT) through the detection of specific bacterial markers in DNA samples extracted from patients' faeces.

The RAID-CRC Screen qPCR Kit *in vitro* diagnostic test is based on the qPCR analysis of a panel of faecal bacteria indicative of both favourable and unfavourable conditions of intestinal health. The panel is used in asymptomatic individuals older than 50 years to rule out colorectal cancer. The RAID-CRC Screen qPCR Kit is based on proprietary technology that detects a combination of 6 bacterial markers tested in faecal samples: B46, B48, *Faecalibacterium prausnitzii*, *Gemella morbillorum*, *Bacteroides fragilis*, and Eubacteria. The qPCR kit allows amplifying and quantifying the characteristic gene fragments of the mentioned microorganisms. Results are provided in qualitative and quantitative form. The product is not automatic. The intended user is a laboratory professional.

Test principles

The RAID-CRC Screen qPCR Kit is optimised for quantitative PCR (qPCR) assays in multiplex using specific primers and fluorescent-labelled probes. It is an easy-to-use tool that offers reproducible results with high sensitivity, specificity, and broad dynamic range. This product is based on 5' exonuclease activity of DNA polymerase. During DNA amplification, this enzyme cleaves the probe bound to the complementary DNA sequence, separating the quencher dye from the reporter. This reaction generates an increase in the fluorescent signal which is proportional to the quantity of the hydrolysed target sequence. This fluorescence can be measured on real-time PCR platforms.

The RAID-CRC Screen qPCR Kit requires three different qPCR assays for each sample to obtain a diagnostic. A total of 250x3 reactions can be performed with each kit. The master mix is provided as a

ready-to-use, stabilised 4X formulation that includes all components for qPCR. The primers and probes are provided lyophilised in separate tubes. Three positive controls are also provided in separate tubes to check the correct performance of each qPCR run.

Requirements for RAID-CRC Screen use

This RAID-CRC Screen qPCR Kit has been optimized for the analysis of DNA extracted from faecal samples that fulfil the following conditions:

- Faecal samples must come from asymptomatic individuals equal to or older than 50 (population at intermediate risk of suffering from colorectal cancer (CRC)).
- Faecal samples must be free of antibiotics the month prior to deposition.
- Faecal sample must come from a person who has not undergone a colonoscopy in the previous month.
- Faecal sample must come from a person who has not had surgical resections of any part of the digestive tract.
- Faecal samples from pregnant women are not accepted.
- This test must be only applied when a positive result of FIT (cut-off of 100 ng of haemoglobin per mL using the Eiken Chemical tube collector) is obtained. **Note**: In case of using another FIT cut-off value, please contact the manufacturer (soporte@goodgut.eu).
- FIT determination must be performed with any equipment with this specific function from Eiken Chemical. FIT units must be expressed in ng of haemoglobin per mL.
- Faecal samples must be treated within the first 48 hours after sample collection. Within this period stool sample must be collected with the Eiken Chemical FIT collector.
- The FIT collection tube (Eiken Chemical) should be stored at 2ºC to 8ºC until FIT analysis.
- After FIT determination, DNA extraction must be performed within the first 18 days after sample collection with this collector.
- For the DNA extraction, since the faecal sample is diluted in the FIT collector solution a preliminary sample preparation needs to be done following the next steps:

    1. Homogenise FIT collector by inverting it manually several times.
    2. Transfer FIT collector content into a 1.5 mL tube (expect between 1 mL to 1.5 mL).
    3. Centrifuge the 1.5 mL tubes for 10 minutes at 4,000 xG.

4. Discard the supernatant (expect to keep between 100 µL to 200 µL of the initial volume).
5. Homogenise the pellet by pipetting.
6. Transfer the pellet into the beads tube provided in the DNeasy Powersoil Pro Kit and proceed following the manufacturer's instructions. **Note**: instead of using 250 mg of soil in Step 1, introduce the volume resuspended from the FIT collector fluid.

Kit Contents

Table 1. Components included in the RAID-CRC Screen qPCR Kit.

RAID-CRC Screen qPCR Kit (250x3 | 10 µL/reaction)	
Multiplex Master Mix 4X (Contains: Taq DNA Polymerase, Antibody, Guard, Buffer, dNTP mix [dATP, dCTP, dGTP, dTTP])	3 x 625 µL
Primer SCR_A_f1	80 µL*
Primer SCR_A_r1	80 µL*
Probe SCR_A_FAM (contains the fluorochrome FAM and the quencher BHQ1)	120 µL*
Primer SCR_A_f2	180 µL*
Primer SCR_A_r2	180 µL*
Probe SCR_A_HEX (contains the fluorochrome HEX and the quencher BHQ1)	80 µL*
Primer SCR_A_f3	180 µL*
Primer SCR_A_r3	180 µL*
Probe SCR_A_ROX (contains the fluorochrome ROX and the quencher BHQ2)	100 µL*
Primer SCR_B_f1	180 µL*
Primer SCR_B_r1	180 µL*
Probe SCR_B_HEX (contains the fluorochrome HEX and the quencher BHQ1)	80 µL*
Primer SCR_B_f2	160 µL*
Primer SCR_B_r2	160 µL*
Probe SCR_B_CY5 (contains the fluorochrome CY5 and the quencher BHQ2)	120 µL*
Primer SCR_C_f1	180 µL*
Primer SCR_C_r1	180 µL*
Probe SCR_C_FAM (contains the fluorochrome FAM and the quencher BHQ1)	160 µL*
Positive Control A (contains a mixture of the qPCR amplification products of SCR_A)	185 µL

RAID-CRC Screen qPCR Kit (250x3 | 10 µL/reaction)	
Positive Control B (contains a mixture of the qPCR amplification products of SCR_B)	185 µL
Positive Control C (contains a mixture of the qPCR amplification products of SCR_C)	185 µL
RNase-free water	3 x 1.9 mL

* Volume indicated to resuspend the lyophilized oligonucleotide with Tris-HCl (pH 7.4 for primers and 8.1 for probes) to obtain a concentration of 2.5 µM for marker 1 tubes of multiplex SCR_A and 5.0 µM for the rest of the markers.

Reagents, materials, and equipment not supplied

The following list includes reagents, materials, and equipment that are required for the analysis of RAID-CRC Screen but are not included in the RAID-CRC Screen qPCR Kit.

- Eiken Chemical FIT collection tube
- DNA extraction kit (to check compatibility see Annex 1)
- Thermocycler and/or Real-Time PCR instrument (to check compatibility see Annex 2)
- Tris-HCl pH 7.4 Buffer (for primers resuspension)
- Tris-HCl pH 8.1 Buffer (for probes resuspension)
- Microcentrifuge tubes
- Strips tubes for PCR or qPCR and optical tube strips caps (8 x strip)
- Filter tips
- Vortex
- Centrifuge for 1.5 mL tubes
- Micropipettes (0.5 – 10 µL, 10 – 100 µL, and 100 – 1000 µL)
- Powder-free disposal gloves

Transport and storage conditions

RAID-CRC Screen qPCR Kits are shipped in cool conditions (2-8ºC). Upon receipt the master mix and the positive control should be stored at -15ºC to -30ºC in a constant-temperature freezer and protected from light. It is recommended to make several aliquots of the positive control to avoid undergoing more than 3 freezing/thawing cycles. Lyophilised primers and probes can be stored at room temperature until being resuspended in Tris-HCl pH 7.4 buffer for primers and pH 8.1 for probes. Once resuspended they should be stored at -15ºC to -30ºC in a constant-temperature freezer and protected from light.

Safety Information

- For professional *in vitro* use only (professional users only).
- Do not use after the expiration date.
- Design a unidirectional workflow. It should begin in the Extraction Area and then move to the Amplification and Detection Area. Do not return samples, equipment, and reagents to the area in which the previous step was performed.
- Follow Good Laboratory Practices. Wear protective clothing, use disposable gloves, protective goggles, and a mask. Do not eat, drink, or smoke in the working area. Once you finish the test wash your hands.
- Discard all the consumables and the qPCR reagents into the biological container.
- Regular decontamination of commonly used equipment is recommended, especially micropipettes and working surfaces.
- **Note**: There are no specific risks for the professional user, except those usual in an analysis laboratory.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample preparation waste.

Quality Control

In accordance with GoodGut's ISO13485-certified Quality Management System, each lot of RAID-CRC Screen qPCR Kit is tested against predetermined specifications to ensure activity, efficiency, and sensitivity. The quality certificate can be found the Professional area of GoodGut website: https://professionalarea.goodgut.eu/.

Reagents Information

Table 2. Information of the reagents included in the RAID-CRC Screen qPCR Kit.

Component	Description
Multiplex Master Mix 4X	
DNA Polymerase	DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. DNA Polymerase is provided in an inactive state and has no enzymatic activity at room temperature. The enzyme is activated by a 1-minute, 95ºC incubation step.
Multiplex qPCR Buffer	Contains Tris-HCl, KCl, NH_4Cl, $MGCl_2$, and additives enabling fast cycling.

Component	Description
dNTP mix	Contains dATP, dCTP, dGTP, and dTTP of ultra-pure quality.
Primers (forward and reverse)	Contains 6 primers sets purified using HPLC.
Probes	Contains 6 probes purified using HPLC.
Positive controls SCR	Each one contains a different pool of qPCR amplification products depending on the qPCR assay indicated, which go through a quality control process including size verification by capillary electrophoresis and sequence identification by mass spectrometry.

RAID-CRC Screen qPCR Kit accessories

GoodGut-Test™ web platform (https://goodgut-test.eu) must be used to obtain the RAID-CRC Screen diagnostic. The access to the platform is provided separately when the RAID-CRC Screen qPCR product is acquired. The user manual is provided together with a DEMO of how the web platform works to professional laboratory users.

The recommended computer configuration for the use of the GoodGut-Test™ web platform is detailed in Table 3.

Table 3. Recommended computer configuration for the use of GoodGut-Test™ web platform.

	For WINDOWS	For MAC
SCALE	125%	125%
SCREEN RESOLUTION	1920 x 1080	1920 x 1080
SCREEN ORIENTATION	Horizontal	Horizontal

Internet access is required to use the GoodGut-Test™ web platform. It can be used with Google Chrome, Google Edge, and Mozilla Firefox browsers.

RAID-CRC Screen qPCR Kit Protocol

This protocol must be followed to obtain RAID-CRC Screen results.

SAMPLE TREATMENT

Faecal samples must be treated within the first 48 hours after sample collection. Upon arrival, the sample must be collected with the Eiken Chemical FIT collector and stored between 2ºC and 8ºC. After a minimum of two days, the FIT value is analysed. Once the FIT value is known, the DNA can be

extracted, and up to 18 days may pass between collecting the sample with the FIT collection tube and extraction as long as the sample is stored between 2ºC and 8ºC.

Results obtained with the RAID-CRC Screen qPCR Kit are only reliable when using compatible DNA extraction kits and/or automated extractors (for compatibility check see Annex 1).

The sample information must be entered in the GoodGut-Test™ web platform (https://goodgut-test.eu/) following the User Manual that is provided to the user once the RAID-CRC Screen qPCR Kit has been purchased and in the professional area of the GoodGut website (https://professionalarea.goodgut.eu/). The sample information includes the requirements that must be met to be suitable for analysis and a sample code to correctly track its traceability.

qPCR PROTOCOL

The amount of primers/probe and template, as well as the parameters (temperatures (annealing), cycle number, and step times), have been optimised for an optimal yield and specificity of the multiplex assay.

Before starting, resuspend the primers and probes with the volumes of Tris-HCl indicated in the protocol (kit content section). In terms of the primers, Tris-HCl pH must be 7.4 and in terms of the probes, it must be 8.1. **Note**: For a proper resuspension of primers and probes, after the Tris-HCl buffer addition, incubate the tubes at room temperature for 1 hour or overnight at 4ºC, keeping them in the same recipient. Once resuspended they should be stored at -15ºC to -30ºC in a constant-temperature freezer and protected from light.

To obtain the diagnostic, two multiplex and a singleplex qPCR assays for each sample are required: Screen A, Screen B, and Screen C.

1. Determine and separate the number of strips and caps/tubes for required reactions including samples and controls for each qPCR assay (material not supplied in the RAID-CRC Screen qPCR Kit). A positive control and a no-template control (NTC) should be included in each qPCR assay. **Note**: Each qPCR has its own positive control.
2. Thaw Multiplex Master Mix 4X, primers, probes, and the positive controls.

Screen A qPCR multiplex assay:

3. Add the following components to a microcentrifuge tube (Table 4). We recommend preparing a n x 1.1 volumes of Mix (where n is the number of reactions), in order to minimise pipetting errors. Minimise the exposure of the fluorescent-labelled probe to light. **Note**: The number of

reactions to be performed, at the same time, must be equal to or lower than the number of reactions permitted in the thermocycler.

Table 4. Reaction mix setup for RAID-CRC Screen A qPCR assay (per reaction).

Component	Final Concentration	Volume/reaction
Multiplex Master Mix 4X	1X	2.50 µL
Primer SCR_A_f1	50 nM	0.20 µL
Primer SCR_A_r1	50 nM	0.20 µL
Probe SCR_A_FAM	60 nM	0.24 µL
Primer SCR_A_f2	300 nM	0.60 µL
Primer SCR_A_r2	300 nM	0.60 µL
Probe SCR_A_HEX	100 nM	0.20 µL
Primer SCR_A_f3	300 nM	0.60 µL
Primer SCR_A_r3	300 nM	0.60 µL
Probe SCR_A_ROX	150 nM	0.30 µL
RNase-free water	-	1.96 µL
Total reaction volume		8 µL

4. Mix the reaction gently and centrifuge it briefly. Dispense 8 µL into the qPCR tubes recommended by your thermocycler manufacturer.
5. Add 2 µL of the DNA samples to the individual qPCR tubes that contain the reaction mix. Add also 2 µL of the positive control specified for Screen A qPCR multiplex assay to the tube reserved for this control and leave a tube only with the reaction mix as a no template control (NTC). Close the qPCR tubes with the optical caps provided.

Screen B qPCR multiplex assay:

6. Add the following components to a microcentrifuge tube (Table 5). We recommend preparing a n x 1.1 volumes of Mix (where n is the number of reactions), in order to minimise pipetting errors. Minimise the exposure of the fluorescent-labelled probe to light. **Note**: The number of reactions to be performed, at the same time, must be equal to or lower than the number of reactions permitted in the thermocycler.

Table 5. Reaction mix setup for RAID-CRC Screen B qPCR assay (per reaction).

Component	Final Concentration	Volume/reaction
Multiplex Master Mix 4X	1X	2.50 µL
Primer SCR_B_f1	300 nM	0.60 µL
Primer SCR_B_r1	300 nM	0.60 µL
Probe SCR_B_HEX	100 nM	0.20 µL
Primer SCR_B_f2	250 nM	0.50 µL
Primer SCR_B_r2	250 nM	0.50 µL
Probe SCR_B_CY5	200 nM	0.40 µL
RNase-free water	-	2.70 µL
Total reaction volume		8 µL

7. Mix the reaction gently and centrifuge it briefly. Dispense 8 µL into the qPCR tubes recommended by your thermocycler manufacturer.

8. Add 2 µL of the DNA samples to the individual qPCR tubes that contain the reaction mix. Add also 2 µL of the positive control specified for Screen B qPCR multiplex assay to the tube reserved for this control and leave a tube only with the reaction mix as a no template control (NTC). Close the qPCR tubes with the optical caps provided.

Screen C qPCR singleplex assay:

9. Add the following components to a microcentrifuge tube (Table 6). We recommend preparing a n x 1.1 volumes of Mix (where n is the number of reactions), in order to minimise pipetting errors. Minimise the exposure of the fluorescent-labelled probe to light. **Note**: The number of reactions to be performed, at the same time, must be equal to or lower than the number of reactions permitted in the thermocycler.

Table 6. Reaction mix setup for RAID-CRC Screen C qPCR assay (per reaction).

Component	Final Concentration	Volume/reaction
Multiplex Master Mix 4X	1X	2.50 µL
Primer SCR_C_f1	300 nM	0.60 µL
Primer SCR_C_r1	300 nM	0.60 µL
Probe SCR_C_FAM	250 nM	0.50 µL
RNase-free water	-	3.80 µL
Total reaction volume		8 µL

10. Mix the reaction gently and centrifuge it briefly. Dispense 8 µL into the qPCR tubes recommended by your thermocycler manufacturer.
11. Add 2 µL of the DNA samples to the individual qPCR tubes that contain the reaction mix. Add also 2 µL of the positive control specified for Screen C qPCR singleplex assay to the tube reserved for this control and leave a tube only with the reaction mix as a no template control (NTC). Close the qPCR tubes with the optical caps provided.
12. Program your thermocycler according to Table 7, the 3 qPCR assays have the same program. **Note**: Selection of the specific channels (targets) for fluorogenic data acquisition can be performed during the combined annealing/extension step: FAM, HEX, and ROX for Screen A; HEX and CY5 for Screen B; and FAM for Screen C.

Table 7. Thermal cycling protocol for RAID-CRC Screen qPCR assays.

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

13. Place the qPCR tubes in the real-time thermocycler and start the run. **Note**: all the samples and controls of the same kind of qPCR assay (Screen A or Screen B or Screen C) must be analysed in the same qPCR run. In case of using more than one thermocycler and/or qPCR instrument for the analysis of the sample (i.e., Screen A analysed in thermocycler 1 and Screen B in thermocycler 2) make sure that the same model is being used.

ANALYSIS AND INTERPRETATION OF THE RESULTS

1. Perform data analysis. The analysis of the samples is done using the software of the used real-time PCR equipment according to the manufacturer's instructions for use. **Note**: Before performing data analysis, select the preestablished analysis settings for each primers + probe system (i.e., baseline settings and threshold values) according to the 'RAID-CRC Screen Technical Specifications' (this information is provided when the RAID-CRC Screen qPCR Kit is acquired and can be found in the Professional Area on the GoodGut website (https://professionalarea.goodgut.eu/)

2. To obtain the RAID-CRC Screen diagnostic, the results obtained during each multiplex qPCR assay run (including positive and negative controls) must be introduced in the GoodGut-Test™ web platform (https://goodgut-test.eu/) following the User manual. The results must be uploaded to the platform using the specific RAID-CRC Screen multiplex excel file that must contain sample code, dye, and the raw Ct data (Cq). The excel file template can be downloaded in the platform following the User manual.

Positive Control

The Positive Control is used to ensure the correct performance of the qPCR run. After setting the analysis settings, the Ct obtained in the Positive Control must be comprised within the Ct range established in the 'RAID-CRC Screen Technical Specifications'. When the Ct value of the Positive Control falls outside the accepted range values the results will not be reliable. The GoodGut-Test™ platform informs if the positive controls are accepted or rejected. If the positive controls are rejected, the sample analysis must be repeated.

No Template Control (NTC)

The No Template Control (NTC) is used to ensure that the reaction mix is not contaminated. After setting the analysis settings the Ct obtained in the NTC must be higher than the limit accepted values established in the 'RAID-CRC Screen Technical Specifications'. When the NTC Ct value is lower than the limit accepted value, the results will not be reliable. The GoodGut-Test™ web platform informs if the NTCs are accepted or rejected. If the NTCs are rejected, the sample analysis must be repeated.

Note: The lot-specific 'RAID-CRC Screen Technical Specifications' of your kit and the GoodGut-Test™ web platform User Manual will be provided separately once the kit is purchased and can also be found in the professional area of the website. GoodGut website https://professionalarea.goodgut.eu/.

If an incident occurs, defined as any failure or problem that has occurred in this *In Vitro* Medical Device during its use or later and may have serious consequences for health, please contact the manufacturing laboratory: GoodGut S.L.U. e-mail: test@goodgut.eu.

Symbol description

REF	Reference or catalogue number
VOL	Amount of liquid or reagent in the vial or bottle
	Read the instructions for use

GoodGut SRN: ES-MF-000000229

RAID-CRC qPCR Kit
Variant: RAID-CRC Screen qPCR Kit
Basic UDI-DI: 8437023437RAIDCRCKC
UDI-DI: (01)08437023437018

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The information reported in this document may vary due to continuous technological updates.

ANNEX 1: COMPATIBILITY OF THE DNA EXTRACTION KIT AND AUTOMATED EQUIPMENT

The DNA extraction kit and the automated extractors that can be used to obtain reliable diagnostics in RAID-CRC Screen are the following:

DNeasy Powersoil Pro DNA extraction kit from Qiagen (**manual extraction**)

- Kit Reference: 47014, QIAGEN
- Before proceeding with the DNA extraction, the following instructions must be followed:
    1. Homogenise FIT collector by inverting it manually several times.
    2. Transfer FIT collector content into a 1.5 mL tube (expect between 1 mL to 1.5 mL).
    3. Centrifuge the 1.5 mL tubes for 10 minutes at 4,000 xG.
    4. Discard the supernatant (expect to keep between 100 µL to 200 µL of the initial volume).
    5. Homogenise the pellet by pipetting.
    6. Transfer the pellet into the beads tube provided in the DNeasy Powersoil Pro Kit and proceed following the manufacturer's instructions. **Note**: instead of using 250 mg of soil in Step 1, introduce the volume resuspended from the FIT collector fluid.

QIAcube from Qiagen (**automated extractor**)

- Use the DNeasy Powersoil Pro DNA extraction kit from Qiagen with the automated extractor QIAcube Connect from QIAGEN.
- Before proceeding with the DNA extraction, the following instructions must be followed:
    1. Homogenise FIT collector by inverting it manually several times.
    2. Transfer FIT collector content into a 1.5 mL tube (expect between 1 mL to 1.5 mL).
    3. Centrifuge the 1.5 mL tubes for 10 minutes at 4,000 xG.
    4. Discard the supernatant (expect to keep between 100 µL to 200 µL of the initial volume).
    5. Homogenise the pellet by pipetting.
    6. Transfer the pellet into the beads tube provided in the DNeasy Powersoil Pro Kit and proceed following the manufacturer's instructions. **Note**: instead of using 250 mg of soil in Step 1, introduce the volume resuspended from the FIT collector fluid.

ANNEX 2: COMPATIBILITY OF THE REAL TIME PCR EQUIPMENT

RAID-CRC Screen multiplex/singleplex can be performed in the thermocyclers equipped with a low-profile block listed below.

AriaDx (Agilent Technologies)

- The analysis of the samples is performed with the software included in the real-time PCR equipment and according to the manufacturer's instructions for use.
- Before performing data analysis, select the preestablished analysis settings for each *primers + probe* set (i.e., baseline settings and threshold values) according the 'Technical specifications of RAID-CRC Screen qPCR Kit'.

CFX96 (BioRad)

- The analysis of the samples is performed with the software included in the real-time PCR equipment and according to the manufacturer's instructions for use.
- Specifications for analysing the results using CFX96 software:
    - Select BR White in plate type.
    - Apply the fluorescence drift correction.
- Before performing data analysis, select the preestablished analysis settings for each *primers + probe* set (i.e., baseline settings and threshold values) according the 'Technical specifications of RAID-CRC Screen qPCR Kit'.

Note: The specific 'RAID-CRC Screen Technical Specifications' for your specific lot are provided separately when acquiring the kit and are also found on the Professional area of GoodGut website https://professionalarea.goodgut.eu/.