DNA-TECHNOLOGY

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IVD

For professional use only

ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

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1. INTENDED USE

The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** is intended for research and diagnostic applications. The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** is an *in vitro* Nucleic Acid Test (NAT) – microbiota-detection-based product. The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** is designed for detection of colon associated bacteria DNA (*Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, Verrucomicrobia, Euryarchaeota* filums), including *Candida* fungi, as well as gene of methicillin-resistance *Staphylococcus spp.* (mecA), *Cl.difficile* with enterotoxins A and B (tcdA, tcdB), *Str.agalactiae* with invasiveness marker gene (srr2) by real-time PCR in DNA preparations obtained from children's faeces sample.

Target patient group: children under 14.

Indications for the use: necessity to assess the composition of the microbiota of the large intestine in the course of therapeutic and diagnostic measures.

It is recommended that the examination be carried out no earlier than two days after the end of the intake of enterosorbents, pro- and prebiotics.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit.**

The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** can be used in clinical and diagnostic laboratories of medical institutions and research practice.

Potential users: personnel qualified in molecular diagnostics methods and working in the clinical and diagnostic laboratory.

It is necessary to apply the kit only as directed in this instruction for use.

2. METHOD

Method: polymerase chain reaction (PCR) with detecting of the results in real time; semi-quantitative multiplex analysis.

The result is expressed as the decimal logarithm (Lg) of the number of genome equivalents of microorganisms and pathogenicity or resistance factors in 1.0 g of clinical material (feces sample).

The implemented PCR method is based on amplification of a target DNA sequence. The process of amplification includes repeating cycles of thermal DNA denaturation, annealing of primers with complementary sequences and their extension by Taq-polymerase.

To increase the sensitivity and specificity of amplification reaction, the use of a hot-start is provided. Hot-start is provided by reaction mixture preparation consisting of two layers separated by a layer of paraffin or the use of Taq-polymerase blocked by antibodies. The polymerase chain reaction starts only when paraffin is melted or thermal dissociation of a complex of Taq polymerase and antibodies is happened. It excludes non-specific annealing of primers to targets DNA in the initial heating of the tube.

The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** is based on fluorescent modification of the PCR method.

The PCR-mix contains target-specific probes, each of them bearing reporter fluorescent dyes and quencher molecules. When specific product is formed, DNA probes are disintegrated and the quencher molecule stops affecting the fluorescent dye. Thus, the level of fluorescence increases and it is detected by the thermocycler data collection unit. As a result of probe activation fluorescence increases proportionally to target sequence amplification. The amount of disintegrated probes increases proportionally to target sequence amplification. The intensity of fluorescence is measured at every cycle of reaction in real time with a Real-time PCR thermo cycler.

DNA probes used to detect products of amplification of genome fragments of the microorganisms being identified include the fluorescent tags Fam, Rox and Cy5.

DNA probes for the detection of amplification products of internal control (IC) contains fluorescent dye Hex.

The application of four fluorescent dyes makes it possible to register the results of different amplification reactions taking place simultaneously in one tube.

The PCR-mix in tube 1 of strip № 1 (Fam channel) includes a mixture for determining the Lg TBL value to determine whether the amount of DNA obtained is sufficient for the assay.

The PCR-mix contain internal control (IC) for the estimation of the PCR efficiency.

The PCR-mix in tube №1, strip 1 and tube №2, strip 2 contains additional probe with Rox dye label – "Marker". It tags the strip orientation. Upon completion of run, software defines actual position of the strip (by means of "marker" position) relative to the position preset by the operator and in case of mismatch warns an operator. Table 1 shows the detection channels of amplification products.

Table 1. Detection channels and color marking of the mixes

Strip type	Strip tube №	Detection channel				Mix color marking
		Fam	Hex	Rox	Cy5	
Strip № 1	1	TBL	IC	Marker	*Candida* spp.	Blue
	2	*Bacteroides* spp.	IC	*Lactococcus lactis*	*Bifidobacterium* spp.	Colorless (package S)/ light pink (package A)
	3	*Faecalibacterium prausnitzii*	IC	*Bif.adolescentis*	*Dialister* spp./ *Alisonella* spp./ *Megaspherae* spp./ *Veillonella* spp.	
	4	*Methanobrevibacter* spp.	IC	*Bif. longum* subsp.*infantis*	*Parabacteroides spp.*	
	5	*Akkermansia muciniphila*	IC	*Bif. longum* subsp.*longum*	*Butyricimonas* spp.	
	6	*Desulfovibrio* spp.	IC	*Bif.bifidum*	*Coriobacteriia*	
	7	*Clostridium leptum gr.*	IC	*Candida albicans*	*Lactobacillaceae*	
	8	*Lachnospiraceae*	IC	*Bif. catenulatum* subsp.	*Clostridium difficile gr.*	
Strip № 2	1	-	IC	*Enterococcus spp.*	*Erysipelotrichaceae*	
	2	*E.coli*	IC	Marker	*Enterobacterales*	Blue
	3	*Peptoniphilaceae*	IC	*Bif. animalis subsp.lactis*	*Pseudomonas* spp.	Colorless (package S)/ light pink (package A)
	4	*Alistipes* spp.	IC	*Bif.dentium*	*Fusobacteriaceae*	
	5	*Prevotella* spp.	IC	*Bif.breve*	*Clostridium perfringens gr.*	
	6	*Str.agalactiae*	IC	srr2	*Streptococcus* spp.	
	7	*St. aureus*	IC	mecA	*Staphylococcus* spp.	
	8	tcdA, tcdB	IC	-	*Clostridioides difficile*	
Package S amplification mix tubes contain paraffin.						

The automatic analysis available on "DNA-Technology" made instruments: DTlite or DTprime REAL-TIME Thermal Cyclers for **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of the software is available for download at https://www.dna-technology.com/software.

3. CONTENT

The detailed description of content is represented in Table 2, 3.

Table 2. The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** content, package S (standard) for R1-P815-S3/6EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix. Strip №1	Colorless or blue transparent liquid under waxy white fraction	1920 µL (20 µL in each tube)	12 8-tube strips
Paraffin sealed PCR-mix. Strip №2	Colorless or blue transparent liquid under waxy white fraction	1920 µL (20 µL in each tube)	12 8-tube strips
Taq-polymerase solution	Colorless transparent liquid	2.0 mL (500 µL in each tube)	4 tubes
Mineral oil	Colorless transparent viscous oily liquid	4.0 mL (1.0 mL in each tube)	4 tubes
Positive control	Colorless transparent liquid	320 µL	1 tube
Strip's caps	24 8-caps		

Table 3. The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** content, package A, for R1-P815-XA/5EU

Reagent	Description	Total volume	Amount
PCR-mix Stream	Slightly pink or blue transparent liquid	1920 µL (120 µL in each tube)	2 8-tube strips
PCR-buffer Stream-K	Colorless transparent liquid	1200 µL (600 µL in each tube)	2 tubes
TechnoTaq MAX polymerase	Colorless transparent viscous liquid	120 µL (60 µL in each tube)	2 tubes
DNA dilution buffer	Colorless transparent liquid	4.0 mL (1.0 mL in each tube)	4 tubes
Positive control	Colorless transparent liquid	200 µL (100 µL in each tube)	2 tubes
Strips	10 8-tube strips		
Strip's caps	2 8-caps		

All components are ready to use and do not require additional preparation for operation.

The kit is intended for single use and is designed for 12 detections for package S and 24 detections for package A (one run of 24 samples or two runs of 12 samples), including the analysis of unknown samples, positive controls and negative controls.

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

4.1. Specimen collection

- Sterile containers to collect clinical material.

4.2. DNA extraction and PCR

Preamplification-specimen and control preparation area:

- Biological safety cabinet class II;
- Vortex mixer;
- Refrigerator;
- Nucleic acid extraction kit ("DNA-Technology" made **PREP-NA PLUS** (REF P-002/2EU), **PREP-MB MAX** (REF P-103-A/8EU) extraction kits are recommended);
- High speed centrifuge (RCF(g) no less than 16000);
- Solid-state thermostat (temperature range 40-95 °C);
- Tube rack for 1.5 mL tubes;
- 1.5 mL tubes;
- Physiological saline solution 0.9% NaCl (Sterile);
- Lysozyme;
- Distilled water;
- Single channel pipettes (dispensers covering 20-1000 µL volume range);
- Electric laboratory aspirator with trap flask for the removal of supernatant;
- RNase and DNase free filtered pipette tips (volume 200 µL, 1000 µL);
- RNase and DNase free pipette tips for aspirator with trap flask;
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator;
- Vortex mixer;
- Vortex rotor for 0.2 mL strips;
- Tube rack for 0.2 mL strips and 1.5 mL tubes;
- Single channel pipettes (dispensers covering 2.0-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- DTstream M1 dosing device or its analogue;
- DTstream M4 dosing device or its analogue;
- RNase and DNase free filtered pipette tips (volume 200 µL) for DTstream M1/M4 dosing device or its analogue;
- DTpack plate sealer ("DNA-Technology Research & Production", LLC);

- Centrifuge (RCF(g) no less than 500) with microplate adapter;
- Polymer thermal film for 384-well microplate sealing;
- 384-well PCR microplate;
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Post-Amplification – Amplification detection area:

- Real-time PCR thermal cycler.

Software:

The most recent version of the DT thermal cyclers software can be downloaded from https://www.dna-technology.com/software.

The OS supported: all versions of Windows starting from 7.

5. TRANSPORT AND STORAGE CONDITIONS

Expiry date – 12 months from the date of production.

All components of **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** except TechnoTaq MAX polymerase must be stored at temperatures from 2 °C to 8 °C during the storage period. TechnoTaq MAX polymerase must be stored at temperatures from minus 18 °C to minus 22 °C during the storage period. The PCR-mixes for amplification must be stored out of light during the storage period.

The excessive temperature and light can be detrimental to product performance.

The kit has to be transported in thermoboxes with ice packs by all types of roofed transport at temperatures corresponding to storage conditions.

Transportation of the kit, except the TechnoTaq MAX polymerase, is allowed in termobox with ice packs by all types of roofed transport at temperatures from 2 °C to 25 °C but no more than 5 days and should be stored at temperatures from 2 °C to 8 °C immediately on receipt.

It is allowed to transport the TechnoTaq MAX polymerase in termobox with ice packs by all types of roofed transport at temperatures up to 25 °C but no more than 5 days and should be stored at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

Shelf-life of the kit following the first opening of the primary container:

- components of the kit except TechnoTaq MAX polymerase should be stored at temperatures from 2 °C to 8 °C during the storage period;
- TechnoTaq MAX polymerase should be stored at temperatures from minus 18 °C to minus 22 °C during the storage period;
- PCR-mixes for amplification should be stored at temperatures from 2 °C to 8 °C and out of light during the storage period;

The kits stored in under undue regime should not be used.

An expired **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** should not be used.

We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

The conformity of the **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

Contact our official representative in EU by quality issues of the **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit**.

6. WARNINGS AND PRECAUTIONS

Only personnel trained in the methods of molecular diagnostics and the rules of work in the clinical and diagnostic laboratory are allowed to work with the kit.

Handle and dispose all biological samples, reagents and materials used to carry out the assay as if they were able to transmit infective agents. The samples must be exclusively employed for certain type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. Pipettes used to handle reagents must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA**.** Avoid direct contact with the biological samples reagents and materials used to carry out the assay. Wear powder-free surgical gloves. Wear protective clothing (work clothes and personal protective equipment) working with microorganisms classified as particularly pathogenic. The protective clothing and personal protective equipment must comply with the work to be performed and health and safety requirements. Avoid producing spills or aerosol. Any material being exposed to biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121 °C before disposal.

Molecular biology procedures, such as nucleic acids extraction, amplification and detection require qualified staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.

All oligonucleotide components are produced by artificial synthesis technology according to internal quality control protocol and do not contain blood or products of blood processing.

Positive control is produced by artificial synthesis technology. Positive control does not include parts of infectious agents.

All the liquid solutions are designed for single use and can not be used more than once in amplification reactions. Plastic tubes do not contain phthalates. Do not breathe gas/fumes/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with eyes. Only use the reagents provided in the kit and those recommended by manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits. All laboratory equipment, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, etc., as well as reagents should be strictly stationary. It is not allowed to move them from one room to another. Equip separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions. Wear lab coats, gloves and tools, which are exclusively employed for the extraction/preparation of the amplification reaction and for the amplification/detection of the amplification products. Never transfer lab coats, gloves and tools from the area designed for amplification/detection of the amplification products to the area designed for extraction/preparation of amplification reactions. Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be exclusively employed for this specific purpose. Remove PCR waste only in a closed form. Remove waste materials (tubes, tips) only in a special closed container containing a disinfectant solution. Work surfaces, as well as rooms where NA extraction and PCR are performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work.

Do not open the tubes after amplification. Work surfaces, as well as rooms where PCR is performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work. Waste materials are disposed of in accordance with local and national standards. All surfaces in the laboratory (work tables, test tube racks, equipment, etc.) must be treated daily with disinfecting solution.

Emergency actions

Inhalation: Inhalation of the PCR-mix contained within this kit is unlikely, however care should be taken.

Eye Contact: If any component of this kit enters the eyes, wash eyes gently under potable running water for 15 minutes or longer, making sure that the eyelids are held open. If pain or irritation occurs, obtain medical attention.

Skin Contact: If any component of this kit contacts the skin and causes discomfort, remove any contaminated clothing. Wash affected area with plenty of soap and water. If pain or irritation occurs, obtain medical attention.

Ingestion: If any component of this kit is ingested, wash mouth out with water. If irritation or discomfort occurs, obtain medical attention.

Do not use the kit:

- When the transportation and storage conditions are breached;
- When the reagents' appearance does not respond to the kit passport;
- When the kit components packaging is breached;
- After the expiry date provided.

Significant health effects are **NOT** anticipated from routine use of this kit when adhering to the instructions listed in the current manual.

7. SAMPLES

The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** is designed to detect DNA extracted from human faeces (including meconium).

Interfering substances

The presence of PCR inhibitors in a sample may cause controversial (uncertain) results. The sign of PCR inhibition is the simultaneous absence of internal control and specific product of amplification.

PCR inhibitors are the presence of hemoglobin, bilirubin, cholesterol, triglycerides, mucus (mucin) and pharmaceuticals, in the DNA sample as a result of incomplete removal during the extraction of DNA from a biomaterial sample containing an impurity of blood, as well as the presence of isopropyl alcohol and methyl acetate in the DNA sample as a result of incomplete removal of washing solutions during sample preparation.

The maximum concentrations of interfering substances are indicated in the table below.

Biological material	Interfering substance	Interfering substance concentration
Endogenous substances		
Faeces	Bilirubin	684 µmol/L
	Cholesterol	13 mmol/L
	Hemoglobin	0.35 mg/mL
	Triglycerides	37 mmol/L
	Mucin	20%
Exogenous substances		
Faeces	Isopropyl alcohol	10%
	Methyl acetate	10%
	Ibuprofen suppositories	5%
	Espumisan emulsion	5%
	Viferon suppositories	10%

Impurities contained in the biomaterial sample, such as mucus, blood, local medicines, including those that are contained in rectal suppositories should be removed during the DNA extraction using sample preparation kits. To reduce the count of PCR inhibitors, it is necessary to follow the principles of taking biological material. Suspecting a large count of PCR inhibitors in the sample, it is recommended to choose DNA extraction methods that allow to remove PCR inhibitors from the sample as much as possible. It is not recommended to use express methods of DNA extraction.

General requirements

In order to obtain correct results, the quality of the biological material sample taken for assay, its storage, transport and pre-treatment are of great importance.

Inappropriate sampling may result in doubtful results and sample collection may need to be repeated.

Sample collection

ATTENTION! Before DNA extraction pre-processing of biological material samples is needed.

Faeces (meconium)

Samples of faeces or meconium with mass (volume) 1.0-3.0 g (1.0-3.0 mL) are transferred to a sterile dry flask by a single-use filtered pipette tip or single-use shovel.

After sample collection the flask is tightly closed with a lid and marked.

Transportation and storage of the samples

Faeces samples

Faeces samples or meconium can be transported and stored:

- at room temperature from 18 °C to 25 °C no more than 6 hours;
- at temperature from 2 °C to 8 °C no more than 3 days.

Faeces suspension with glycerin:

- at minus 20 °C for one week;
- at minus 70 °C for prolonged period.

Sample preparation

Faeces (meconium) - preparation of the suspension

1. Put approximately 0.1-0.2 g (mL) of faeces into the 1.5 mL tube with 1.0 mL of sterile saline solution.
2. Vortex the tube for 5-10 seconds.

If it is not possible to examine the material within 24 hours and/or if long-term storage is necessary, glycerin at a final concentration of 10-15% is added to faeces suspension in sterile isotonic sodium chloride solution. Samples prepared in this manner are frozen only after thorough homogenization and exposure to glycerin for 30-40 minutes.

Further processing of the suspension is carried out in accordance with the instruction for the DNA extraction kit from the corresponding biomaterial.

8. PROCEDURE

DNA extraction from biological material

For DNA extraction it is recommended to use a **PREP-NA PLUS** and **PREP-MB MAX** extraction kit manufactured by “DNA-Technology”, or similar reagents that have medical device registration certificates and are intended for the examined biomaterial for subsequent DNA PCR assay, with the use of which 300 µL of isolated DNA will be obtained.

It is strictly necessary to treat the test samples with lisozyme before the DNA extraction procedure.

8.1 Prepare a lysozyme solution with a concentration of 100 µg/µL.

8.1.1. In a 1.5 mL Eppendorf-type tube, weigh the lysozyme powder in an amount of (N+1) x 2 mg, where N is the number of test samples;

8.1.2. Add (N+1) x 20 µL of distilled water to the lysozyme suspension, where N is the number of test samples;

8.1.3. Resuspend for 3-5 seconds on a vortex mixer until the crystals are completely dissolved;

8.1.4. Spin down the drops from the cap for 1-3 seconds in a vortex mixer.

The lysozyme solution is ready to use.

8.2 Sample processing

8.2.1. Centrifuge tubes with fecal suspension samples at RCF(g) 13000 for 30 seconds at room temperature (18 °C to 25 °C) to settle the debris at the bottom of the tube.

8.2.2. Mark one 1.5 mL tube for each test sample and negative control "C-".

ATTENTION! To prevent contamination, only open the cap of the tube you are working with (sample/reagent adding) and close it afterwards. It is not allowed to work with several tubes with opened caps at the same time.

8.2.3. Add 100 µL of the middle fraction from the fecal suspension tubes to each corresponding tubes. No samples should be added to the "C-" tube.

8.2.4. Add 100 µL of sterile physiological saline solution or the negative control included in the kit for nucleic acid extraction to the "C-" tube in the volume specified in the instruction of the corresponding kit.

8.2.5. Add 20 µL of lysozyme solution (see 8.1) to the marked sample tubes and to the "C-" tube.

8.2.6. Shake the tubes for 3-5 seconds on a vortex mixer.

8.2.7. Centrifuge the tubes for 1-3 seconds in a vortex mixer.

8.2.8. Incubate the tubes for 60 minutes at room temperature (18 °C to 25 °C) or for 30 minutes at 37 °C. During incubation shake the tubes gently 2-3 times for 3-5 seconds on a vortex mixer.

8.2.9. Centrifuge the tubes for 60 seconds in a vortex mixer.

The samples are ready for DNA extraction with the use of **PREP-NA PLUS** and **PREP-MB MAX** extraction kits.

DNA extraction is carried out in accordance with the instruction for extraction kits. DNA extraction kits for subsequent usage of DNA in PCR are recommended. The quality control of the extraction is carried out by the system of internal control (IC).

ATTENTION! Independently of DNA extraction kit used, a negative control sample should go through all stages of DNA extraction. Physiological saline solution or negative control sample from an extraction kit can be used as a negative control sample in volumes as indicated.

8.3 Preparing PCR for package S:

ATTENTION! The reagents and tubes should be kept away from direct sun light.

ATTENTION! When using package S, strips, strictly observe the completeness of the strips and caps for them. Do not use the caps for the strips of the other kits!

8.3.1. Mark one strip № 1 and one strip № 2 with paraffin sealed PCR-mix for each test sample, negative control "C-" and positive control "C+".

Example: 2 samples to be tested. Mark 4 strips № 1 and 4 strips № 2 (8 strips total): one strip № 1 and № 2 for <u>each</u> of 2 test samples, one strip № 1 and № 2 for "C-", and one strip № 1 and № 2 for "C+" (see Table 4).

Table 4– Example of strip marking for PCR

Samples	Strip №	Strip ID
Sample 1	1	“strip № 1”
	2	“strip № 2”
Sample 2	3	“strip № 1”
	4	“strip № 2”
C-	5	“strip № 1”
	6	“strip № 2”
C+	7	“strip № 1”
	8	“strip № 2”

8.3.2. Shake the tube with Taq-polymerase solution in vortex mixer for 3-5 seconds, then centrifuge at low speed for 1-3 seconds.

8.3.3. Add 10 µL of Taq-polymerase solution into each tube of marked strips without damaging the paraffin layer.

8.3.4. Add one drop (~20 µL) of mineral oil into each strip tube. Close the strips.

8.3.5. Shake the tubes with DNA preparation, “C-” and “C+” in vortex mixer for 3-5 seconds, then centrifuge at low speed for 1-3 seconds.

ATTENTION! In case of using **PREP-MB MAX DNA Extraction Kit**. The DNA samples must stand in a magnetic rack while adding DNA. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 3-5 seconds in a vortex mixer.

ATTENTION! Open the strip, add DNA sample (or control sample), then close the strip before proceeding to the next strip to prevent contamination. Close the strips tightly. Use filter tips.

8.3.6. Add 5.0 µL of DNA preparation extracted from the sample into each tube of the corresponding marked strips (except for “C-” and “C+”) without damaging the paraffin layer.

8.3.7. Add 5.0 µL of negative control which has passed the DNA extraction step into each strip tube marked “C-” without damaging the paraffin layer.

8.3.8. Add 5.0 µL of positive control into each strip tube marked “C+” without damaging the paraffin layer.

8.3.9. Centrifuge all the strips at low speed for 1-3 seconds.

8.3.10. Set the strips into the Real-time Thermal Cycler.

8.3.11. Launch the operating software for DT instrument[1]. Add corresponding test[2], specify the number and ID’s of the samples, positive and negative control samples. Specify the position of the strips in the thermal unit (see 8.3.10) and run PCR. See Table 5.

[1] Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.
[2] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

Table 5. The PCR program for DTlite and DTprime Thermal Cyclers (package S)

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	80	0	30	1		Cycle
	94	1	30			
2	94	0	30	5		Cycle
	64	0	15		√	
3	94	0	10	45		Cycle
	64	0	15		√	
4	94	0	5	1		Cycle
5	10[1]		…	Holding		Holding

√ - optical measurement
[1] – holding at 25°C is allowed

8.4 Preparing PCR for package A:

Preparation and performance of PCR using a DTstream dosing device.

ATTENTION! The reagents and tubes should be kept away from direct sun light.

8.4.1 Thoroughly mix the content of PCR-buffer Stream-K and TechnoTaq MAX polymerase tubes on a vortex mixer and at low speed for 3-5 seconds. To carry out 24 detections, 2 tubes with PCR-buffer Stream-K and 2 tubes with TechnoTaq MAX polymerase are used simultaneously. To carry out 12 detections, 1 tube with PCR-buffer Stream-K and 1 tube with TechnoTaq MAX polymerase are used simultaneously.

ATTENTION! TechnoTaq MAX polymerase should be taken out of the freezer immediately before use.

8.4.2 If using the “Basic setting method”, prepare TechnoTaq MAX polymerase solution and test DNA samples according to the dosing device user manual. If using the “Integral setting method”, no preconditioning of the TechnoTaq MAX polymerase solution and test DNA samples is required.

8.4.3 Centrifuge strips with PCR-mix Stream at low speed for 1-3 seconds.

8.4.4 Shake tubes with positive and negative control for 3-5 seconds on vortex mixer, then centrifuge at low speed for 1-3 seconds.

8.4.5 When using the "Basic setting method," set up strips with PCR-mix Stream leading from the mixture’s color code (the first tube of strip № 1 and the second tube of strip № 2 are marked with blue buffer), tubes with prepared TechnoTaq MAX polymerase solution, positive control and negative control, and a new 384-well microplate on the DTstream workbench.

8.4.6 When using the "Integral setting method," set up strips with PCR-mix Stream leading from the mixture’s color code (the first tube of strip № 1 and the second tube of strip № 2 are marked with blue buffer), tubes with PCR-buffer Stream-K, TechnoTaq MAX polymerase, DNA dilution buffer, positive control and negative control, and a new 384-well microplate on the DTstream workbench

ATTENTION! In case of using **PREP-MB MAX DNA Extraction Kit**. The DNA samples must stand in a magnetic rack while adding DNA. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 3-5 seconds in a vortex mixer.

8.4.7 Set up the tubes with DNA samples on DTstream workbench according to the chosen setting

method (“basic” or “integral”) and to the dosing device user manual.

8.4.8 Open the strips with PCR-mix Stream after carefully removing the protection seal and carry out the dosing of the components according to the user manual.

8.4.9 Place the 384-well microplate carefully, without shaking, into the DTpack plate sealer carrier after the program on DTstream is finished.

8.4.10 Seal the 384-well microplate with thermal film according to the DTpack user manual.

8.4.11 Centrifuge 384-well microplate at RCF(g) 500 for 30 seconds.

8.4.12 Place a 384-well microplate in the detection thermal cycler unit.

8.4.13 Launch the operating software for DT instrument[3]. Add corresponding test[4], specify the number and ID’s of the samples, positive and negative control samples. Specify the position of the tubes in the thermal unit (see 8.4.12) and run PCR. See Table 6.

Table 6. The PCR program for DTlite and DTprime Thermal Cyclers (package A)

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	80	0	05	15		Cycle
	94	0	05			
2	94	5	00	1		Cycle
3	94	0	30	5		Cycle
	64	0	15		√	
4	94	0	10	45		Cycle
	64	0	15		√	
5	94	0	5	1		Cycle
6	10[1]		…	Holding		Holding
√ - optical measurement [1] – holding at 25°C is allowed						

9. CONTROLS

The **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** contains positive control sample. Positive control is a cloned part of the bacterial genome. It is produced with genetic engineering techniques and characterized by automatic sequencing. The PCR-mix from the kit includes the internal control (IC). IC is an artificial plasmid intended to assess the quality of PCR performance. To reveal possible contamination a negative control is required.

ATTENTION! A negative control sample should go through all stages of DNA extraction. Physiological saline solution can be used as a negative control sample in volumes indicated in supplied instructions.

The test result is considered valid when:

[3] Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.
[4] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

- the exponential growth of the fluorescence level for the specific product is present, in this case the internal control is not taken into account;
- the exponential growth of the fluorescence level for the specific product is absent and for internal control is present.

The test result is considered invalid when the exponential growth of the fluorescence level for the specific product and for internal control are not observed.

If positive control (C+) does **not** express growing fluorescence of the specific product or positive result, it is required to repeat the whole test. It may be caused by inhibitors, operation error or violation of storage and handling.

If negative control (C-) expresses growing fluorescence of the specific product or positive result, all tests of the current batch are considered false. Decontamination is required.

10. DATA ANALYSIS

Registration of the PCR results are held in automatic mode. The general principles of interpreting results are shown in Table A.1 of Annex A.

The graph will show the fluorescence dependence of the number of cycle on all detecting channels for each tube in the thermoblock.

The table will show the sample ID, decimal logarithms of concentrations (Lg) on corresponding channels and interpretation of the amplification results ("+" or "-").

It is possible to create and print a report based on the analysis results.

After the end of the amplification program the software compares predetermined order of tubes with the real localization of the Rox marker and in case of mismatch warns an operator. In this case the operator should check the localization of the strips in a thermoblock and correct identifiers of tubes in the protocol.

1 When analyzing the results, the values of total bacterial load[5] (TBL, the first tube of strip № 1, Fam channel) and internal control (IC, tubes 1-8 of strips № 1 and № 2, Hex channel) must be considered:

   – To control the quality of sampling, the TBL indicator is used, which also allows to determine whether the amount of DNA obtained is sufficient for the assay. A TBL value less than 6.0 Lg should be interpreted as an insufficient amount of material. In this case it is recommended to repeat the DNA extraction or to repeat the sampling (performed sequentially).

   – To assess the quality of DNA extraction, Internal control (IC) is used. If the IC is not present in one or more tubes in the strip and at the same time there are the absence of specific positive results in these tubes, the result in these tubes is considered invalid due to incorrect conduction of PCR. In this case repeating of PCR amplification, or reextraction of DNA, or resampling is needed (performed sequentially).

2 In test samples containing DNA of the analytes being detected, the software will record an exponential increase in the fluorescence level on the corresponding channel (Fam, Rox, or Cy5) in the corresponding tube. In the table, the line with the name of the corresponding analyte will indicate the result of the assay (decimal logarithm of the number of genome equivalents in 1.0 g of feces (Lg GE/g) and a histogram).

3 In samples of biological material not containing the DNA of the analytes being detected, when amplification is performed, an exponential increase in the fluorescence level in the corresponding tube for the designated detection channels will be absent or present below the background value, but present for internal control (IC) for the Hex channel. In the table, the line with the name of

[5] The term previously used is total bacterial mass (TBM).

the corresponding analyte will indicate the test result (“-”).

4 In tube 1 of strip № 1, Lg ≤4.5 on the Fam channel and Lg ≤4.0 on the Cy5 channel are not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

5 In tube 2 of strip № 1, Lg ≤4,0 on the Rox channel is not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

6 In tube 3 of strip № 1, Lg ≤4,0 on the Cy5 channel is not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

7 In tube 4 of strip № 1, Lg ≤4,0 on the Cy5 channel is not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

8 In tube 6 of strip № 1, Lg ≤4,0 on the Cy5 channel is not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

9 In tube 7 of strip № 1, Lg ≤4,0 on the Fam and Cy5 channel are not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

10 In tube 8 of strip № 1, Lg ≤4,0 on the Fam channel is not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

11 In tube 1 of strip № 2, Lg ≤4,0 on the Rox and Cy5 channels are not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

12 In tube 2 of strip № 2, Lg ≤4,0 on the Fam and Cy5 channels are not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

13 In tube 3 of strip № 2, Lg ≤4,0 on the Fam and Cy5 channels are not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

14 In tube 4 of strip № 2, Lg ≤4,0 on the Fam channel is not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

15 In tube 5 of strip № 2, Lg ≤4,0 on the Fam and Cy5 channels are not taken into account by the software, and the table to the right, in the column “Result (Lg)” will show the results of the assay (“-”).

16 In tubes 6 and 7 of strip № 2, Lg ≤4,0 on the Cy5 channel is not taken into account by the software, and the table to the right, in the column "Result (Lg)" will show the results of the assay (“-”).

17 For negative and positive control, the results shown in Table 7 should be obtained. In the negative control sample the IC Lg value must be indicated. In the positive control sample the IC Lg value is not considered.

18 If the results for the negative control differ from the values specified in Table 7, the results of the whole production series are considered unreliable. In this case it is necessary to carry out special measures to identify and eliminate possible contamination.

19 A positive control included in the kit is used to control the efficiency of PCR. If all reaction conditions are met, the amount of target DNA determined in the control sample should be within the range shown in Table 6. If the values for a positive control sample differ from those specified in Table 7, the whole batch of samples must be amplified again.

Table 7. The results of the test for negative and positive control samples

Strip type	Strip tube №	Detected indicator	C- (Lg)	C+ (Lg)
Strip № 1	1	TBL	Not stated or ≤4.5	5.0-7.0
		Candida spp.	-	5.0-7.0
	2	*Bacteroides* spp.	-	5.0-7.0
		Lactococcus lactis	-	5.0-7.0
		Bifidobacterium spp.	-	5.0-7.0
	3	*Faecalibacterium prausnitzii*	-	5.0-7.0
		Bif.adolescentis	-	5.0-7.0
		Dialister spp.,*Alisonella* spp., *Megaspherae* spp.,*Veillonella* spp.	-	5.0-7.0
	4	*Methanobrevibacter* spp.	-	5.0-7.0
		Bif. longum subsp.infantis	-	5.0-7.0
		Parabacteroides spp.	-	5.0-7.0
	5	*Akkermansia muciniphila*	-	5.0-7.0
		Bif. longum subsp. longum	-	5.0-7.0
		Butyricimonas spp.	-	5.0-7.0
	6	*Desulfovibrio* spp.	-	5.0-7.0
		Bif.bifidum	-	5.0-7.0
		Coriobacteriia	-	5.0-7.0
	7	*Clostridium leptum gr.*	-	5.0-7.0
		Candida albicans	-	5.0-7.0
		Lactobacillaceae	-	5.0-7.0
	8	*Lachnospiraceae*	-	5.0-7.0
		Bif. catenulatum subsp.	-	5.0-7.0
		Clostridium difficile gr.	-	5.0-7.0
Strip № 2	1	*Enterococcus* spp.	-	5.0-7.0
		Erysipelotrichaceae	-	5.0-7.0
	2	*E.coli*	-	5.0-7.0
		Enterobaterales	-	5.0-7.0
	3	*Peptoniphilaceae*	-	5.0-7.0
		Bif. animalis subsp.lactis	-	5.0-7.0
		Pseudomonas spp.	-	5.0-7.0
	4	*Alistipes* spp.	-	5.0-7.0
		Bif.dentium	-	5.0-7.0
		Fusobacteriaceae	-	5.0-7.0
	5	*Prevotella* spp.	-	5.0-7.0
		Bif.breve	-	5.0-7.0
		Clostridium perfringens gr.	-	5.0-7.0
	6	*Str.agalactiae*	-	5.0-7.0
		srr2	-	5.0-7.0
		Streptococcus spp.	-	5.0-7.0
	7	*St.aureus*	-	5.0-7.0
		mecA	-	5.0-7.0
		Staphylococcus spp.	-	5.0-7.0
	8	tcdA, tcdB	-	5.0-7.0
		Clostridioides difficile	-	5.0-7.0

20 If the amplification of a specific product (Lg indicated) is positive in the Fam, Rox or Cy5 channels in the negative control “C-”, the results of the whole production series are rejected. In this case, special measures must be taken to detect and eliminate possible contamination.

11. SPECIFICATIONS

a. The analytical **specificity** of the **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** was assessed by bioinformatics analysis using available on-line databases with up-to-date comprehensive genetic information. The specific oligonucleotides used in the test were checked against GenBank database sequences. None of the sequences showed sufficient similarity for unspecific detection.

The samples of human biological material with DNA of the analytes detected are to be registered positive for specific product through the declared detection channels.

The samples of human biological material free of the detected analytes' DNA are to be registered negative for specific product through the declared detection channels.

For each test in the kit, there are not cross non-specific results with all other tests from the kit and non-specific positive results of amplification in the presence of other microorganisms or human DNA in concentration up to 1.0×10^8 copies/mL of the sample.

b. Analytical **sensitivity** of the **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit** is 25 copies of DNA per amplification tube ($5.0x10^3$ copies per 1.0 mL of extracted DNA and $1.5x10^5$ copies per 1.0 g of feces). Sensitivity is determined by the analysis of serial dilutions of the laboratory control sample (LCS) and artificial control samples of feces suspensions with an added mixture of cultures of analytes detected.

Sensitivity depends the extraction kit used for DNA extraction and the final elution volume (dilution) of the extracted DNA.

The specified values of the detection limits of the **ENTEROFLOR Kiddy** PCR kit for microorganism cultures were obtained during DNA extraction with the recommended **PREP-MB MAX** extraction kit produced by "DNA-Technology TS", LLC (elution volume 300 µL).

c. Diagnostic characteristics

Indicator	Diagnostic characteristics	
	Diagnostic sensitivity (%)	**Diagnostic specificity (%)**
Akkermansia muciniphila	100% (95.26 – 100)	100% (96.61 – 100)
Alistipes spp.	100% (95.80 – 100)	100% (96.27 – 100)
*Bacteroide*s spp.	100% (97.16 – 100)	100% (93.51 – 100)
Bifidobacterium adolescentis	100% (93.84 – 100)	100% (97.07 – 100)
Bifidobacterium animalis subsp. *lactis*	100% (94.40 – 100)	100% (96.95 – 100)
Bifidobacterium bifidum	100% (95.44 – 100)	100% (96.52 – 100)
Bifidobacterium breve	100% (94.04 – 100)	100% (97.05 – 100)
Bifidobacterium catenulatum subsp.	100% (92.45 – 100)	100% (97.32 – 100)
Bifidobacterium dentium	100% (93.40 – 100)	100% (97.18 – 100)
Bifidobacterium longum subsp. infantis	100% (87.66 – 100)	100% (97.65 – 100)
Bifidobacterium longum subsp. *longum*	100% (96.34 – 100)	100% (95.70 – 100)
Bifidobacterium spp.	100% (97.79 – 100)	100% (81.47 – 100)
*Butyricimona*s spp.	100% (97.20 – 100)	100% (93.28 – 100)
Candida albicans	100% (66.37 – 100)	100% (97.90 – 100)
Candida spp.	100% (94.87 – 100)	100% (96.79 – 100)
Clostridioides difficile	100% (84.56 – 100)	100% (97.73 – 100)
Clostridium difficile gr	100% (97.63 – 100)	89,66% (72.65-97.81)
Clostridium leptum gr.	100% (97.36 – 100)	100% (92.13 – 100)
Clostridium perfringens gr.	100% (96.11 – 100)	100% (95.98 – 100)
Coriobacteriia	100% (97.26 – 100)	100% (92.89 – 100)
Desulfovibrio spp.	100% (95.94 – 100)	100% (96.15 – 100)
E. coli	100% (97.38 – 100)	100% (91.96 – 100)
Enterobaterales	100% (97.72 – 100)	100% (85.18 – 100)
Enterococcus spp.	100% (97.71 – 100)	100% (85.75 – 100)
Erysipelotrichaceae	100% (97.49 – 100)	100% (90.75 – 100)
Faecalibacterium prausnitzii	100% (96.76 – 100)	100% (94.94 – 100)
Fusobacteriaceae	100% (90.26 – 100)	100% (97.52 – 100)
Lachnospiraceae	100% (97.18 – 100)	100% (93.40 – 100)
Lactobacillaceae	100% (97.49 – 100)	100% (90.75 – 100)
Lactococcus lactis	100% (96.41 – 100)	100% (95.60 – 100)
mecA	100% (91.96 – 100)	100% (97.38 – 100)
Dialister spp./*Alisonella* spp./ *Megaspherae* spp./ *Veillonella* spp.	100% (97.52 – 100)	94,44% (81.34-99.32)
Methanobrevibacter spp.	100% (83.16 – 100)	100% (97.76 – 100)
Parabacteroides spp.	100% (96.76 – 100)	100% (94.94 – 100)
Peptoniphilaceae	100% (95.80 – 100)	100% (96.27 – 100)
Prevotella spp.	100% (96.15 – 100)	100% (95.94 – 100)
Pseudomonas spp.	100% (89.42 – 100)	100% (97.57 – 100)
srr2	100% (59.04 – 100)	100% (97.93 – 100)
Staphylococcus aureus	100% (93.02 – 100)	100% (97.24 – 100)
Staphylococcus spp.	100% (96.58 – 100)	100% (95.32 – 100)
Streptococcus agalactiae	100% (92.13 – 100)	100% (97.36 – 100)
Streptococcus spp.	100% (97.90 – 100)	100% (66.37 – 100)
tcdA tcdB	100% (69.15 – 100)	100% (97.89 – 100)

d. Within- batch and between- batch precision

Within-batch precision – 100% (83.16–100%).

Between- batch precision – 100% (83.16–100%).

12. TROUBLESHOOTING

Table 8. Troubleshooting

	Result	Possible cause	Solution
C+	-	Operation error PCR inhibition Violation of storage and handling requirements	Repeat whole test Dispose current batch
C-	+	Contamination	Dispose current batch Perform decontamination procedures
IC	Invalid	PCR inhibition	Repeat whole test Resample

If you face to any undescribed issues contact our customer service department regarding quality issues with the kits:

Phone: +7(495) 640.16.93

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com/support

13. QUALITY CONTROL

The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

- observation of quality management in manufacturing of IVDD products;
- creation of values for customers;
- maintenance of the best service quality and customer management.

Contact our customer service by quality issues of **ENTEROFLOR Kiddy REAL-TIME PCR Detection Kit**.

Technical support:

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14. KEY TO SYMBOLS

IVD	*In vitro* diagnostic medical device		Date of manufacture
	Temperature limit		Consult instructions for use
Σ	Contains sufficient for <n> tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
VER	Version	CONTROL +	Positive control
	Caution		

Annex A

Results interpretation principles

Detection, data analysis and logarithm of microbiota DNA concentrations calculation are made by software automatically. In this supplementary results interpretation principles are described.

Table A.1

Samples	Detection channel		Results interpretation
	Fam, and/or Rox, and/or Cy5	Hex	
	Result (Lg)		
1	Lg value indicated/not indicated on one/several channel(s)	Lg value not considered	Fragment of the analyte DNA determined on the corresponding detection channel is detected/not detected.
2	Lg value not indicated on any channels	Lg value indicated	Fragment of the analyte DNA determined on the corresponding detection channel not detected
3	Lg value not indicated on any channels	Lg value not indicated	Unreliable result*

* PCR with the existing DNA preparation must be repeated, or DNA must be isolated and PCR performed again, or another sampling must be carried out (carried out sequentially).