GA-map® | **Dysbiosis Test Lx v2**

# INSTRUCTIONS FOR USE (IFU)

Dysbiosis Test Lx v2 – 96 Tests

REF 2156 (2157, 2158)

Revision 2

CE IVD

Genetic Analysis

Scan the QR-code or type in the link below to watch the **GA-map® Dysbiosis Test Lx v2 – Lab Instruction Video**!

https://qrco.de/bcP5cH

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**Please note: The test is for research use only in the US – not for use in diagnostic procedures.**

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## DEFINITIONS OF SYMBOLS

| | | | |
|---|---|---|---|
|  | Manufacturer |  | Temperature storage limitation |
| LOT | Lot number |  | Expiry date |
| PSF | Probe adjustment factor |  | Single use |
| REF | Catalogue number |  | Procedure can be paused |
| RUO | Research Use Only | CE | Meets the applicable provision according to the EU Directive 98/79/EC on IVDs |

## WARNINGS AND PRECAUTIONS

Fecal samples should be treated as potentially infectious material and require the use of BSL-2 grade laboratory equipment and precautions. This involves the use of appropriate PPE, biological safety cabinet, proper waste disposal and risk-minimizing routines for sample handling.

Appropriate skin and eye protection should be worn during usage of the DNA isolation kit (mag™ maxi, LGC genomics). Do not use bleach for decontamination of liquid waste from the extraction process.

The operators must have general skills in molecular biology laboratory techniques to perform the GA-map® Dysbiosis Test Lx v2.

## PRINCIPLE OF ANALYSIS

The GA-map® Dysbiosis Test Lx v2 is a diagnostic test that maps the intestinal microbiota profile for a selected set of bacteria and is for research use only.

The GA-map® platform uses probes that target variable regions (V3 to V7) of the bacterial 16S rRNA gene to characterize and identify bacteria present (Casén C *et al*. (2015) Aliment Pharmacol Ther.). The targets are identified in a molecular multiplex assay that utilizes the Single Nucleotide Primer Extension (SNuPE) technology patented by Professor Knut Rudi (US6617138). A unique algorithm takes advantage of all the data generated by the detection of the SnuPE products to determine dysbiosis level in the sample. The algorithm is incorporated in the GA-map® Dysbiosis Analyzer software that accompanies the test.

## INTENDED USE

GA-map® Dysbiosis Test Lx v2 is intended to be used as a fecal gut microbiota DNA analysis tool to identify and characterize dysbiosis in adults.

### Indications for use

- Determine if a sample is non-dysbiotic or dysbiotic (and degree of dysbiosis) according to cut-off given in the GA-map® Dysbiosis Test Lx v2.
  In patients where organic disorders have been ruled out, the test can be used for identification of dysbiosis in:
    - IBS samples

    - IBD samples
    - symptomatic non-IBD patients (patients with negative colonoscopy results)
- Establish a gut microbiota profile given bacteria levels compared to a normobiotic reference in:
    - IBS samples
    - IBD samples
    - Symptomatic non-IBD samples (patients with negative colonoscopy results)
    - samples with functional gastrointestinal disorders
- Monitor dysbiosis during treatment of IBS or non – IBD or IBD.

## CONTENT OF THE REAGENT KIT

The volumes provided in the GA-map® Dysbiosis Test Lx v2 kit are sufficient for 96 reactions, including controls. The reagents are provided in two boxes; Box A and Box B.

| Part number | Component name | Volume (µl) |
|---|---|---|
| **2157** | **Box A** | |
| 2205 | GA-map® PCR MasterMix | 3 × 1400 |
| 4005 | GA-map® PCR polymerase | 95 |
| 6005 | GA-map® End-Labeling polymerase | 150 |
| 2204 | GA-map® End-Labeling MasterMix | 3 × 1250 |
| 2405 | GA-map® Biotin | 3 × 88 |
| 1102 | GA-map® End-Labeling ctrl | 70 |
| 1302 | GA-map® Hybridization ctrl | 20 |
| 1402 | GA-map® Kit ctrl pos | 38 |
| 1502 | GA-map® Kit ctrl neg | 38 |
| 2404 | GA-map® ExoI | 485 |
| 2404 | GA-map® rSAP | 485 |
| **2158** | **Box B** | |
| 2411 | GA-map® dH2O | 2000 |
| 9003 | GA-map® Bead set | 84 |
| 2305 | GA-map® SAPE | 150 |
| 2426 | GA-map® Hybridization Buffer | 15000 |
| 2427 | GA-map® Detection Buffer | 30000 |

## SHELF LIFE AND STORAGE OF REAGENT KIT

The shelf life of the reagent kit is set to nine months from date of production. The expiry date is indicated on the box label and the content should not be used after the expiry date. The kit can be reused maximum three times.

| Kit box | Storage temperature | Maximum freeze-thaw cycles |
|---|---|---|
| GA-map® Dysbiosis Test Lx v2, Box A | <-15°C | 3* |
| GA-map® Dysbiosis Test Lx v2, Box B** | 2-8 °C | NA |

* The GA-map® PCR MasterMix, End-Labeling MasterMix and Biotin are provided in three single-use aliquots.
** The GA-map® Hybridization buffer and Detection buffer should be equilibrated to ambient temperature before use.

## MATERIALS REQUIRED BUT NOT PROVIDED

### General materials and equipment

- 10µl, 100µl and 1000µl single channel precision pipettes with disposable tips
- 10µl, 100µl and 300µl multichannel precision pipettes with disposable tips
- Dispenser pipette w/disposable tips (volumes to be dispensed are 20, 40, 200, 270, 720µl)
- Centrifuge for quick spin down of 96 well PCR plate
- Microcentrifuge for quick spin down of reagents and microcentrifuge tubes
- Vortex mixer with speed of ~2800 rpm
- Microcentrifuge tubes with 1.5 and/or 2ml, and 5ml capacity
- Tube for mixing of larger volumes up to 50ml
- Microtiter sealing tape for 96-well plate
- Reagent reservoir with capacity of ≥25ml
- Ice or cooling elements for keeping reagents cold on bench
- Freezing block for use during handling of enzymes

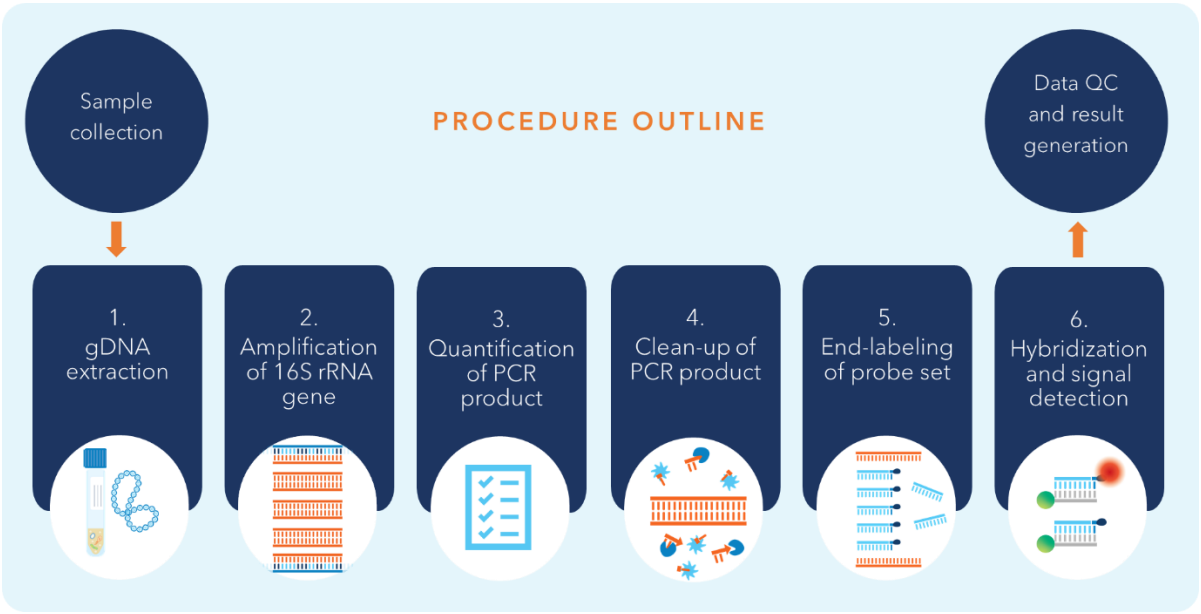
### Specific materials and equipment

Equipment and materials that are specific for each of the steps in the procedure are listed in the Lab Procedure section.

# GENERAL ASSAY INFORMATION

## PROCEDURE OUTLINE

The GA-map® Dysbiosis Test Lx v2 lab procedure is divided into six main steps, which are described in more detail in the Lab Procedure section. The outline and instructions must be followed carefully.



## SAMPLE STORAGE

The recommended storage conditions for fecal samples depend on the type of starting material selected; dry/native sample or sample on eNAT™ buffer.

| Sample material | Storage temperature | Maximum storage time | Maximum freeze-thaw cycles |
|---|---|---|---|
| Dry fecal sample | RT | 5 days plus<br>12h at ≤-15°C * | 1 |
| Fecal sample on eNAT™ buffer | RT (<40°C)** | 14 days | NA |
| | 2-8°C | 4 weeks | NA |
| | <-15°C | Prolonged storage | 2 |

* Dry fecal sample must be stored for minimum 12h at ≤-15°C prior to genomic DNA (gDNA) extraction.
** Samples on eNAT™ buffer should be kept at +4°C after reception. For storage >4 weeks, the tubes should be frozen ≤-15°C.

The sample intermediates are products of the different steps in the lab procedure. The procedure can be paused at several different steps, as indicated by the maximum storage time of the sample intermediates. The procedure description also contains several "procedure can be paused" symbols, which indicate possibility for overnight pauses.

| Sample intermediate | Storage temperature | Maximum storage time | Maximum freeze-thaw cycles |
|---|---|---|---|
| gDNA undiluted and 1:50 dilution | 2-8°C | 3 weeks | NA |
| | <-15°C | Prolonged storage | 9 |
| 16S rRNA PCR product | 2-8 °C | 48 hours | NA |
| 16S rRNA PCR product, ExoI/rSAP-treated | 2-8 °C | 7 days | NA |
| End-Labeling product | 2-8 °C | 2.5 hours | NA |
| | <-15°C | 2 weeks | 1 |
| Hybridization product | RT, protected from light | 24 hours | NA |

## ASSAY QUALITY CONTROL SCHEME

An overview of the different controls included in the assay (both required and optional) is given in the table below.

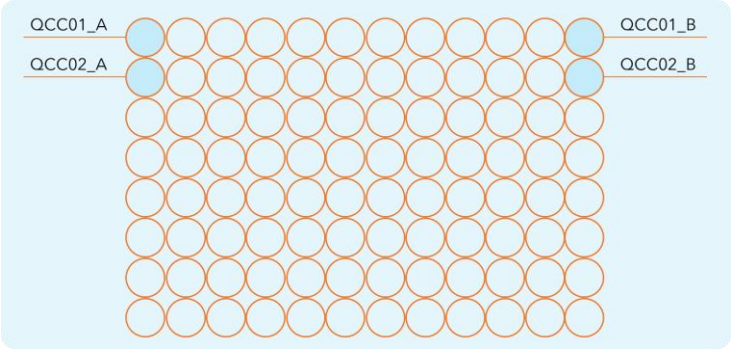
| Control name | Control ID | Comment | Included from step |
|---|---|---|---|
| Extraction control positive | QCC01 | Fecal sample (optional) | Step 1 – Genomic DNA extraction |
| Extraction control negative | QCC02 | Option A: S.T.A.R. buffer<br>Option B: Lysis Buffer BLM | Step 1 – Genomic DNA extraction |
| GA-map® Kit ctrl pos | QCC23 | Included in kit | Step 2 – Amplification of the bacterial 16S rRNA gene |
| GA-map® Kit ctrl neg | QCC33 | Included in kit | Step 2 – Amplification of the bacterial 16S rRNA gene |

| | | | |
|---|---|---|---|
| PCR control negative | QCC05 | The water used to dilute gDNA | Step 2 – Amplification of the bacterial 16S rRNA gene |
| End-Labeling control negative | QCC29 | Use GA-map® dH2O | Step 5 – End-Labeling of probe set |
| GA-map® End-Labeling ctrl | QCC30 | Included in kit | Step 5 – End-Labeling of probe set |
| GA-map® Hybridization ctrl | HYC01 | Included in kit; added to all samples and controls | Step 6 – Hybridization and signal detection |

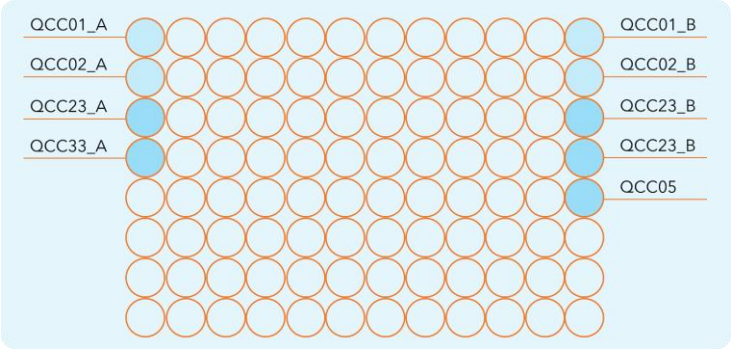
## PLATE SETUP

Assay controls should be arranged in the 96-well microtiter plates as illustrated in the figure below. The samples can be placed in any other well on the plate. All steps should be performed by working in columns, from column 1 to column 12, and an 8-channel pipette should be used whenever applicable. The arrangement and naming of assay controls is especially important during step 6 of the assay, as the result-generating software requires these controls to perform data QC.

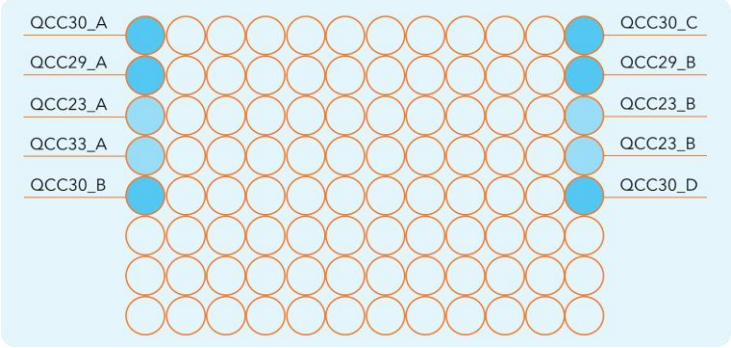
### Step 1 - Genomic DNA extraction




### Step 2 - Amplification of the bacterial 16S rRNA




### Step 5 - End-Labeling of Probe set and Step 6 - Hybridization and signal detection



### GENERAL PROCEDURE NOTES

- Before start, it is recommended that the user confirms that required equipment, materials and reagents are available for each step of the procedure.
- A plate setup with controls and samples should be prepared for each step of the procedure and reagent volumes should be calculated according to the sample number. All reagents in the kit are provided in at least 10% access to allow for surplus volume when making mastermixes.
- Appendix 4 – GA-map® Dysbiosis Test Lx v2 Run log (available upon request) can be used for plate setup and volume calculation.
- Components from different lots of the GA-map® Dysbiosis Test Lx v2 kit should not be mixed.
- Reagents from Box A, except for enzymes, should be thawed on ice before use and kept on ice during handling.
- All enzymes should be kept on a freezing block during handling. Due to high viscosity, the enzymes should be briefly spun down (not vortexed) before use, then slowly aspirated without pipetting up and down.
- The reagents from Box B should be kept at ambient temperature (15 to 25°C) during handling.
- Before use, all reagent tubes except enzymes should be vortexed at 2800 rpm for 3 seconds and briefly spun down.
- For all centrifugation steps (tubes and plates), unless otherwise specified, use a brief spin down up to 100 x g.
- The 96-well plates and reagents from Step 2 - Amplification of the bacterial 16S rRNA gene to Step 5 - End-Labeling of Probe set should be kept on ice during handling.
- The 96-well plates and all reagents except for the GA-map® Hybridization ctrl (HYC01), in Step 6 - Hybridization and Signal detection should be kept at ambient temperatures.

## SAMPLE COLLECTION

### SAMPLING PROCEDURE

Sample collection kits can be obtained from Genetic Analysis AS. Alternatively, it is recommended to use a commercial fecal sampling kit that includes the necessary material. Two different sample collection systems are available for use with the GA-map® Dysbiosis Test Lx v2:

**Option A**: Dry sampling

Use the dry sample collection kit from Genetic Analysis AS or the equipment listed below:

- Sampling tube, CE marked, empty (sterile, 20-30ml, no prefilled buffer).
- Sampling spoon (sterile/clean, often the spoon is attached to the lid of the sampling tube).
- Fecal sampling container, e.g. sampling tray or Med Auxil sampling paper (sterile/clean).

Sampling procedure (Option A):

1. Sampling can be performed at any convenient place, at any time of the day.
2. Sampling shall be done from one single bowel movement.
3. Collect the fecal sample in a collecting unit. Do not to mix urine with the fecal sample, and never collect the sample directly from the toilet.
4. With a sterile spoon, collect 10-15ml feces into the sampling tube from at least 10 different sites of the fecal portion.
5. Tighten the tube well.

6. Mark the tube with name/ID and sampling date.
7. Secure the sample by placing the tube into a secondary container (tube or bag with absorbing unit).
8. Place the secured sample in an addressed envelope/bag/box and ship the sample as directed.

**Note!** Dry fecal samples should be stored at ambient temperature (15-25°C) for no more than 5 days before freezing.

**Option B**: Buffer-based sampling

Use the buffer-based sample collection kit from Genetic Analysis AS or the equipment listed below:

- eNAT™ tube w/ regular FLOQSwab from Copan, cat no: 608CS01R (1ml) or 606CS01R (2ml)
- Fecal sampling container, e.g. sampling tray or Med Auxil sampling paper (sterile/clean).

Sampling procedure (Option B):

1. Sampling can be performed at any convenient place, at any time of the day.
2. Sampling shall be done from one single bowel movement.
3. Collect the fecal sample in a collecting unit. Do not to mix urine with the fecal sample, and never collect the sample directly from the toilet.
4. Open the package containing sample tube and swab and remove the swab stick holding above the red line to avoid contamination.
5. Dip the swab into the sample and roll until the swab is saturated with a thin layer of material. Do not over-saturate the swab! Only a small amount of sample material is needed for analysis.
6. Place the swab inside the sample tube and break off the top part of the stick (above the red line). Securely close the tube by tightening the cap and shake until the sample appears homogenous.
7. Mark the tube with name/ID and sampling date.
8. Secure the sample by placing the tube into a secondary container (tube or bag with absorbing unit).
9. Place the secured sample in the addressed envelope/bag/box and ship the sample as directed.

**Note**! After sampling, fecal samples on eNAT™ buffer can be stored at 2-8 °C for up to 4 weeks, or at ambient temperature for up to 14 days before analysis.

**Note**! Other types of transport buffers/reagents are not validated for use with the GA-map® Dysbiosis Test Lx v2. Use of other buffer types may result in a bias in the results.

## SAMPLE RECEIPT AT SITE OF ANALYSIS

Upon receipt of fecal samples, the shipment should be checked for leakage from the tubes or damage, and visually assessed for presence of mucus and blood. Storing of the fecal samples is specific for the sample collection system used:

**Option A**: Dry sampling

- Must be stored at ≤-15°C prior to genomic DNA (gDNA) extraction, as these samples require one freeze-thaw cycle prior to gDNA extraction.

**Option B**: Buffer-based sampling

- Samples collected on eNAT™ buffer should be stored at 2-8 °C or at ambient temperatures (<40°C) until analysis.
- If the recommended time from sampling to analysis will be exceeded, the sample tube should be frozen at ≤-15°C upon receipt.

## LABORATORY PROCEDURE

### STEP 1 - GENOMIC DNA EXTRACTION

The preparation of samples and addition of negative extraction controls is specific for the sample collection system used:

**Option A**: Dry sampling, see specific protocol and notes in the following section.
**Option B**: Buffer-based sampling see specific protocol and notes in the following section.

Specific materials and reagents required are listed in the table below.

| Materials and equipment | Requirement | Recommended alternative |
|---|---|---|
| Bead beater | FastPrep-96™ Homogenizer w/96-well plate insert (116010500), MP Biomedicals | |
| DNA extraction robot | KingFisher Flex - 96 deep well head (5400630), w/magnetic micro-plate Separator, installed with KF Flex 96 KF heating block (24075420), ThermoFisher | MagMAX™Express-96, w/magnetic micro-plate Separator (4456933), ThermoFisher |
| Water bath | Capacity: deep well 96 plate<br>Temperature: 65°C | Any |
| Centrifuge | Capacity: Lysing Matrix-096 E well plate (plate height 6 cm) (1300 x g) | Any |
| Centrifuge* | Any for 2ml tubes (2500 x g) | Any |
| Decapper | For lysing Matrix-E screw lids | 8-channel Screw Cap Decapper (4105MAT), ThermoFisher |
| Lysing Matrix-E tubes | Lysing Matrix-E tubes (2450), Genetic Analysis | |
| Deep well plate | KingFisher Deepwell 96 Plate (95040450 or A48305 or 95040455), ThermoFisher | |
| Deep well tip comb | KingFisher 96 tip comb for DW magnets (97002534 or A48438), ThermoFisher | |
| Elution plate | KingFisher 96 KF microplate (97002540), ThermoFisher | |
| Microtiter plate for dilution | Capacity: 96-well, ≥250µl | Any |
| Adhesive PCR plate seals | For 96-well plate | Adhesive PCR Plate Seals, (AB0558), ThermoFisher |
| Stirring rod* | | Any |
| Pipette tip, wide orifice | | Any |
| DNA extraction reagent kit | mag™ maxi (LGC DNA isolation kit) 288 tests, (40430), LGC Genomics | |
| Ethanol | 96 % rectified | Any |
| S.T.A.R buffer* | S.T.A.R buffer 300ml, (03 335 208 001), Roche | |

| | | |
|---|---|---|
| Sterile, Nuclease free water (H2O) | | Any |
| Extraction control positive (optional)* | | Fecal sample |
| Extraction control negative, **Option A**: dry samples (QCC02) | S.T.A.R buffer 300ml, (03 335 208 001), Roche | |
| Extraction control negative, **Option B**: samples on eNAT™ buffer (QCC02) | Lysis buffer BLM, (from mag™ maxi), LGC Genomics | |

*Not applicable if using the buffer-based option for sample collection (Option B)

## Preparing the sample and reagents for gDNA extraction

**Note!** Protease from LGC DNA isolation kit must be prepared in accordance with LGC DNA isolation kit instruction before use. Thaw on ice before use. The BML2 buffer must be prepared by adding Acetone in accordance with LGC DNA isolation kit instruction before use.

**Option A**: Preparation of dry samples

1. Take out frozen samples to thaw for 30-120 minutes
2. Prepare one 2.0ml test tube containing 1200µl S.T.A.R. buffer per fecal sample to be extracted (including additional tubes for positive and negative extraction controls).
3. Mix feces with a stirring rod to make the sample homogeneous.
4. Using an inoculating loop, transfer feces equivalent of 400µl displaced buffer to the 2.0ml test tube containing 1200µl S.T.A.R. buffer, to increase the total volume in the test tube to 1600µl.
5. Vortex sample tubes well.
6. Incubate at room temperature for 60 minutes. Use the 60 minutes incubation period to prepare plates for extraction.
7. Vortex and centrifuge fecal tubes briefly by pulsing up to approximately 2500 x g.
8. Transfer 600µl sample supernatant to Lysing Matrix-E tubes.
9. Continue with Performing gDNA extraction, step 1.

**Option B**: Preparation of samples on eNAT™ buffer

1. If frozen, samples on Copan eNAT™ tubes should be thawed for ≥15 minutes.
2. Vortex the sample tubes to make feces homogeneous.
3. If using 1ml Copan eNAT™ tubes: Add 200µl Elution buffer to the Lysing Matrix-E tubes and then transfer 400µl sample from the Copan tubes to the Lysing Matrix-E tubes.
   **OR:**
   If using 2ml Copan eNAT™ tubes: Transfer 600µl sample from the Copan tubes to the Lysing Matrix-E tubes.
4. Continue with Performing gDNA extraction, step 1.

## Performing gDNA extraction

1. Make sure to balance the FastPrep-96. Process the Lysing Matrix-E tubes with samples twice in the FastPrep-96 at 1800 rpm for 40 seconds with a 40 second pause between runs.
2. Centrifuge samples at 1300 x g for 5 minutes in a deep well plate centrifuge.
3. Prepare Lysis protease mix according to table below. Mix by pipetting.

**Note!** If using samples collected on eNAT™ buffer, remember to include an additional well for negative extraction control.

| Component in Lysing mix | µl per well |
|---|---|
| Lysis buffer BLM (LGC Genomics) | 250 |
| Protease (LGC Genomics) | 20 |
| **Total volume** | **270** |

4. Transfer 270µl Lysis protease mix to all wells according to plate setup.
5. Transfer 250µl sample supernatant from the centrifuged samples to the wells with Lysis protease mix. Mix well by pipetting.

**Note!** When analyzing samples collected on eNAT™ buffer, 250µl Lysis buffer BLM is added as negative extraction control.

6. Cover plate with adhesive PCR film. Make sure the film is thoroughly sealed.
7. Carefully place the deep well plate to float in the water bath and incubate at 65°C for 15 minutes.
8. During the incubation, finalize preparation of buffer plates according to the table below and the plate setup. Seal the plates with microtiter sealing tape until use.

| Plate Type | Plate # | Content | Volume |
|---|---|---|---|
| Deep well Plate | 1 | MagMAX particles (vortex thoroughly before use) | 20µl |
| | | Ethanol | 200µl |
| | 2 | Washing buffer BLM 1 | 720µl |
| | 3 | Washing buffer BLM 2* | 720µl |
| | 4 | Washing buffer BLM 2* | 720µl |
| Elution plate | 5 | Elution buffer BLM | 200µl |
| Elution plate | 6 | Tip comb placed in KF microplate | N/A |

9. Remove the deep well plate from the water bath. If any drop of buffer is visible under the sealing tape after incubation, pulse spin before removing the film.
10. Add 400µl protease treated sample to plate#1 according to plate setup. Mix well by pipetting.
11. Turn on the magnetic separator, find the GAmap program and press Start. The robot will request plate#6 first, then plate#5 and so on. Unseal the plates before placing them in the machine. The procedure will take approximately 40 minutes.

| Instrument name | Program name | Program settings |
|---|---|---|
| MagMAX™Express-96 | GAmap_v1 | Appendix 1 (available upon request) |
| KingFisher Flex | GAmap_v2 | Appendix 2 (available upon request) |

12. When the program is finished, remove plate#5 (Elution plate). Seal the plate with microtiter sealing tape and centrifuge the plate for 1 minute at 1000 x g. Place the plate on ice.
13. Discard plates #1, #2, #3, #4, and #6.
14. From plate#5, transfer 100µl of the gDNA (avoid transferring the brown debris in the bottom) into a 96-well microtiter plate.

15. Dilute the gDNA 1:50 by mixing 5µl gDNA and 245µl nuclease free water. Mix well.

If pausing at this step, the gDNA can be stored at 2-8°C for up to 3 weeks (or alternatively at ≤-20°C for long term storage).

## STEP 2 - AMPLIFICATION OF THE BACTERIAL 16S RRNA GENE

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step and other specific materials and reagents required are listed in the tables below.

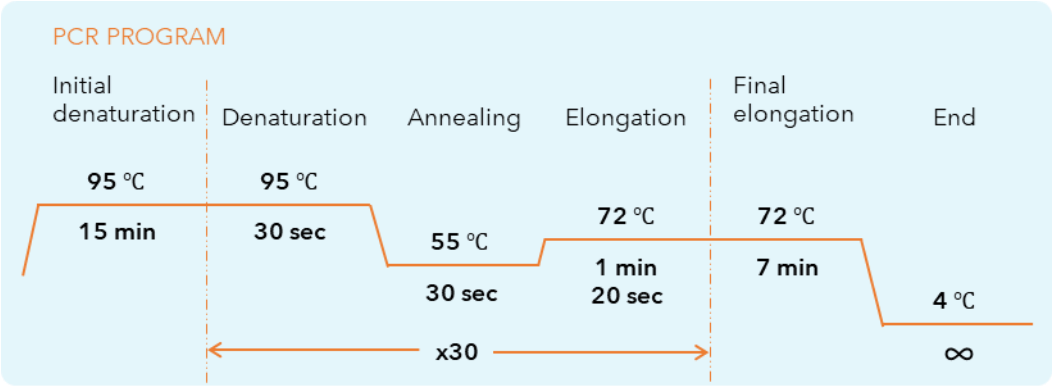
| GA-map® reagent | Storage | Handling method |
|---|---|---|
| GA-map® PCR MasterMix ② | GA-map® Dysbiosis Test Lx v2, Box A | Thaw on ice before use, keep on ice during handling. |
| GA-map® Kit ctrl pos (QCC23) | | |
| GA-map® Kit ctrl neg (QCC33) | | |
| GA-map® PCR polymerase | GA-map® Dysbiosis Test Lx v2, Box A | Keep on a freezing block during handling. |
| **Other materials and equipment** | **Requirement** | **Recommended alternative** |
| Thermal cycler | Veriti™ 96-Well Thermal Cycler, ThermoFisher **or** T100 Thermal Cycler, Bio-Rad Laboratories | Other systems must be validated for GA-map® |
| 96-well microtiter plate | PCR grade, with suitable 8-cap strips | Any |
| PCR control negative | Sterile, Nuclease-free H2O, same as used for gDNA dilution | Any |

### Performing the amplification of bacterial 16S rRNA gene

1. Keep thawed reagents and the diluted gDNA on ice/ice block.
2. Prepare the PCR mix according to the table below. Mix by vortexing and briefly spin down.

| Component in PCR mix | µl per well |
|---|---|
| GA-map® PCR MasterMix | 19.25 |
| GA-map® PCR polymerase | 0.75 |
| **Total volume** | **20.0** |

3. Dispense 20µl of the PCR mix into a 96-well microtiter PCR plate according to plate setup. Keep on ice.
4. Add to the PCR plate according to plate setup:
   - 5µl of the 1:50 diluted gDNA (samples and controls)
   - 5µl of the kit controls and PCR control negative
5. Mix well by pipetting.
6. Cover the plate with lid PCR 8-strips.
7. The plate can be vortexed to ensure proper mixing.
8. Briefly pulse spin the plate up to 100 x g.
9. Initiate the PCR-program and load the plate on the thermal cycler.




If pausing at this step, the PCR-product can be stored at 2-8°C for up to 48 hours.

## STEP 3 - QUANTIFICATION OF PCR PRODUCT

The quality of the PCR product must be assessed to ensure optimal performance in further downstream processes. This quality control step is important for the functionality of the GA-map® Dysbiosis Test Lx v2.

The quantitative method used for measuring dsDNA is optional, but the recommended material and equipment is presented in the table below.

| Materials and equipment | Recommendations |
|---|---|
| DNA quantification system | FLUOstar OMEGA Microplate Reader, BMG LabTech |
| | Qubit™ 3.0 Fluorometer, ThermoFisher |
| Assay for quantitative DNA measurements | Quant-iT™ 1X dsDNA HS Assay (Q33232), ThermoFisher |
| | Quant-iT™ PicoGreen™ dsDNA Assay Kit (P11496), ThermoFisher |
| | Qubit™ dsDNA HS assay kit (Q32854), ThermoFisher |

Note that the PCR yield should be within the concentration limits presented below.

| Sample type | Lower limit | Upper limit |
|---|---|---|
| Fecal samples and positive controls (QCC01, QCC23 and QCC33) | ≥17 ng/µl | <50 ng/µl |
| Negative controls (QCC02 and QCC05) | - | <5 ng/µl |

See Troubleshooting section if PCR product is confirmed to be outside the lower or upper limits.

## STEP 4 - CLEAN-UP OF PCR PRODUCT

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step and other materials and reagents required are listed in the tables below.

| GA-map® reagent | Storage | Handling method |
|---|---|---|
| GA-map® rSAP | GA-map® Dysbiosis Test Lx v2, Box A | Keep on a freezing block |
| GA-map® ExoI | | |
| **Other materials and equipment** | **Requirement** | **Recommended alternative** |
| Thermal cycler | Veriti™ 96-Well Thermal Cycler, ThermoFisher **or** T100 Thermal Cycler, Bio-Rad Laboratories | Other systems must be validated for GA-map® |

### Performing clean-up of PCR product

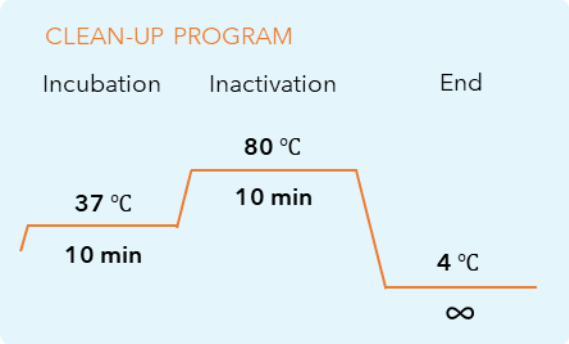
1. Vortex and briefly spin down the 96-well plate containing 16S rRNA PCR product.
2. Keep thawed reagents and the PCR product on ice/ice block.
3. Prepare the ExoI/rSAP mix according to the table below. Mix by pipetting and briefly spin down.

| Component in ExoI/rSAP mix | µl per µl PCR product | Example: µl per well when using 23µl PCR product |
|---|---|---|
| GA-map® rSAP | 0.2 | 4.6 |
| GA-map® ExoI | 0.2 | 4.6 |
| **Total volume** | **0.4** | **9.2** |

4. Add the appropriate volume of ExoI/rSAP mix to the PCR products. Mix by pipetting.
5. Cover the plate with PCR 8-strips.

**Note!** Due to the viscosity of the ExoI and rSAP enzymes, additional mixing using a vortexer is recommended. Insufficient mixing may lead to a bias in the results!

6. Briefly pulse spin the plate to 100 x g.
7. Initiate the clean-up program and load the plate on the thermal cycler.




If pausing at this step, the ExoI/rSAP-treated PCR product can be stored at 2-8°C for up to 7 days.

## STEP 5 - END-LABELING OF PROBE SET

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step and other materials and reagents required are listed in the tables below.

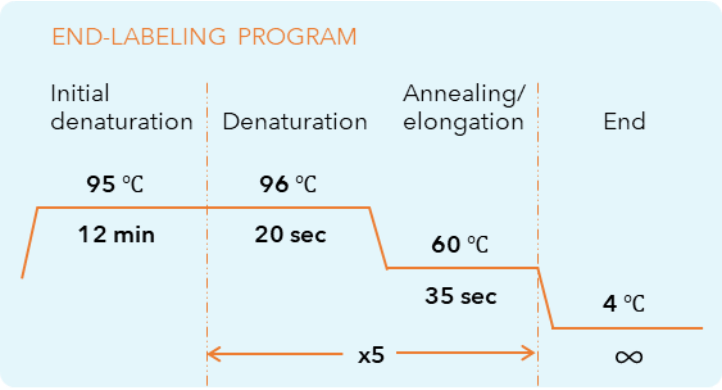
| GA-map® reagent | Storage | Handling method |
|---|---|---|
| GA-map® End-Labeling MasterMix (2) | GA-map® Dysbiosis Test Lx v2, Box A | Thaw on ice before use, keep on ice during handling |
| GA-map® Biotin (2) | | |
| GA-map® End-Labeling ctrl (QCC30) | | |
| GA-map® End-Labeling polymerase | GA-map® Dysbiosis Test Lx v2, Box A | Keep on a freezing block during handling |
| GA-map® $dH_2O$ (QCC29) | GA-map® Dysbiosis Test Lx v2, Box B | Keep on ice during handling |
| **Other materials and equipment** | **Requirement** | **Recommended alternative** |
| Thermal cycler | Veriti™ 96-Well Thermal Cycler, ThermoFisher **or** T100 Thermal Cycler, Bio-Rad Laboratories | Other systems must be validated for GA-map® |
| 96-well microtiter plate | PCR grade, with suitable 8-cap strips | Any |

### Performing End-Labeling of probe set

1. Vortex and briefly spin down the 96-well plate containing ExoI/rSAP-treated PCR product.
2. Keep thawed reagents and the ExoI/rSAP-treated PCR product on ice/ice block.
3. Prepare End-Labeling mix according to the table below. Mix by vortexing and briefly spin down.

| **Component in End-Labeling mix** | **µl per well** |
|---|---|
| GA-map® End-Labeling MasterMix | 17.5 |
| GA-map® Biotin | 1.25 |
| GA-map® End-Labeling polymerase | 1.25 |
| **Total volume** | **20** |

4. Dispense 20µl of the End-Labeling mix into a 96-well microtiter PCR plate according to plate setup. Keep on ice.
5. Add to the End-Labeling plate according to plate setup:
   - 5µl of the ExoI/rSAP-treated PCR samples and kit controls
   - 5µl of the End-Labeling controls
6. Mix well by pipetting.
7. Cover the plate with PCR 8-strips.
8. Briefly pulse spin the plate to 100 x g.
9. Initiate the End-Labeling program and load the plate on the thermal cycler.




10. While the samples are in the thermal cycler, proceed to preparation for Step 6 – Hybridization and Signal detection.
11. Directly after completion of the End-Labeling program, transfer the PCR plate from the cycler to an ice block.

**NOTE!** The End-Labeling product is labile. To prevent unspecific signals, keep the End-Labeling product on an ice block during handling and storage. Ensure that the time between End-Labeling (this step) and Hybridization (next step) is less than 2.5 hours!

Alternatively, if stopping at this step, the End-labeling product can be stored at -20°C for up to two weeks.

## STEP 6 - HYBRIDIZATION AND SIGNAL DETECTION

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step and other materials and reagents required are listed in the tables below.

| GA-map® reagent | Storage | Handling method |
|---|---|---|
| GA-map® Hybridization ctrl (HYC01) | GA-map® Dysbiosis Test Lx v2, Box A | Thaw on ice before use, keep on ice during handling |
| GA-map® $dH_2O$ | GA-map® Dysbiosis Test Lx v2, Box B | Ambient temperature (15-25°C) |
| GA-map® Bead set | | |
| GA-map® SAPE | | |
| GA-map® Hybridization Buffer | | |
| GA-map® Detection Buffer | | |
| **Materials and equipment** | **Requirement** | **Recommended alternative** |
| Thermal cycler | Veriti™ 96-Well Thermal Cycler, ThermoFisher **or** T100 Thermal Cycler, Bio-Rad Laboratories | Other systems must be validated for GA-map® |
| Signal detection platform | Luminex® 200™ w/ xPONENT software 4.2 or higher | |
| Magnetic ring plate separator | 96-well with minimum capacity of 100µL and | Permagen™ 96-Well Ring Magnet Plate (S380) |

| | | |
|---|---|---|
| | compatible with v-bottom plates | |
| 96-Well Polycarbonate PCR Microplates | Corning™ Thermowell™ 96-Well Polycarbonate PCR Microplates, Model P (6509), Corning/Costar | |
| Sealing film | Microseal® 'A' (MSA5001), Bio-Rad Laboratories | |

## Performing Hybridization and SAPE-labeling

**NOTE!** All reagents required for this step (except for the GA-map® Hybridization ctrl) are to be kept at ambient temperature. The hybridization plate should also be kept at ambient temperature during preparation and during the washing steps. Lower temperatures may lead to a drop in signal intensities.

1. Vortex and briefly spin down the 96-well plate containing End-Labeling product. Keep on ice/ ice block.
2. Make sure the Luminex® 200 system is calibrated and ready for use.
3. Prepare the Hybridization ctrl (HYC01) dilution according to the table below. Choose "Option 1" if the total number of wells is below 50 and "Option 2" if the total number of wells exceeds 50.

**Note!** Hybridization ctrl dilution should be fresh. Make new dilution for each run.

| Component in Hybridization ctrl (HYC01) dilution | Option 1 (<50 wells) µl | Option 2 (>50 wells) µl |
|---|---|---|
| GA-map® $dH_2O$ | 495 | 990 |
| GA-map® Hybridization ctrl (HYC01) | 5 | 10 |
| **Total volume** | **500** | **1000** |

4. Prepare the Hybridization bead mix according to the table below.

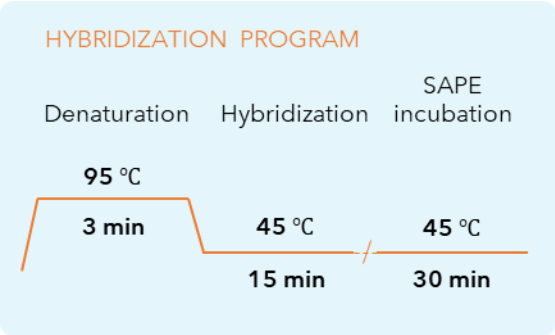
**Note!** The GA-map® Bead set must be resuspended by vortexing for ≥10 seconds.

| Component in Hybridization bead mix | µl per well |
|---|---|
| GA-map® Hybridization Buffer | 32.2 |
| Hybridization ctrl (HYC01) dilution | 7 |
| GA-map® Bead set | 0.8 |
| **Total volume** | **40** |

5. Resuspend the prepared Hybridization bead mix by vortexing for 10 seconds.
6. Dispense 40µl of the Hybridization bead mix in a 96-well Corning® microplate according to plate setup. Vortex the Hybridization bead mix thoroughly between each 24 wells.
7. Transfer 10µl of the End-Labeling product to the Corning® microplate according to plate setup. Mix well by pipetting.

**Note!** Due to the different salt concentrations of the reaction components, additional mixing is recommended. Mix by pipetting up and down minimum 6 times upon distribution of the End-Labeling product. Insufficient mixing may lead to a bias in the results!

8. Cover the plate with Microseal® 'A' film.
9. Initiate the Hybridization program and load the plate into the thermal cycler. Set a timer to 18 minutes.




10. Prepare fresh Reporter mix according to the table below.

| Component in Reporter mix | µl per well |
|---|---|
| GA-map® Hybridization Buffer | 23.8 |
| GA-map® SAPE | 1.2 |
| **Total volume** | **25** |

11. After completion of the 18 minutes (Denaturation and Hybridization), keep the plate at 45°C in the thermal cycler and add 25µl of the Reporter mix to each well. Mix gently by pipetting.
12. Cover the plate with a new Microseal® 'A' film, close the lid and leave the program running for an additional 30 minutes.

## Performing washing and signal detection

1. While the plate is incubating, prepare for scan on the Luminex 200:
    a. In xPONENT, create a new batch from the protocol "GA-map Dysbiosis v2" version 1.0 (Appendix 3, available upon request).
    b. Import sample and control IDs to the respective wells and optional additional information.
    c. Note that some wells in columns 1 and 12 are reserved for controls: ensure the names of the control IDs are correct.
    d. Make sure the sample and control IDs correspond to the correct positions on the plate.

**Note!** The plate wash is a time sensitive step. Limit the time the wells are left without buffer to ≤3 minutes. Beads drying out may lead to signal loss!

2. When the 30 minutes reporter hybridization step has completed, take the plate out of the thermal cycler and place on a magnetic ring plate separator for 60 seconds.
3. After the beads have collected on the side of the wells, carefully remove the hybridization/ reporter solutions.
4. With the plate still positioned on the magnet, add 75µl of GA-map® Detection Buffer and remove the buffer shortly afterwards.
5. Remove the plate from the magnetic ring plate and add 75µl of GA-map® Detection Buffer to each well. Mix by pipetting until all beads are resuspended (approximately 10 times).

**Note!** Insufficient resuspension after plate wash may lead to low bead count errors. Perform a visual check of the plate to ensure the beads are properly resuspended. In case of a visible band of beads across the wells, repeat the resuspension process in step 5 above.

Prior to scan, the Hybridization-product can be stored at ambient temperature for up to 24 hours in the dark. Resuspend the beads by pipetting up and down a few times before continuing the process.

6. Move the plate to the Luminex analyzer.
7. Click the Run button to start analysis and confirm by pressing OK.
8. When the run is complete, export the results in a .csv format:
    a. From the Results > Saved Batches tab, select the batch and click Exp Results.
    b. Save file in the comma separated values (.csv) format. This file can be processed in the GA-map® Dysbiosis Analyzer software for quality check and result generation.
9. Remove the plate and discard it according to local regulations.

# RESULT GENERATION AND INTERPRETATION

## DATA QC AND RESULT GENERATION

The GA-map® Dysbiosis Analyzer software analyses the CSV files generated by the Luminex 200 instrument ("Lx200", Luminex Corp.) and performs the following tasks:

1. **Plate QC check**: The GA-map® Dysbiosis Analyzer software checks that all the required quality controls are present in the uploaded csv file and that the plate QC parameters are met. It is therefore important to use the controls and names specified in the Plate setup section. If the plate QC check does not pass, the plate is invalid, and no sample results can be reported from the run.

2. **Sample QC check**: If the plate is found valid, the individual samples are evaluated. Samples that do not meet the raw data signaling level QC parameters will be flagged as invalid.

3. **Data normalization and result generation for each sample:**
    a. DI score
    b. Bacteria Abundance Scores
    c. Bacteria profiles

4. **Report generation**: For all samples with a valid QC status, a report can be generated.

Please refer to the GA-map® Dysbiosis Analyzer Software Manual for detailed instruction on how to use the software and generate reports.

## INTERPRETATION OF RESULTS

Several report form templates are available with the GA-map® Dysbiosis Test Lx v2. Generally, the test results are presented in three levels as described below. The Dysbiosis Index (DI) scale is CE-marked, while the Bacteria Profiles and Bacteria Abundance Table are not. Refer to the report form supplements for a more detailed description of each section.

**Dysbiosis Index scale**: To determine the degree of dysbiosis compared to a normal healthy reference population, the test result is given as a Dysbiosis Index score (DI) consisting of five levels ranging from 1 to 5, where 1 and 2 are non- dysbiotic and 3 to 5 are dysbiotic. DI score 3 is considered mildly dysbiotic, while scores 4 to 5 are severely dysbiotic.

**Bacteria Profiles**: Several different functional profiles are reported, each representing a set of unique bacteria signatures. The profiles are reported as either Balance or Imbalance followed by a comment.

**Bacteria Abundance Table**: The Bacteria Abundance Score of 48 preselected bacteria markers relative to the value of a normal healthy reference population. The relative abundance of each bacteria marker can be normal, reduced or elevated.

## PERFORMANCE CHARACTERISTICS

### ANALYTICAL PERFORMANCE CHARACTERISTICS

Precision: Precision analysis for GA-map® Dysbiosis Test Lx v2 show acceptable levels of repeatability and reproducibility. All samples had a standard deviation ≤0.2 for DI score.

### DIAGNOSTIC PERFORMANCE CHARACTERISTICS

An independent validation study has been performed to investigate the classification performance of GA-map® Dysbiosis Test Lx v2. The validation study comprised a total of 213 IBD and non-IBD samples from fecal sample recruitment hospitals in Norway, Sweden and Denmark. A separate clinical validation on a healthy normal cohort collected in Germany was performed using 54 fecal samples. All samples were from unique feces donors not included in previous development of GA-map® Dysbiosis Test Lx v2.

The performance characteristics (distribution of normobiotic and dysbiotic per sample category) are shown below.

| | IBD | non-IBD | Healthy normal |
|---|---|---|---|
| No of samples DI>2 | 87 | 65 | 9 |
| No of samples DI<2 | 33 | 28 | 45 |
| Total no of samples | 120 | 93 | 54 |
| % DI>2 | 73 | 70 | 17 |
| % DI<2 | 28 | 30 | 83 |

## LIMITATIONS OF GA-MAP® DYSBIOSIS TEST LX V2

- GA-map® Dysbiosis Test Lx v2 has not been documented for use to discriminate between IBS, IBD, and other types of gastro-intestinal disorders.
- GA-map® Dysbiosis Test Lx v2 is intended for use with patients at the age between 18 and 70 years.
- Repeated freeze-thaw cycles of the fecal sample may change microbiota composition.
- The analysis method, including the sample collection and extraction method, must be performed as described to generate valid GA-map® Dysbiosis Test Lx v2 results. Alternative methods require validation.

## TOO HIGH/LOW DNA CONCENTRATION FOR FECAL SAMPLE

If the PCR quantification results for a single fecal sample is outside the accepted range, the sample in question must be re-analysed, either from PCR through preparing a new dilution or performing a new extraction. In case of repeated failure for the same sample upon re-extraction, the patient must be asked to donate a new sample.

## TOO HIGH/LOW DNA CONCENTRATION FOR CONTROL SAMPLE

If the PCR quantification results for GA-map® Kit ctrl pos (QCC23), the GA-map® Kit ctrl neg (QCC33) and/or the PCR control negative (QCC05) are outside the accepted range, the PCR setup should be repeated from the amplification step. A failed kit control suggests a failure in the amplification itself (e.g. PCR master mix or temperature cycling), not with the extracted gDNA samples.

If the PCR quantification results for extraction controls (QCC01 and/or QCC02) outside the accepted range, the analysis should be repeated from genomic gDNA extraction.

## QC ERRORS IN THE GA-MAP® DYSBIOSIS ANALYZER SOFTWARE

Any of the following error messages might occur when analyzing the data in the GA-map® Dysbiosis Analyzer.

| Error message | Interpretation | Possible causes | Suggested action |
|---|---|---|---|
| *Kit control #1/ Kit control #2/ End-labeling ctrl positive* not found on plate | QCC23/ QCC33/ QCC30 controls are not detected on the plate. | Incorrect naming of the Control IDs in the .csv file; see assay quality control scheme and plate setup for correct naming. | Open the .csv file as a .txt file and manually correct the naming<br>OR<br>Locate the run under "Saved Batches" in the xPONENT software and select "Replay batch" using correct naming. |
| | | Controls are omitted from analysis or sample list; see assay quality control scheme and plate setup for correct control scheme. | If one or more controls were not included in the analysis, repeat the analysis with the correct controls. |
| *Kit control #1* and/or *Kit control #2* profile error | QCC23 and/or QCC33 controls have a DI score outside the accepted range. | Error in the 16S PCR or subsequent steps of the analysis. | Repeat the procedure from Step 2 (Amplification of the bacterial 16S rRNA gene) for all samples and controls. |
| *End-labeling ctrl positive* total signal above/below the limit | QCC30 total signal too high/low. | Error in the End-Labeling or subsequent steps of the analysis. | Repeat the procedure from Step 5 (End-Labeling of Probe set) for all samples and controls. |

| | | | |
|---|---|---|---|
| *End-labeling ctrl negative* total signal above the limit | QCC29, background signals are too high. | Error in the End-Labeling or subsequent steps of the analysis. | Repeat the procedure from Step 5 (End-Labeling of Probe set) for all samples and controls. |
| Hybridization error for […] | HYC01 has too high/low signal in the affected sample(s). | Error in the Hybridization step or subsequent steps of the analysis. | Repeat the procedure from Step 5 (End-Labeling of Probe set) or Step 6 (Hybridization and signal detection) for the affected samples and all controls. |
| Universal target error for […] | UNI05 has too low signal in the affected sample(s). | Error in the Step 2 (Amplification of the bacterial 16S rRNA gene) or subsequent steps of the analysis. | Repeat the procedure from Step 2 (Amplification of the bacterial 16S rRNA gene) for affected samples and all controls. |
| Low bead count for […] | One or more of the probes for given sample have bead count below the lower limit. | Too little of the GA-map® Bead set added during preparation of the Hybridization plate.<br><br>Loss of beads or incomplete resuspension of beads during the washing step. | Repeat the procedure from Step 5 (End-Labeling of Probe set) or Step 6 (Hybridization and signal detection) for the affected samples and all controls. |

# APPENDICES

Appendix 1: GAmap_v1.kf2

Appendix 2: GAmap_v2.bdz

Appendix 3: GA-map Dysb.Test Lx v2[1.0].lxt2

Appendix 4: GA-map Dysbiosis Test Lx v2 Run log.xlsm

All appendices are available upon request from support@genetic-analysis.com

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