GA-map® Dysbiosis Test Lx v2

For Research Use Only.
Not for use in diagnostic procedures.

Pour la recherche uniquement. Ne pas utiliser à des fins diagnostiques.

CE

REF 2156 (2157, 2158)

RUO 96 Tests

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This document is also available from support@genetic-analysis.com.

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1 Definition of symbols

Product labels and used 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		

2 Warnings and precautions

Fecal samples should be treated as potentially infectious material until inactivation 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.

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

3 Principle of analysis

The GA-map® Dysbiosis Test Lx v2 is a test that allows mapping of the intestinal microbiota profile for a selected set of bacteria and is used to characterize dysbiosis in samples.

The GA-map® Dysbiosis Test Lx v2 is based on advances in DNA profiling using probes targeting 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.

4 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.

4.1 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
- Establish a gut microbiota profile given bacteria levels compared to a normobiotic reference in:
    - IBS samples
    - IBD samples
    - Symptomatic non-IBD samples

5 Composition

The volumes provided in the GA-map® Dysbiosis Test Lx v2 kits are sufficient for 96 reactions, including controls. The reagents are provided in two boxes, in addition to one set of external buffers. Box A contains reagents for the PCR and end-labeling reaction, as well as control material. Box B contains the GA-map® Bead set, GA-map® SAPE and GA-map® dH_2O. The external reagents are GA-map® Hybridization Buffer and GA-map® Detection Buffer. See Table 1 for part numbers and volume overview.

Table 1. Overview of content in the GA-map® Dysbiosis Test Lx v2; box A, box B and external reagents.

Part no.	Brand name	Comment	Volume (µl)
2157	**Box A (Store at <-15°C)**		
2205	GA-map® PCR MasterMix	single use	3 × 1400
4005	GA-map® PCR polymerase		95
6005	GA-map® End-Labeling polymerase		150
2204	GA-map® End-Labeling MasterMix	single use	3 × 1250
2405	GA-map® Biotin	single use	3 × 88
1102	GA-map® End-Labeling ctrl	QCC30	70
1302	GA-map® Hybridization ctrl	HYC01	20
1402	GA-map® Kit ctrl pos	QCC23	38
1502	GA-map® Kit ctrl neg	QCC33	38
2404	GA-map® ExoI		485
2404	GA-map® rSAP		485
2158	**Box B (Store at 2-8 °C)**		
2411	GA-map® dH_2O		2000
9003	GA-map® Bead set		84
2305	GA-map® SAPE		150
NA	**External (Store at ambient temperature, 15-25 °C)**		
2426	GA-map® Hybridization Buffer		8000
2427	GA-map® Detection Buffer		2 × 12000

6 Storage, handling and shelf life

6.1 Storage and shelf life

GA-map® Dysbiosis Test Lx v2 Box A should be stored at <-15°C while Box B should be stored at 2 to 8°C. The external buffers should be stored at ambient temperature.

The GA-map® Dysbiosis Test Lx v2 kit has a shelf life of 9 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 GA-map® kit can be used three times after opening. Freeze/thaw cycles should be avoided for the GA-map® PCR mm, the GA-map® End-labeling mm and the GA-map® Biotin. Three tubes of each have been provided for single use.

Table 2 gives an overview of conditions for storage and reuse conditions for the main kit components and sample intermediates.

Table 2. Overview of storage and reuse conditions of reagents and samples intermediates.

Reagent/Sample	Storage temperature	Maximum storage time	Maximum freeze thaw cycles
GA-map® Dysbiosis Test Lx v2, Box A	<-15°C	See expiry date on the box label	3
GA-map® Dysbiosis Test Lx v2, Box B	2-8 °C	See expiry date on the box label	NA
GA-map® Dysbiosis Test Lx v2, External buffers	RT	See expiry date on each tube label	NA
Dry fecal sample	RT	5 days plus 12h at ≤-15°C *	1
Fecal sample on eNAT™ buffer	RT (<40°C)**	14 days	2
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/SAP-treated	2-8 °C	7 days	NA
End-labeling product	2-8 °C	2.5 hours	NA
Hybridization product	RT, protected from light	24 hours	NA

* 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 >14 days, the tubes should be frozen ≤-15°C.

The procedure can be paused at several different steps, as indicated by the maximum storage time of the sample intermediates in Table 2. The procedure description contains several “stop symbols”, which indicates possibility for overnight pauses.

6.2 General reagent handling procedures

- Before start, it is recommended that the user confirms that required equipment, materials and reagents are available for each step.
- Kit components from different kitlots should not be mixed.
- Reagents from Box A should be thawed on ice before use and kept on ice during handling.
- The reagents from Box B and the external reagents 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. For all centrifugation steps (tubes and plates), unless otherwise specified, use a brief spin down up to 100xg.
- 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 96-well plates and reagents from section 9.2 Amplification of the bacterial 16S rRNA gene to 9.5 End-Labeling of Probe set should be kept on ice during handling.
- The 96-well plates and all reagents with the exception of the GA-map® Hybridization ctrl (HYC01), in Step 9.6 Hybridization and Signal detection should be kept at ambient temperatures.

7 Assay outline and setup

7.1 Procedure outline

A general outline of the procedure is shown in Figure 1.

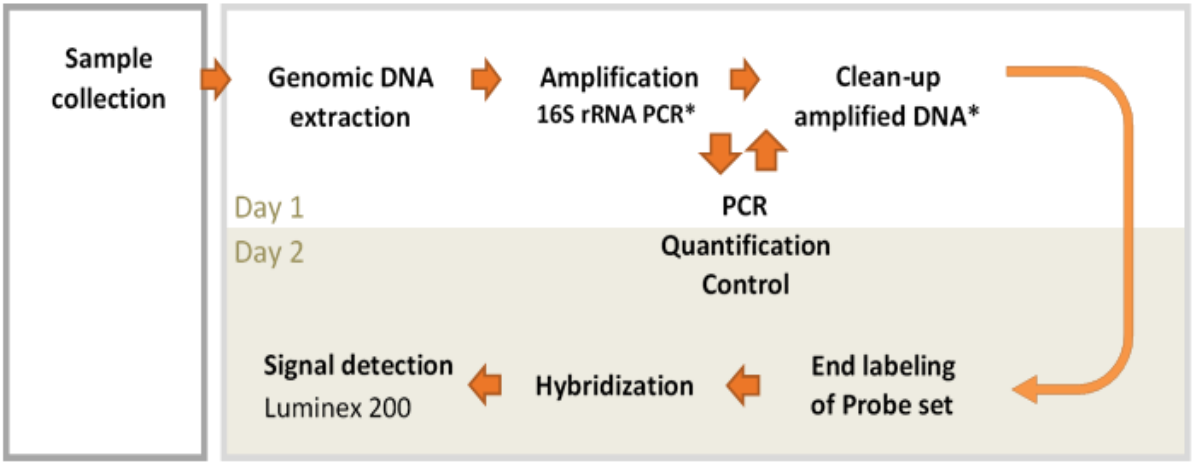

Figure 1. Outline of the GA-map® procedure for feces sample preparation and running of GA-map® Dysbiosis Test Lx v2.

The GA-map® Dysbiosis Test Lx v2 procedure comprises the following steps:

- Fecal sample collection (validated protocol); see Section 8
- Genomic DNA extraction (validated protocol); see Section 9.1
- Amplification of the bacterial 16S rRNA gene; see Section 9.2
- PCR Quantification Control; see Section 9.3
- Clean-up amplified DNA; see Section 9.4
- End-Labeling of Probe set; see Section 9.5
- Hybridization and signal detection; see Section 9.6
- Data QC and result generation; see Section 9.7

The steps are described in more detail in the following pages. Please follow instructions carefully.

7.2 Assay quality control scheme

GA-map® Kit ctrl pos and GA-map® Kit ctrl neg control samples are included in the kit as positive and negative sample controls. The GA-map® End-Labeling ctr is included as a positive control for the probe labeling step, while water is used for negative control of the same step. Negative controls are also recommended for the genomic DNA extraction and PCR step. In addition, it is recommended to include a fecal sample as a positive control for the Genomic DNA extraction step. An overview of the different controls included in the assay (both required and recommended) is given in Table 3.

Table 3. Overview of the GA-map® Dysbiosis Test Lx v2 quality control scheme.

Brand name	Control ID	Comment	Included in/from
Extraction control positive	QCC01	Fecal sample (recommended)	9.1 Genomic DNA extraction
Extraction control negative	QCC02	Option A: S.T.A.R. buffer Option B: Lysis Buffer BLM (recommended)	9.1 Genomic DNA extraction
GA-map® Kit ctrl pos	QCC23	Included in kit	9.2 Amplification of the bacterial 16S rRNA gene
GA-map® Kit ctrl neg	QCC33	Included in kit	9.2 Amplification of the bacterial 16S rRNA gene
PCR control negative	QCC05	Use same water as that used to dilute gDNA (recommended)	9.2 Amplification of the bacterial 16S rRNA gene
End-Labeling control negative	QCC29	Use GA-map® dH2O	9.5 End-Labeling of Probe set
GA-map® End-Labeling ctrl	QCC30	Included in kit	9.5 End-Labeling of Probe set
GA-map® Hybridization ctrl	HYC01	Included in kit; added to all samples and controls	9.6 Hybridization and Signal detection

7.3 Plate setup

Assay controls should be arranged in the 96-well microtiter plates as illustrated in Figure 2. 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.

a) Genomic DNA extraction (section 9.1)

	1	2	3	4	5	6	7	8	9	10	11	12
A	QCC01_A											QCC01_B
B	QCC02_A											QCC02_B
C												
D												
E												
F												
G												
H												

b) Amplification of the bacterial 16S rRNA (section 9.2)

	1	2	3	4	5	6	7	8	9	10	11	12
A	QCC01_A											QCC01_B
B	QCC02_A											QCC02_B
C	QCC23_A											QCC23_B
D	QCC33_A											QCC33_B
E												QCC05_A
F												
G												
H												

c) End-labeling of Probe set (section 9.5) and Hybridization and signal detection (section 9.6)

	1	2	3	4	5	6	7	8	9	10	11	12
A	QCC30_A											QCC30_C
B	QCC29_A											QCC29_B
C	QCC23_A											QCC23_B
D	QCC33_A											QCC33_B
E	QCC30_B											QCC30_D
F												
G												
H												

Figure 2 Control sample layout for steps a) Genomic DNA Extraction; b) Amplification of the bacterial 16S rRNA; c) End-labeling of Probe set, Hybridization and signal detection.

8 Sample collection

8.1 Sampling

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 using a sterile 20-30ml tube: se details in section 8.1.1
Option B: Buffer-based sampling on Copan eNAT™: se details in section 8.1.2

8.1.1 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).
- Sampling tray (sterile/clean).

Sampling procedure

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. Note name, sampling date, and sampling time on the tube.
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 to 25°C) for no more than 5 days before freezing.

8.1.2 Option B: Buffer-based sampling

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

- eNAT™ tube w/ FLOQSwab (1 mL) from Copan, cat no 608CS01R
- Fecal sampling paper from Med Auxil (sterile/clean).

Sampling procedure

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.

Note: 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 tighten the cap and shake the closed tube until the sample appears homogenous.
7. Note name, sampling date, and sampling time on the tube.
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 temperature +4 to +40°C 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.

8.2 Sample receipt at site of analysis

Storing of the fecal samples is specific for the sample collection system used:

Option A: Dry sampling, see details in section 8.2.1
Option B: Buffer-based sampling on Copan eNAT™, see details in section 8.2.2

8.2.1 Option A: Dry samples

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

8.2.2 Option B: Buffer-based samples

- Samples collected on eNAT™ buffer should be stored at +4 or at ambient temperatures (<40°C) until analysis.
- In case of >14 days from sampling to analysis, the sample tube should be frozen at ≤-15°C upon receipt.

9 Laboratory procedure

9.1 Genomic DNA extraction

Reagents required for this step as well as materials and lab equipment are listed in Table 4.

The initial steps of the gDNA extraction process (sample lysis), is specific for the sample collection system used:

Option A: Dry sampling: se details in section 9.1.1
Option B: Buffer-based sampling on Copan eNAT™: se details in section 9.1.2

Table 4. Materials and reagents for gDNA extraction

		Required	Recommended
Lab equipment	Biological safety cabinet		Any suitable brand
	Bead beater	FastPrep-96™ Homogenizer w/96-well plate insert, MP Biomedicals	
	DNA extraction robot	KingFisher Flex with 96 deep well head, ThermoFisher	MagMAX™Express-96, w/magnetic micro-plate Separator, ThermoFisher
	Water bath	65°C	Any suitable brand
	Centrifuge	Capable for lysing matrix-96 E well plate (**NOTE plate height 6 cm**) (3500rpm/1300rcf)	
	Centrifuge	Capable for 96-well plate (spin down only)	
	Centrifuge	Any for 2ml feces -tubes (5000 rpm/2655rcf)	
	Microcentrifuge		Any microcentrifuge for 2 ml tubes, spin down only
	Vortex		Any suitable brand
	Decapper w/ bench top stand		Any suitable brand
	Dispensing pipette		Eppendorf Multipette® E3/E3x
	Single-channel pipette		Any suitable brand
	Multi-channel pipette		Any suitable brand
	Ice or cooling elements for keeping reagents/samples cold on bench.	Any	
Material	Lysing Matrix-E tubes	Lysing Matrix-E tubes (2450), Genetic Analysis	
	Microtiter plate, deep well	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, min 250 µl		Any suitable brand
	Lysing Matrix-E tubes	Lysing Matrix-E tubes (2450), Genetic Analysis	
	Tube for Lysis Protease mix: 5, 15 or 50 mL		Any suitable brand
	Adhesive PCR plate seals		Adhesive PCR Plate Seals, (AB0558), ThermoFisher
	Microtiter sealing tape		Any suitable brand
	Stirring rod		Any suitable brand
	Pipette tip, wide orifice		Any suitable brand
Reagent	DNA extraction reagent kit	mag™ maxi (LGC DNA isolation kit) 288 tests, (40430), LGC Genomics	
	Ethanol 96 % rectified		Any suitable brand
	S.T.A.R buffer (n.a. for Option B: buffer-based sampling)	S.T.A.R buffer 300ml, (03 335 208 001), Roche	
	Sterile, Nuclease free water (H_2O)		Any suitable brand
Control	Extraction control positive		Fecal sample. (To be supplied by lab, if desired)
	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

Preparing the sample and reagents for gDNA extraction

1. Make sure all elements in Table 4 are prepared and available.

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.

2. Prepare a sample setup with control samples placed according to Figure 2A. Extraction control negative is added at different steps depending on the sample collection system used:
   - Option A: Dry samples. Extraction control negative is added in step 1, section 9.1.1.
   - Option B: Samples on eNAT™ buffer. Extraction control negative is added during *Performing gDNA extraction*, step 5.
3. Take out frozen fecal samples for thawing.
   - Dry samples should be thawed for 30-120 minutes
   - If frozen, Copan eNAT™ tubes should be thawed for ≥15 minutes
4. Make sure that the samples are not mixed with blood or mucus.
5. Continue with preparation of samples for lysis according to **Option A** or **Option B**, depending on the sample type.

9.1.1 Option A: Preparation of dry samples

1. 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).
2. Make sure the samples are fully thawed.
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 1600ul.
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 rcf.
8. Transfer 600µl sample supernatant to Lysing Matrix-E tubes.
9. Continue with *Performing gDNA extraction*, step 1.

9.1.2 Option B: Preparation of samples on eNAT™ buffer

1. Make sure the samples are fully thawed.
2. Vortex the sample tubes to make feces homogeneous.
3. Add 200µl Elution buffer to the Lysing Matrix-E tubes.
4. Transfer 400µl sample from the Copan tubes to the Lysing Matrix-E tubes.
5. 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 rcf for 5 minutes in a plate centrifuge.
3. Prepare Lysis protease mix according to Table 5. Mix by pipetting.

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

Table 5. Lysis protease mix

Component	µl per well
Lysis buffer BLM (LGC Genomics)	250
Protease (LGC Genomics)	20
Total volume	**270**

Prepare mix for n + 1 samples + 10%, where n = the number of fecal samples + positive & negative extraction controls

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

Note! When analyzing samples collected on eNAT™ buffer, 250µl Lysis buffer BLM is added as negative extraction control; see Table 3 and Figure 2.

6. Mix well by pipetting.
7. Cover plate with adhesive PCR film. **Make sure the film is thoroughly sealed.**
8. Carefully put the deep well plate with the samples on the 65°C water (floating). Close the lid of the water bath.
9. Incubate in the water bath at 65°C for 15 minutes.
10. During bath incubation, finalize preparation of buffer plates according to Table 6.

Table 6. Plates numbers and buffers volumes for gDNA extraction

Plate Type	Plate #	Content	Volume
Deepwell 96 Plate	1	MagMAX particles	20µl
		Ethanol	200µl
		Protease treated sample (step 16)	400µl
	2	Washing buffer BLM 1	720µl
	3	Washing buffer BLM 2*	720µl
	4	Washing buffer BLM 2*	720µl
96 KF microplate	5	Elution buffer BLM	200µl
96 tip comb	6	Tip comb placed in KF microplate	N/A

* Prepare BLM 2 according to manufacturer's instructions using Acetone

11. Vortex the MagMAX particles thoroughly before use to fully resuspend. Add 20µl MagMAX particles into the wells of a 96 deep well plate (plate#1) according to the prepared sample setup.
12. Add 200µl ethanol. Seal the plate with microtiter sealing tape immediately.
13. Continue to prepare buffer plates.
14. Cover the plates with microtiter sealing tape.
15. 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.
16. Add 400µl protease treated sample to each well on plate#1. Mix well by pipetting.
17. Turn on the magnetic separator, find the correct program according to Table 7 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.

Table 7. Program for each gDNA extraction robot.

Instrument name	Program
MagMAX™Express-96	GAmap_v1
KingFisher Flex	GAmap_v2

18. When the program is finished, remove plate#5 (Elution plate). Seal plate with microtiter sealing tape and centrifuge the plate for 1 minute at 1000 rcf. Place the plate on ice.
19. Discard plates #1, #2, #3, #4, and #6.
20. From plate#5, transfer 100µl of the gDNA (avoid transferring the brown debris in the bottom) into a 96-well microtiter plate. Undiluted gDNA can be stored long term at ≤-20°C.
21. Dilute the gDNA 1:50 by mixing 5µl gDNA and 245µl nuclease free water. Mix well.

22. If stopping at this step, seal and store gDNA at 2 to 8°C for no more than 3 weeks (or alternatively at ≤-20°C for long term storage) until further use.

9.2 Amplification of the bacterial 16S rRNA gene

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step are listed in Table 8. Other materials and reagents required are listed in Table 9.

NOTE! Avoid freeze/thaw cycles with the GA-map® PCR MasterMix.

Table 8. GA-map® Dysbiosis Test Lx v2 kit reagents required for Amplification step.

Reagent brand name	Storage	Handling method
GA-map® PCR MasterMix	GA-map® Dysbiosis Test Lx v2, Box A	Thaw on ice. Mix by vortexing and briefly spin down before use. Keep on ice during handling.
GA-map® Kit ctrl pos (QCC23)		
GA-map® Kit ctrl neg (QCC33)		
GA-map® PCR polymerase		Gently flick the tube to mix and briefly spin down before use. Keep on a freezing block during handling.

Table 9. Other materials and reagents for Amplification step.

		Required	Recommended
Lab equipment	Thermal cycler with 96-well head and heated lid	Veriti™ 96-Well Thermal Cycler, ThermoFisher or T100 Thermal Cycler, Bio-Rad Laboratories	Other systems must be validated for GA-map®
	Plate centrifuge		Any suitable brand
	Microcentrifuge		Any suitable brand for 2 ml tubes, spin down only
	Vortexer		Any suitable brand
	Dispensing pipette		Eppendorf Multipette® E3/E3x
	Multi-channel pipette		Any suitable brand
	Single-channel pipette		Any suitable brand
	Ice or cooling elements for keeping reagents/samples cold on bench.	Any	
Material	Microtiter plate PCR grade		Any suitable brand
	Lid PCR 8-strip		Any suitable brand
	Microcentrifuge tube 1.5/2.0/5.0 mL		Any suitable brand
	PCR control negative		Sterile, Nuclease-free H_2O, same as used for gDNA dilution

Preparing for amplification:

1. Make sure all elements in Table 8 and Table 9 are prepared and available. Thaw and keep all reagents on ice.
2. Prepare a sample setup with control samples placed according to Figure 2B.
3. Calculate the volume of PCR reagents needed for the number of samples to be amplified according to Table 10.
4. Place the 1:50 diluted gDNA template from Step 9.1 on ice.

Performing the amplification of bacterial 16S rRNA gene:

1. Prepare the PCR mix according to Table 10. Mix by vortexing and briefly spin down the mix.

Table 10. PCR mix

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

Include 10% extra volume when calculating.

2. Place a 96-well PCR plate on ice and dispense 20µl of the PCR mix into the appropriate wells according to sample setup.
3. Add 5µl templates (1:50 diluted gDNA) and control samples according to plate setup. Mix by pipetting up and down before and after transfer.
4. Cover the plate with lid PCR 8-strips.
5. To ensure proper mixing, vortex each corner of the plate for 3 seconds.
6. Briefly pulse spin the plate up to 100 x g.
7. Initiate the PCR-program described in Table 11 and load the samples on the thermal cycler when the temperature has reached 80°C.

Table 11. gDNA Amplification program*

Initial denaturation	Denaturation	Annealing	Elongation	Final elongation	End
		30 cycles			
95°C	**95°C**	**55°C**	**72°C**	**72°C**	**4°C**
15 min	30sec	30 sec	1min 20sec	7 min	∞

*Set heated lid to 105°C

8. Store the PCR-product at 2 to 8°C until next step, no longer than 48 hours.

9.3 PCR quantification control

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. However, note that the PCR yield should be above the concentration corresponding to the lower limit as measured by GA's validated methods: Quant-iT™ 1X dsDNA HS Assay kit, Quanti-iT™ PicoGreen™ dsDNA Assay Kit or Qubit™ dsDNA HS Assay Kit.

The criteria are shown in Table 12.

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

Table 12. Quality control criteria for PCR product

	Lower limit	Upper limit
DNA conc. for samples and positive controls (QCC01, QCC23 and QCC33)	≥19 ng/µl	<50 ng/µl
DNA conc. for negative controls (QCC02 and QCC05)	-	<5 ng/µl

The quantitative measurement can be performed directly after completion of the amplification of the 16S rRNA gene. Alternatively, an aliquot of the PCR product can be transferred to a new plate and measured during the Hybridization step (section 9.6). Store PCR product as stated in Section 6.1.

Keep the PCR product on ice during handling.

9.4 Clean-up amplified DNA

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step are listed in Table 13. Other materials and lab equipment are listed in Table 14.

Table 13. GA-map® Dysbiosis Test Lx v2 kit reagents required for Clean-up amplified DNA

Reagent brand name	Storage	Handling method
GA-map® rSAP	GA-map® Dysbiosis Test Lx v2, Box A	Keep on a freezing block
GA-map® ExoI		

Table 14. Materials and lab equipment for Clean-up amplified DNA

		Required	Recommended
Lab equipment	Thermal cycler with 96-well head and heated lid	Veriti™ 96-Well Thermal Cycler, ThermoFisher or T100 Thermal Cycler, Bio-Rad Laboratories	Other systems must be validated for GA-map®
	Plate centrifuge		Any suitable brand
	Microcentrifuge		Any suitable brand for 2 ml tubes, spin down only
	Multi-channel pipette		Any suitable brand
	Single-channel pipette		Any suitable brand
	Ice or cooling elements for keeping reagents/samples cold on bench.	Any	
Material	Lid PCR 8-strip		Any suitable brand
	Microcentrifuge tube 1.5/2.0 mL		Any suitable brand
	Reservoir tray		Any suitable brand

Preparing for clean-up of amplified DNA:

1. Make sure all elements in Table 13 and Table 14 are prepared and available.
2. Calculate the volume of clean-up reagents needed for the number of samples and volume to be treated with ExoI/rSAP mix in accordance with Table 15.
3. Vortex and briefly spin down the 96-well plate containing 16S rRNA PCR product from Step 9.2. Work on ice.

Performing clean-up of amplified DNA:

1. Prepare the ExoI/rSAP mix according to Table 15. Mix by pipetting and briefly spin down.

Table 15. ExoI/rSAP mix

Enzyme	µl per µl PCR prod.	Example: Volume per well when using 23µl PCR prod.*
GA-map® rSAP	0.2	4.6
GA-map® ExoI	0.2	4.6
Total volume	**0.4**	**9.2**

Include 10% extra volume when calculating. *If removed 2µl for DNA quantification control.

2. Transfer the appropriate volume of ExoI/rSAP mix to the 16S rRNA PCR products. Mix by pipetting.
3. 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. Vortex each corner of the plate for 3 seconds. Insufficient mixing may lead to a bias in the results!

4. Briefly pulse spin the plate to 100 x g.
5. Initiate the PCR program described in Table 16 and load the samples on the thermal cycler when it has reached 30°C.

Table 16. Clean-up amplified DNA program

Incubation	Inactivation	End
37°C	**80°C**	**4°C**
10 min	10 min	∞

*Set heated lid to 105°C

6. Store the ExoI/rSAP treated PCR product at 2 to 8°C until next step, no longer than 7 days.

9.5 End-Labeling of Probe set

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step are listed in Table 17. Other materials and lab equipment are listed in Table 18.

NOTE! Avoid freeze/thaw cycles with the GA-map® End-Labeling MasterMix and the GA-map® Biotin.

Table 17. GA-map® Dysbiosis Test Lx v2 kit reagents required for End-Labeling of Probe set.

Reagent brand name	Storage	Handling method
GA-map® End-Labeling MasterMix	GA-map® Dysbiosis Test Lx v2, Box A	Thaw on ice before use, keep on ice during handling
GA-map® Biotin		
GA-map® End-Labeling ctrl (QCC30)		
GA-map® End-Labeling polymerase		Keep on a freezing block
GA-map® dH_2O	GA-map® Dysbiosis Test Lx v2, Box B	Keep on ice during handling

Table 18. Materials and lab equipment for End-Labeling.

		Required	Recommended
Lab equipment	Thermal cycler with 96-well head and heated lid	Veriti™ 96-Well Thermal Cycler, ThermoFisher or T100 Thermal Cycler, Bio-Rad Laboratories	Other systems must be validated for GA-map®
	Plate centrifuge		Any suitable brand
	Microcentrifuge		Any suitable brand for 2 ml tubes, spin down only
	Vortex		Any suitable brand
	Dispensing pipette		Eppendorf Multipette® E3/E3x
	Multi-channel pipette		Any suitable brand
	Single-channel pipette		Any suitable brand
	Ice or cooling elements for keeping reagents/samples cold on bench.	Any	
	Ice block for PCR plate	Any	
Material	Microtiter plate		Any suitable brand
	Lid PCR 8-strip		Any suitable brand
	Microcentrifuge tube 1.5/2.0 mL		Any suitable brand

Preparing for end-labeling:

1. Make sure all elements in Table 17 and Table 18 are prepared and available.
2. Prepare a sample setup with control samples placed according to Figure 2C.
3. Calculate the volume of end-labeling reagents needed for the number of samples and controls in accordance with Table 19.
4. Vortex and briefly spin down the 96-well plate containing ExoI/rSAP treated PCR product from Step 9.4. Work on ice.

Performing End-Labeling:

1. Prepare End-labeling mix according to Table 19. Mix by vortexing and briefly spin down.

Table 19. End-labeling mix.

Component	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**

Include 10% extra volume when calculating.

2. Place a new 96-well plate on ice and dispense 20µl of the End-labeling mix to each well according to plate layout.
3. Add 5µl of ExoI/rSAP treated samples and controls from the PCR plate, in addition to 5µl End-labeling controls according to the plate layout. Mix well by pipetting.
4. Cover the plate with PCR 8-strips.
5. Briefly pulse spin the plate to 100 x g.
6. Initiate the PCR-program described in Table 20 and load the plate on the thermal cycler when the temperature has reached 80°C.

Table 20. End-Labeling program*

Initial denaturation	Denaturation	Annealing/elongation	End
	5 cycles		
95°C	96°C	60°C	4°C
12 min	20sec	35sec	∞

*Set heated lid to 105°C

7. While the samples are in the thermal cycler, proceed to preparation for section 9.6 Hybridization and Signal detection.
8. 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!

9.6 Hybridization and Signal detection

GA-map® Dysbiosis Test Lx v2 kit reagents required for this step are listed in Table 21. Other materials and lab equipment are listed in Table 22.

Table 21. GA-map® Dysbiosis Test Lx v2 kit reagents required for Hybridization and Signal detection.

Reagent brand name, Part no	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 to 25°C)
GA-map® Bead set		
GA-map® SAPE		
GA-map® Hybridization Buffer	GA-map® Dysbiosis Test Lx v2, External ragents	
GA-map® Detection Buffer		

Table 22. Materials and reagents for Hybridization and Signal detection.

		Required	Recommended
Lab equipment	Signal detection platform	Luminex® 200™ with xPONENT 4.2 or higher software	
	Thermal cycler with 96-well head and heated lid	Veriti™ 96-Well Thermal Cycler, ThermoFisher or T100 Thermal Cycler, Bio-Rad Laboratories	Other systems must be validated for GA-map®
	Magnetic ring plate separator	96-well with minimum capacity of 100 µL and compatible with v-bottom plates	Permagen™ 96-Well Ring Magnet Plate (S380)
	Plate centrifuge		Any suitable brand
	Microcentrifuge		Any suitable brand
	Vortex		Any suitable brand
	Dispensing pipette		Eppendorf Multipette® E3/E3x
	Multi-channel pipette		Any suitable brand
	Single-channel pipette		Any suitable brand
	Ice or cooling elements for keeping reagents/samples cold on bench.	Any	
Material	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	
	Tube for hybridisation bead mix, (min 4 ml)		5ml tube (0030 119.401), Eppendorf
	Microcentrifuge tube 1.5/2.0 mL		Any suitable brand
	Reservoir tray		Any suitable brand
Reagent	Luminex® 100/200™ Performance Verification Kit	Luminex® 100/200™ Performance Verification Kit (LX200-CON-K25), Luminex	
	Luminex® 100/200™ Calibration Kit	Luminex® 100/200™ Calibration Kit (LX200-CAL-K25), Luminex	

Preparing for hybridization and signal detection:

1. Make sure all elements in Table 21 and Table 22 are prepared and available.

NOTE! All reagents required for this step (except for the GA-map® Hybridization ctrl) is 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.

2. Prepare a sample setup with control samples placed according to Figure 2C.
3. Use Table 23, Table 24 and Table 26 to calculate volumes of the different reagents needed for the number of samples and controls
4. Vortex and briefly spin down the 96-well plate containing End-labeling product from Step 9.5. Keep the plate on an ice block.
5. Make sure the Luminex® 200™ is calibrated and ready for use.

Performing probe and reporter hybridization:

1. Prepare the Hybridization ctrl (HYC01) dilution according to Table 23. 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.

Table 23. Hybridization ctrl dilution

Component	**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**

2. Mix the first two components from Table 24.
3. Resuspend the GA-map® Bead set by vortexing for 10 seconds.
4. Transfer the proper volume of GA-map® Bead set to finalize preparation of Hybridization bead mix, according to Table 24.

Table 24. Hybridization bead mix

Component	µl per well
GA-map® Hybridization Buffer	32.2
Hybridization ctrl dilution*	7
GA-map® Bead set	0.8
Total volume	**40**

Include 10% extra volume when calculating. *Diluted according to Table 23.

5. Resuspend the prepared Hybridization bead mix by vortexing for 10 seconds.
6. Place a new 96-well Corning® microplate at ambient temperature and dispense 40µl of the Hybridization bead mix to each well according to sample setup in using a single-channel dispenser. 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 layout. 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 and start the Hybridization program as indicated in Table 25. Insert the plate into the thermal cycler when the temperature has reached 80°C. Set a timer to 18 minutes**.**

Table 25. Hybridization program

Denaturation	Hybridization	Incubation
95°C	**45°C**	**45°C**
3 min	15 min	30 min (after addition of reporter mix)

*Set heated lid to 105°C

9. Prepare fresh Reporter mix according to Table 26.

Table 26. Reporter mix

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

Include 25% extra volume when calculating volume.

10. After 18 minutes of 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.
11. 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.
   b. Import sample IDs to the respective wells and optional additional information.
      Note that some wells in columns 1 and 12 are reserved for controls. See Figure 2C.
   c. Ensure the names of the control samples are in accordance with Table 3 and Figure 2.
   d. Make sure the sample-/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 75 µl of GA-map® Detection Buffer and remove the buffer shortly afterwards.
5. Remove the plate from the magnetic ring plate and add 75 µl 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.

6. Prior to scan, the Hybridization-product can be stored up to 24 hours in the dark at ambient temperature. In case of storing the plate for >1 hour, resuspend the beads by pipetting up and down a few times before continuing the process.
7. Move the plate to the Luminex analyzer.
8. Click the **Run** button to start analysis and confirm by pressing **OK**.
9. 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.
      The file can now be used on GA-map® Dysbiosis Analyzer software for quality check and patient result generation.
10. Remove the plate and discard it according to local regulations for biological waste disposal.

9.7 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 plate set-up and control names specified in sections 7.2 Assay quality control scheme and 7.3 Plate setup.
   NB: 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 patient report can be generated.

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

9.8 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*.

10 Specific performance characteristics

10.1 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.

10.2 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 samples from fecal sample recruitment hospitals in Norway, Sweden and Denmark. All samples were from unique feces donors not included in previous development of GA-map® Dysbiosis Test Lx v2.

The performance characteristics are shown in the Table 27 below.

Table 27. Distribution of normobiotic and dysbiotic per patient category

	IBD	non-IBD
No of samples DI>2	87	65
No of samples DI<2	33	28
Total no of samples	120	93
% DI>2	73	70
% DI<2	28	30

11 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 v1 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 in order to generate valid GA-map® Dysbiosis Test Lx v2 results. Alternative methods require validation.

12 FAQ/ Troubleshooting

12.1 Too high/low DNA concentration for patient sample

If the PCR quantification results for a single patient 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.

12.2 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.

12.3 QC errors in GA-map® Dysbiosis Analyzer Lx software

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

Table 28. Software error message overview

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 samples in the .csv file; see sections 7.2 Assay quality control scheme and 7.3 Plate setup for correct naming. Controls are omitted from analysis or sample list. See sections 7.2 Assay quality control scheme and 7.3 Plate setup for correct control scheme.	Open the .csv file as a .txt file and manually correct the naming OR Locate the run under "Saved Batches" in the xPonent software and select "Replay batch" using correct naming. 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 section 9.2 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 section 9.5 End-Labeling of Probe set for all samples and controls.
End-labeling ctrl negative	QCC29, background signals are too high.	Error in the End-labeling or subsequent steps of the analysis.	Repeat the procedure from section 9.5 End-Labeling of Probe set for all samples and controls.

total signal above the limit			
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 section 9.5 End-Labeling of Probe set for the affected samples and all controls.
Universal target error for […]	UNI05 has too low signal in the affected sample(s).	Error in the 16S PCR or subsequent steps of the analysis.	Repeat the procedure from section 9.2 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. Loss of beads or incomplete resuspension of beads during the washing step.	Repeat the procedure from section 9.5 End-Labeling of Probe set for the affected samples and all controls.

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