RUO

INSTRUCTION FOR USE

Gastrointestinal Panel PCR Kit

For Research Use Only

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REF MBLGAS010

06 2024

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Document Revision History

Rev.No_Date	Revision Description
Rev.00_June 20, 2024	First Release

CONTENTS

1. INTENDED USE

For Research Use Only (RUO). Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of disease. Furthermore, the test kit is not intended to diagnose infectious animal diseases.

The ***MarinaBiolab Gastrointestinal Panel PCR Kit*** is a multiplexed qualitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-qPCR) test intended for the simultaneous detection and identification of multiple pathogenic nucleic acids in research samples. The ***MarinaBiolab Gastrointestinal Panel PCR Kit*** allows to achieve RT-qPCR result in less than 1 hour. The test is performed to detect gene sequences of the following organisms.

Targets	
Sapovirus (I, II, IV, and V)	*Plesiomonas shigelloides*
Adenovirus F40/41	*Salmonella spp.*
Norovirus GI/GII	*Campylobacter spp.*
Astrovirus	*Enteroaggregative E. coli (EAEC)*
Rotavirus A	*Shiga toxin-producing E. coli (STEC)*
Vibrio vulnificus	*Shigella/Enteroinvasive E. coli (EIEC)*
Vibrio cholerae	*Enteropathogenic E. coli (EPEC)*
Vibrio parahaemolyticus	*Enterotoxigenic E. coli (ETEC)*
Giardia lamblia	*Yersinia enterocolitica*
Entamoeba histolytica	*Clostridioides (Clostridium) difficile toxin A*
Cryptosporidium spp.	*Clostridioides (Clostridium) difficile toxin B*
Cyclospora cayetanensis	*Clostridioides (Clostridium) difficile binary toxin A/B*
Controls	
Human RNase P (IC)	
Bacillus atrophaeus (EC)	
MS2 Bacteriophage (EC)	

2. PRINCIPLE of the PROCEDURE

From the RNA and DNA target regions in the lysed or extracted research samples, the RNA target regions are reverse transcribed into cDNA via reverse transcriptase. Then cDNA and DNA target regions are amplified via real-time PCR instruments using the primer and probe sets in the kit. In the process, the probe anneals a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the real-time PCR instruments. Probes labeled with different fluorophores are used to detect specific amplicons originating from targets and Internal Control.

PCR instruments measure these signals at the end of each amplification cycle in real time and interpret the data to provide a qualitative result for each of the above targets. A positive result for the detection of target RNA or DNA is indicated by the presence of a real-time PCR growth curve and an associated Cq (Quantification Cycle) value.

3. KIT COMPONENTS

The ***MarinaBiolab Gastrointestinal Panel PCR Kit*** consists of four main components:

1. RT-qPCR Enzyme and Buffer Mix (RT-qPCR Master Mix)
2. Forward, Reverse and Probe Oligo Mix (GIP Oligo Mix 1-8)
3. Mix of non-infectious cDNA and DNA from artificial sample including targets in the table below (PC-GIP)
4. DNase/RNase-Free Water (NTC)

The kit components are provided in Table 1-2.

Table 1. Kit components.

Component	Description	Quantity x Volume
		100 rxn **MBLGAS010**
RT-qPCR Master Mix	Ready-to-use mix for RT-qPCR	4 x 1000 µL
GIP Oligo Mix 1-8	Primers and probes complementary to specific regions of the targets in the table above	8 x 250 µL
PC-GIP	Mix of non-infectious cDNA and DNA from artificial sample including targets in the table above	2 x 400 µL
NTC	DNase/RNase-Free Water	2 x 400 µL

Table 2. Oligo Mix target organisms and detection channels.

Vial Name	Target	Channel
GIP Oligo Mix 1	Sapovirus (I, II, IV, and V)	**FAM/Green**
	Human RNase P (IC)	**HEX/VIC/JOE/Yellow**
	-	**ROX/Texas Red/Orange**
	Adenovirus F40/41	**CY5/Red**
GIP Oligo Mix 2	*Giardia lamblia*	**FAM/Green**
	Entamoeba histolytica	**HEX/VIC/JOE/Yellow**
	Cryptosporidium spp.	**ROX/Texas Red/Orange**
	MS2 Bacteriophage (EC)	**CY5/Red**
GIP Oligo Mix 3	*Plesiomonas shigelloides*	**FAM/Green**
	Clostridioides (Clostridium) difficile toxin B	**HEX/VIC/JOE/Yellow**
	Cyclospora cayetanensis	**ROX/Texas Red/Orange**
	Vibrio vulnificus	**CY5/Red**

GIP Oligo Mix 4	Norovirus GI/GII	**FAM/Green**
	Astrovirus	**HEX/VIC/JOE/Yellow**
	-	**ROX/Texas Red/Orange**
	Rotavirus A	**CY5/Red**
GIP Oligo Mix 5	*Campylobacter spp.*	**FAM/Green**
	Vibrio parahaemolyticus	**HEX/VIC/JOE/Yellow**
	Salmonella spp.	**ROX/Texas Red/Orange**
	Vibrio cholerae	**CY5/Red**
GIP Oligo Mix 6	*Enteroaggregative E. coli (EAEC)*	**FAM/Green**
	Shiga toxin-producing E. coli (STEC)	**HEX/VIC/JOE/Yellow**
	-	**ROX/Texas Red/Orange**
	Shigella/Enteroinvasive E. coli (EIEC)	**CY5/Red**
GIP Oligo Mix 7	*Enteropathogenic E. coli (EPEC)*	**FAM/Green**
	Enterotoxigenic E. coli (ETEC)	**HEX/VIC/JOE/Yellow**
	-	**ROX/Texas Red/Orange**
	Bacillus atrophaeus (EC)	**CY5/Red**
GIP Oligo Mix 8	*Clostridioides (Clostridium) difficile toxin A*	**FAM/Green**
	Yersinia enterocolitica	**HEX/VIC/JOE/Yellow**
	-	**ROX/Texas Red/Orange**
	Clostridioides (Clostridium) difficile binary toxin A/B	**CY5/Red**

The oligonucleotide set targeting the human *RNase P* mRNA (Internal Control: IC), *Bacillus atrophaeus* (External Control: EC) and MS2 Bacteriophage (EC) are used to monitor sampling, nucleic acid extraction, reverse transcription, and inhibition of both reverse transcription and qPCR. The kit also contains negative and positive control templates for evaluating the contamination and the RT-qPCR reagent stability, respectively.

4. EQUIPMENT and MATERIALS REQUIRED but NOT PROVIDED

- 2-8°C Refrigerator
- ≤ -20°C Freezer
- ≤ -70°C Freezer (Optional)
- Vortex mixer
- Benchtop centrifuge with rotor for 1.5 mL tubes
- Benchtop mini centrifuge with rotor for PCR strips
- Benchtop plate centrifuge
- Biological Safety Cabinet (BSC)
- PCR cabinet for PCR Setup
- Adjustable Micropipettes: 1-10, 10-100, 100-1000 µL
- Sterile DNase/RNase free micropipettes tips - Compatible with the micropipettes
- Cold tube rack for microfuge tubes (1.5/2 mL) and for PCR tubes (0.1/0.2 mL)
- Disposable, powder-free, nitrile gloves
- Disposable (preferably) laboratory coat
- Surface decontaminants - Freshly diluted 10% bleach solution (0.5% NaClO)
- Applied Biosystems QuantStudio 5, 7, and 12K with Design & Analysis software and consumables
- Bio-Rad CFX96 Touch™/CFX96™ Dx/CFX Opus 96™/CFX Opus 96™ Dx/CFX384 Touch™/CFX Opus 384™ with Maestro software v1.1 and consumables
- Qiagen Rotor-Gene Q 5plex Platform with Rotor-Gene Q series software v2.1.0.9 and consumables
- Roche LightCycler 480 with software and consumables

5. WARNING and PRECAUTIONS

- The ***MarinaBiolab Gastrointestinal Panel PCR Kit*** is designed for research use only and should be used by professionally trained, qualified staff only. All work should be performed using Good Laboratory Practices.
- Biological material used for extraction of nucleic acid should be handled as potentially infectious. When handling biological material appropriate safety precautions are recommended (do not pipet by mouth; wear disposable gloves while handling biological material and performing the test; disinfect hands when finished the test).
- Biological material should be inactivated before disposal (e.g., in an autoclave). Disposables should be autoclaved or incinerated after use.
- Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated areas swabbed with a suitable standard disinfectant or 70% alcohol. Material used to clean spills, including gloves, should be inactivated before disposal (e.g., in an autoclave).
- Disposal of all samples, unused reagents and waste should be in accordance with country, federal, state, and local regulations.
- Microbial contamination of the reagents while taking aliquots should be avoided. It is recommended to use sterile one-way pipettes and tips. Reagents that look cloudy or show any signs of microbial contamination must not be used.
- The kit should be stored away from nucleic acid sources and qPCR amplicons.
- Always check the expiration date on the kit. Do not use expired or incorrectly stored kit.
- The components in the kit should not be mixed with components with different lot numbers or chemicals of the same name but from different manufacturers.
- Kit components should be mixed by gently shaking before use.
- A common concern with PCR-based assays is false positive results caused by contamination of the work area with PCR amplicon. To prevent amplicon contamination:
    - It shall be ensured that separate work areas with their own apparatus are available.
    - Do not open the reaction tubes/plates post amplification to avoid contamination with amplicons.
    - Discard used tubes/plates in a biohazard container immediately after the run has completed.
    - Avoid excessive handling of tubes/plates after test runs.
    - Change gloves after handling a used tubes/plate.

6. HANDLING, STORAGE, and STABILITY

- The ***MarinaBiolab Gastrointestinal Panel PCR Kit*** is shipped on dry ice. If any component except RT-qPCR Master Mix of the kit is not frozen on arrival, or if the outer packaging has been compromised during shipment, please contact **MarinaBiolab** or the local distributors as soon as possible.
- All components should be stored between -25°C and -15°C upon arrival.
- Repeated freezing and thawing of the kit components may result in lower detection quality. The kit can undergo up to 15 freeze/thaw cycles without affecting performance.
- When stored under the specified storage conditions, the kit is stable until the stated expiration date printed on the package. The expiration date of the kit is 12 months from date of manufacture.
- All components must be thawed at ambient temperature for a minimum of 30 minutes before use.
- It is recommended that all components should be kept on ice when setting up the assay mixes.
- The primer and probe mixes contain fluorophore labeled probes and should be protected from direct sunlight or long-term ambient light.
- Do not use expired or incorrectly stored components.

7. TEST PROCEDURE

7.1. Sample Preparation and Nucleic Acid Extraction

The sample material for the isolation of nucleic acid must be sent in appropriate cell collection systems. The performance of the kit strongly depends on the amount and quality of the extracted nucleic acid. It must be ensured that the system used for nucleic acid extraction is compatible with real-time PCR technology.

If the established standard method of the lab is used for nucleic acid isolation, it must be validated by the user.

For frozen samples or frozen extracted nucleic acid, only thaw the number of specimen extracts that will be tested in a single day.

Do not freeze/thaw extracted nucleic acid more than once before testing as each freeze/thaw cycle can decrease the nucleic acid quality. For optimal results, use it directly.

7.2. PCR Reaction Preparation and Processing

- Completely thaw the components at room temperature for a minimum of 30 minutes before each use.
- Place all components on ice once thawed during the whole test procedure.
- Determine the number of reactions and create the PCR plate plan.
- Include the following reactions to the plan:
    - Reactions for each test sample and extraction negative control.
    - PCR control reactions:
        - Positive Control (included in the kit)
        - Negative (No Template) Control (NTC) (included in the kit)
        - No Template Addition Control (NRC)
- Vortex and spin down briefly the components before each use.
- Combine the following components for the number of reactions required plus 10% overage to compensate for pipetting errors:

Table 3. Reaction set-up.

Reaction Mix Component	1X Reaction (µL) per well
RT-qPCR Master Mix	5 µL
GIP Oligo Mix 1-8	2.5 µL
Template Nucleic Acid	2.5 µL
Total Reaction Volume	10 µL

- Add 5 µL of RT-qPCR Master Mix and 2.5 µL of GIP Oligo Mix 1-8 into PCR tubes.
- Add 2.5 µL of the isolated sample into the individual tubes.
- The final reaction mix volume is 10 µL.
- Close the tubes, centrifuge briefly, insert tubes into the real-time PCR instrument and amplify according to the following PCR profile.

Table 4. Amplification profile.

Step	Number of Cycles	Temperature	Time	Data Collection
Reverse Transcription	1	52 °C	5 min	**FAM/Green**, **HEX/VIC/JOE/Yellow**, **ROX/Texas Red/Orange**, **CY5/Red**
Initial Denaturation	1	95 °C	10 sec	
Denaturation	40	95 °C	5 sec	
Annealing/Extension		55 °C	15 sec	

8. INTERPRETATION OF RESULTS

MarinaBiolab Gastrointestinal Panel PCR Kit provides a qualitative result for the presence (Detected) or absence (Not Detected) of the target genes.

8.1. Calculation of Cq Values and Instrument-Specific Requirements

Perform the following instrument settings before evaluating the results.

Table 5. Instrument-specific requirements before evaluating the results.

Instrument	Threshold Level	Other Settings
CFX96 Touch™/CFX96™ Dx/CFX Opus 96™/CFX Opus 96™ Dx/ CFX384 Touch™/CFX Opus 384™ (Bio-Rad)	500 RFU	-
Rotor-Gene Q 5plex Platform (QIAGEN)	0.02 RFU	Dynamic Tube: Active Slope Correct: Active Outlier Removal: 0
QuantStudio™ 5, 7 and 12K (Applied Biosystems™)	Auto	-
Roche LightCycler 480 (Roche)	Auto	-

The shape of the amplification curves should be examined. If a Cq value is assigned to a sample by the instruments' software and the curve is sigmoidal, the Cq value can be used in the final evaluation. *Non-sigmoidal curves should be recorded as negative.*

The result is recorded as positive if Cq≤38 or as established by your lab.

8.2. Overall Validity of Detection

Table 6. Expected performance of controls.

Control Type	Used to Monitor	Signal	
		Target Channel	Internal/External Control Channel
Negative Control	Cross-contamination during extraction and reaction setup	-	-
No template addition	Reagent and/or environmental contamination	-	-
Positive Control	RT-qPCR reaction setup and reagent integrity	+	+
Internal/External Control	To monitor the integrity of nucleic acid extraction and RT-qPCR from each specimen	Not applicable	+

Before analyzing samples results, we recommend verifying if the real-time PCR test is valid. Thus, for each run, please confirm if the results for Positive and Negative controls performed as expected, according to the following criteria:

Table 7. Run validity/positive and negative control pass criteria.

Positive Control		Negative Control		Results	Recommendation
Target Channel	**Internal/External Control Channel**	**Target Channel**	**Internal/External Control Channel**		
+	+	-	-	VALID	Continue to result interpretation of samples.
Any of them is Negative		Not considered		INVALID	Contact the manufacturer, renew the reagents, and repeat the reaction.
Not considered		Any of them is Positive		INVALID	Repeat analysis, paying attention to "Warnings and Precautions" in IFU.

If any control does not perform as described above, the run is considered invalid, and the test is repeated. If the problem persists, contact the manufacturer.

If all the controls are valid, proceed to the interpretation of the results.

8.3. Interpretation of Unknown Specimen Results

The data produced by the instruments can manually be evaluated and reported using their software.

Table 8. Interpretation of unknown specimen results for RNA pathogens.

RNA Pathogens	Internal Control (*RNase P*)	External Control (*MS2*)	Results	Interpretation
Positive (+) (Cq<38)	Positive (+) (Cq<38)	Positive (+) (Cq<38)	Positive for Target	Target RNA is detected
Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Positive for Target	Target RNA is detected
Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.
Positive (+) (Cq<38)	Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.
Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Positive (+) (Cq<38)	Negative for Target	Target RNA is not detected
Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Negative for Target	Target RNA is not detected
Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.
Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.

Table 9. Interpretation of unknown specimen results for DNA pathogens.

DNA Pathogens	Internal Control (*RNase P*)	External Control (*Bacillus atrophaeus*)	Results	Interpretation
Positive (+) (Cq<38)	Positive (+) (Cq<38)	Positive (+) (Cq<38)	Positive for Target	Target DNA is detected
Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Positive for Target	Target DNA is detected
Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.
Positive (+) (Cq<38)	Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.
Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Positive (+) (Cq<38)	Negative for Target	Target DNA is not detected
Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Negative for Target	Target DNA is not detected
Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.
Negative (-) (Cq≥38 or N/A)	Positive (+) (Cq<38)	Negative (-) (Cq≥38 or N/A)	Invalid	Repeat test by re-extracting the sample. If the repeat result remains invalid, consider collecting a new sample.

9. ASSAY LIMITATIONS

- The ***MarinaBiolab Gastrointestinal Panel PCR Kit*** is intended for use by professionally trained, qualified staff only.
- A false negative result may occur if a specimen is improperly collected, transported, or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Spontaneous mutations within the target sequences may result in failure to detect the target sequence. While this risk is mitigated in the test's design, if failure to detect the target is expected it is recommended to test the specimen with a different test that detects different target sequences from the target's genome.
- There is a risk of false positive results due to cross-contamination by target viruses and/or bacteria, their nucleic acids or amplified product, or from non-specific signals in the assay. Attention should be given to the handling of consumables under the Warnings and Precautions section to help minimize this risk.
- This assay is a qualitative test and does not provide a quantitative assessment of the concentration of the detected organism.
- All instruments (e.g., pipettes, real-time cyclers) must be calibrated according to the manufacturer's instructions.

10. PERFORMANCE CHARACTERISTICS

10.1. Analytical Sensitivity (Limit of Detection, LoD)

The LoD was defined as the concentration at which the test produces a positive result >95% of the time. Serial dilutions of the strains were tested and the initial tentative LoD confirmed with twenty (20) replicates. To ensure the accuracy of the LoD determination, if the initial detection rate was 100%, a further twenty (20) replicates were performed at the next lower concentration until ≤95% was achieved. For nucleic acid extraction, simulated research matrix was spiked with strains and loaded onto the Automatic Nucleic Acids Extraction Instrument. The tests were carried out using the CFX96 Touch™ (Bio-Rad) Real-Time PCR system. The confirmed LoDs for the strains tested and the corresponding LoDs for the ***MarinaBiolab Gastrointestinal Panel PCR Kit*** reportable targets are shown in Table 10 below.

Table 10. Summary of LoD study results.

Analyte	Isolate ID/Source	LoD Concentration (copies/mL)	Detected/Total
Sapovirus (I, II, IV, and V)	ATCC VR-3237SD	3.5E+01 copies/mL	**20/20** 100%
Adenovirus F40/41	Zeptometrix 0810084CF	4.8E+01 copies/mL	**20/20** 100%
Norovirus GI	ATCC VR-3234SD	5.1E+01 copies/mL	**20/20** 100%
Norovirus GII	ATCC VR-3235SD	6.2E+01 copies/mL	**20/20** 100%
Astrovirus	ATCC VR-1936	7.3E+01 copies/mL	**20/20** 100%
Rotavirus A	Zeptometrix 0810041CF	6.6E+01 copies/mL	**20/20** 100%
Plesiomonas shigelloides	Zeptometrix 0801899	1.2E+02 copies/mL	**20/20** 100%
Vibrio vulnificus	Zeptometrix 0804349	2.2E+02 copies/mL	**20/20** 100%
Vibrio cholerae	Zeptometrix 0801789	9.8E+01 copies/mL	**20/20** 100%
Vibrio parahaemolyticus	Zeptometrix 0801903	8.7E+01 copies/mL	**20/20** 100%
Salmonella spp.	Zeptometrix 0804268	8.0E+01 copies/mL	**20/20** 100%
Campylobacter spp.	Zeptometrix 0804272	5.5E+01 copies/mL	**20/20** 100%
Enteroaggregative E. coli (EAEC)	Zeptometrix 0801919	1.0E+02 copies/mL	**20/20** 100%

Shiga toxin-producing E. coli (STEC)	Zeptometrix 0801793	1.2E+02 copies/mL	**20/20** 100%
Shigella/Enteroinvasive E. coli (EIEC)	Zeptometrix 0801747	9.8E+01 copies/mL	**20/20** 100%
Enteropathogenic E. coli (EPEC)	Zeptometrix 0801747	8.7E+01 copies/mL	**20/20** 100%
Enterotoxigenic E. coli (ETEC)	Zeptometrix 0801624DNA-10UG	8.0E+01 copies/mL	**20/20** 100%
Yersinia enterocolitica	Zeptometrix 0801734	1.4E+02 copies/mL	**20/20** 100%
Clostridioides (Clostridium) difficile toxin A	ATCC 9689	1.2E+02 copies/mL	**20/20** 100%
Clostridioides (Clostridium) difficile toxin B	ATCC 9689	9.5E+01 copies/mL	**20/20** 100%
Clostridioides (Clostridium) difficile binary toxin A/B	ATCC BAA-1870	8.0E+01 copies/mL	**20/20** 100%
Giardia lamblia	Zeptometrix 0801788	2.1E+02 copies/mL	**20/20** 100%
Entamoeba histolytica	Zeptometrix NATEHI(DS4)-GP	2.2E+02 copies/mL	**20/20** 100%
Cryptosporidium spp.	Zeptometrix 0801700	1.8E+02 copies/mL	**20/20** 100%
Cyclospora cayetanensis	ATCC PRA-3000SD	1.6E+02 copies/mL	**20/20** 100%

10.2. Device Equivalence Study

Device equivalence study was carried out to observe the differences between the results to be obtained using the kit in different instruments. For this purpose, the same LoD determination study was performed again with the Bio-Rad CFX96™ Dx/CFX Opus 96™/CFX Opus 96™ Dx/CFX384 Touch™/CFX Opus 384™, Applied Biosystems QuantStudio 5, 7, and 12K, Qiagen Rotor-Gene Q 5plex Platform and Roche LightCycler 480. Similar test results were obtained with the 1x LoD concentration level of the targets in the "device equivalence study" performed with the other instruments.

10.3. Analytical Reactivity (Inclusivity)

10.3.1. In-Slico Analytical Reactivity

BLAST search of the oligonucleotides was performed on the Sapovirus (I, II, IV, and V), Adenovirus F40/41, Giardia lamblia, Entamoeba histolytica, Cryptosporidium spp., Plesiomonas shigelloides, Cyclospora cayetanensis, Yersinia enterocolitica, Vibrio vulnificus, Norovirus GI/GII, Astrovirus, Rotavirus A, *Campylobacter spp., Vibrio parahaemolyticus, Salmonella spp., Vibrio cholerae, Enteroaggregative E. coli (EAEC), Shiga toxin-producing E. coli (STEC), Shigella/Enteroinvasive E. coli (EIEC), Enteropathogenic E.*

coli (EPEC), Enterotoxigenic E. coli (ETEC), Clostridioides (Clostridium) difficile toxin A, Clostridioides (Clostridium) difficile toxin B, and Clostridioides (Clostridium) difficile binary toxin A/B genome sequences available in the NCBI database, using the Primer-BLAST tool of NCBI.

The aggregated result of all in-silico analyzes performed in NCBI database is provided in Table below. The melting temperatures (Tm) of the oligonucleotide sequences with 1-base mismatch, are still higher than the annealing temperature specified in the PCR cycle parameters of the kit. Hence, the single mismatches in the sequences are not expected to affect the inclusivity of the test.

Table 11. In-silico analysis results performed in the NCBI database.

Target	Primer	Total number of target sequences	Ratio of the sequences without mismatch	Ratio of the sequences with 1 base mismatch	Ratio of the sequences with 2 base mismatches	Ratio of the sequences with 3 base mismatches
Sapovirus I	Sense Primer	1284	97.68%	2.32%	0.00%	0.00%
Sapovirus I	Antisense Primer	1284	98.24%	1.76%	0.00%	0.00%
Sapovirus I	Hydrolysis Probe	1284	98.12%	1.88%	0.00%	0.00%
Sapovirus II	Sense Primer	161	99.10%	0.90%	0.00%	0.00%
Sapovirus II	Antisense Primer	161	98.24%	1.76%	0.00%	0.00%
Sapovirus II	Hydrolysis Probe	161	98.64%	1.36%	0.00%	0.00%
Sapovirus IV	Sense Primer	215	99.20%	0.80%	0.00%	0.00%
Sapovirus IV	Antisense Primer	215	97.45%	2.55%	0.00%	0.00%
Sapovirus IV	Hydrolysis Probe	215	98.60%	1.40%	0.00%	0.00%
Sapovirus V	Sense Primer	131	99.23%	0.77%	0.00%	0.00%
Sapovirus V	Antisense Primer	131	98.84%	1.16%	0.00%	0.00%
Sapovirus V	Hydrolysis Probe	131	99.23%	0.77%	0.00%	0.00%
Adenovirus F40/41	Sense Primer	221	100.00%	0.00%	0.00%	0.00%
Adenovirus F40/41	Antisense Primer	221	99.99%	0.005%	0.00%	0.00%
Adenovirus F40/41	Hydrolysis Probe	218	99.99%	0.005%	0.00%	0.00%
Norovirus GI	Sense Primer	3242	99.60%	0.40%	0.00%	0.00%
Norovirus GI	Antisense Primer	3242	98.42%	1.58%	0.00%	0.00%
Norovirus GI	Hydrolysis Probe	3242	99.20%	0.80%	0.00%	0.00%
Norovirus GII	Sense Primer	15453	98.46%	1.54%	0.00%	0.00%
Norovirus GII	Antisense Primer	15453	97.82%	2.18%	0.00%	0.00%
Norovirus GII	Hydrolysis Probe	15648	97.98%	2.02%	0.00%	0.00%
Astrovirus	Sense Primer	708	100.00%	0.00%	0.00%	0.00%
Astrovirus	Antisense Primer	708	100.00%	0.00%	0.00%	0.00%

Astrovirus	Hydrolysis Probe	1136	93.69%	6.16%	0.15%	0.00%
Rotavirus A	Sense Primer	4340	97.70%	2.30%	0.00%	0.00%
Rotavirus A	Antisense Primer	4340	97.97%	1.93%	0.10%	0.00%
Rotavirus A	Hydrolysis Probe	4644	98.03%	1.97%	0.00%	0.00%
Campylobacter spp.	Sense Primer	628	99.99%	0.005%	0.00%	0.00%
Campylobacter spp.	Antisense Primer	628	99.99%	0.005%	0.00%	0.00%
Campylobacter spp.	Hydrolysis Probe	634	99.99%	0.005%	0.005%	0.00%
Salmonella spp.	Sense Primer	4256	98.24%	1.76%	0.00%	0.00%
Salmonella spp.	Antisense Primer	4256	98.46%	1.54%	0.00%	0.00%
Salmonella spp.	Hydrolysis Probe	4324	97.88%	2.12%	0.00%	0.00%
Plesiomonas shigelloides	Sense Primer	56	99.99%	0.005%	0.00%	0.00%
Plesiomonas shigelloides	Antisense Primer	56	99.99%	0.005%	0.00%	0.00%
Plesiomonas shigelloides	Hydrolysis Probe	54	99.99%	0.005%	0.00%	0.00%
Vibrio cholerae	Sense Primer	195	99.82%	0.18%	0.00%	0.00%
Vibrio cholerae	Antisense Primer	195	99.74%	0.26%	0.00%	0.00%
Vibrio cholerae	Hydrolysis Probe	196	99.99%	0.005%	0.00%	0.00%
Vibrio parahaemolyticus	Sense Primer	345	99.99%	0.005%	0.00%	0.00%
Vibrio parahaemolyticus	Antisense Primer	345	99.99%	0.005%	0.00%	0.00%
Vibrio parahaemolyticus	Hydrolysis Probe	350	99.84%	0.15%	0.01%	0.00%
Vibrio vulnificus	Sense Primer	53	100.00%	0.00%	0.00%	0.00%
Vibrio vulnificus	Antisense Primer	53	100.00%	0.00%	0.00%	0.00%
Vibrio vulnificus	Hydrolysis Probe	52	100.00%	0.00%	0.00%	0.00%
Clostridioides (Clostridium) difficile toxin A	Sense Primer	227	99.99%	0.005%	0.00%	0.00%
Clostridioides (Clostridium) difficile toxin A	Antisense Primer	227	99.99%	0.005%	0.00%	0.00%
Clostridioides (Clostridium) difficile toxin A	Hydrolysis Probe	227	99.99%	0.005%	0.00%	0.00%
Clostridioides (Clostridium) difficile toxin B	Sense Primer	250	100.00%	0.00%	0.00%	0.00%
Clostridioides (Clostridium) difficile toxin B	Antisense Primer	250	99.80%	0.20%	0.00%	0.00%
Clostridioides (Clostridium) difficile toxin B	Hydrolysis Probe	241	100.00%	0.00%	0.00%	0.00%
Yersinia enterocolitica	Sense Primer	37	100.00%	0.00%	0.00%	0.00%
Yersinia enterocolitica	Antisense Primer	37	100.00%	0.00%	0.00%	0.00%

Yersinia enterocolitica	Hydrolysis Probe	38	99.99%	0.005%	0.00%	0.00%
Enteroaggregative E. coli (EAEC) (aap)	Sense Primer	90	98.00%	2.00%	0.00%	0.00%
Enteroaggregative E. coli (EAEC) (aap)	Antisense Primer	90	98.00%	2.00%	0.00%	0.00%
Enteroaggregative E. coli (EAEC) (aap)	Hydrolysis Probe	90	98.00%	2.00%	0.00%	0.00%
Enteroaggregative E. coli (EAEC) (aggR)	Sense Primer	87	100.00%	0.00%	0.00%	0.00%
Enteroaggregative E. coli (EAEC) (aggR)	Antisense Primer	87	100.00%	0.00%	0.00%	0.00%
Enteroaggregative E. coli (EAEC) (aggR)	Hydrolysis Probe	87	100.00%	0.00%	0.00%	0.00%
Shiga toxin-producing E. coli (STEC) (stx1)	Sense Primer	483	99.99%	0.01%	0.00%	0.00%
Shiga toxin-producing E. coli (STEC) (stx1)	Antisense Primer	483	99.99%	0.01%	0.00%	0.00%
Shiga toxin-producing E. coli (STEC) (stx1)	Hydrolysis Probe	484	92.74%	7.26%	0.00%	0.00%
Shiga toxin-producing E. coli (STEC) (stx2)	Sense Primer	1235	96.48%	3.52%	0.00%	0.00%
Shiga toxin-producing E. coli (STEC) (stx2)	Antisense Primer	1235	97.86%	2.14%	0.00%	0.00%
Shiga toxin-producing E. coli (STEC) (stx2)	Hydrolysis Probe	1348	97.84%	2.12%	0.00%	0.00%
Shigella/Enteroinvasive E. coli (EIEC)	Sense Primer	444	99.99%	0.005%	0.00%	0.00%
Shigella/Enteroinvasive E. coli (EIEC)	Antisense Primer	444	99.99%	0.005%	0.00%	0.00%
Shigella/Enteroinvasive E. coli (EIEC)	Hydrolysis Probe	440	100.00%	0.00%	0.00%	0.00%
Enteropathogenic E. coli (EPEC) (eae)	Sense Primer	1317	99.99%	0.005%	0.00%	0.00%
Enteropathogenic E. coli (EPEC) (eae)	Antisense Primer	1317	100.00%	0.00%	0.00%	0.00%
Enteropathogenic E. coli (EPEC) (eae)	Hydrolysis Probe	1321	98.24%	1.76%	0.00%	0.00%
Enteropathogenic E. coli (EPEC) (bfp)	Sense Primer	94	100.00%	0.00%	0.00%	0.00%
Enteropathogenic E. coli (EPEC) (bfp)	Antisense Primer	94	100.00%	0.00%	0.00%	0.00%
Enteropathogenic E. coli (EPEC) (bfp)	Hydrolysis Probe	101	100.00%	0.00%	0.00%	0.00%
Enterotoxigenic E. coli (ETEC) (lt)	Sense Primer	114	100.00%	0.00%	0.00%	0.00%

Enterotoxigenic E. coli (ETEC) (lt)	Antisense Primer	114	100.00%	0.00%	0.00%	0.00%
Enterotoxigenic E. coli (ETEC) (lt)	Hydrolysis Probe	114	100.00%	0.00%	0.00%	0.00%
Enterotoxigenic E. coli (ETEC) (st)	Sense Primer	79	100.00%	0.00%	0.00%	0.00%
Enterotoxigenic E. coli (ETEC) (st)	Antisense Primer	79	100.00%	0.00%	0.00%	0.00%
Enterotoxigenic E. coli (ETEC) (st)	Hydrolysis Probe	78	100.00%	0.00%	0.00%	0.00%
Giardia lamblia	Sense Primer	3410	99.24%	0.76%	0.00%	0.00%
Giardia lamblia	Antisense Primer	3410	99.20%	0.80%	0.00%	0.00%
Giardia lamblia	Hydrolysis Probe	3770	99.14%	0.86%	0.00%	0.00%
Entamoeba histolytica	Sense Primer	206	99.99%	0.005%	0.00%	0.00%
Entamoeba histolytica	Antisense Primer	206	99.99%	0.005%	0.00%	0.00%
Entamoeba histolytica	Hydrolysis Probe	231	99.99%	0.005%	0.00%	0.00%
Cyclospora cayetanensis	Sense Primer	213	100.00%	0.00%	0.00%	0.00%
Cyclospora cayetanensis	Antisense Primer	213	99.99%	0.005%	0.00%	0.00%
Cyclospora cayetanensis	Hydrolysis Probe	251	99.99%	0.005%	0.00%	0.00%
Cryptosporidium spp.	Sense Primer	301	96.46%	3.54%	0.00%	0.00%
Cryptosporidium spp.	Antisense Primer	301	97.40%	2.60%	0.00%	0.00%
Cryptosporidium spp.	Hydrolysis Probe	310	98.46%	1.54%	0.00%	0.00%

10.3.2. Wet-Test Analytical Reactivity

The analytical reactivity (inclusivity) of the ***MarinaBiolab Gastrointestinal Panel PCR Kit*** was demonstrated with a comprehensive panel representing temporal, evolutionary, and geographic diversity for each of the target organisms.

Each sample was tested with the ***MarinaBiolab Gastrointestinal Panel PCR Kit*** in triplicate at an initial concentration 3-fold higher than the LoD determined for each analyte. In cases where the expected targets were not detected in one or more replicates, concentrations at a 3-fold higher level were evaluated.

The individual strains and concentrations at which positive test results were obtained for all three (3) replicates are presented by target organism in Table 12 below.

Table 12. Results of the wet inclusivity test.

Variant/Type/Subtype/Lineage/Genotype/Species	Isolate ID/Source	xLoD Detected
Sapovirus (I, II, IV, and V)	ATCC VR-3237SD	1x
Adenovirus F40/41	Zeptometrix 0810084CF	1x
Norovirus GI	ATCC VR-3234SD	1x
Norovirus GII	ATCC VR-3235SD	1x

Astrovirus	ATCC VR-1936	1x
Rotavirus A	Zeptometrix 0810041CF	1x
Plesiomonas shigelloides	Zeptometrix 0801899	1x
Vibrio vulnificus	Zeptometrix 0804349	1x
Vibrio cholerae	Zeptometrix 0801789	1x
Vibrio parahaemolyticus	Zeptometrix 0801903	1x
Salmonella spp.	Zeptometrix 0804268	1x
Campylobacter spp.	Zeptometrix 0804272	1x
Enteroaggregative E. coli (EAEC)	Zeptometrix 0801919	1x
Shiga toxin-producing E. coli (STEC)	Zeptometrix 0801793	1x
Shigella/Enteroinvasive E. coli (EIEC)	Zeptometrix 0801747	1x
Enteropathogenic E. coli (EPEC)	Zeptometrix 0801747	1x
Enterotoxigenic E. coli (ETEC)	Zeptometrix 0801624DNA-10UG	1x
Yersinia enterocolitica	Zeptometrix 0801734	1x
Clostridioides (Clostridium) difficile toxin A	ATCC 9689	1x
Clostridioides (Clostridium) difficile toxin B	ATCC 9689	1x
Clostridioides (Clostridium) difficile binary toxin A/B	ATCC BAA-1870	1x
Giardia lamblia	Zeptometrix 0801788	1x
Entamoeba histolytica	Zeptometrix NATEHI(DS4)-GP	1x
Cryptosporidium spp.	Zeptometrix 0801700	1x
Cyclospora cayetanensis	ATCC PRA-3000SD	1x

10.4. Analytical Specificity (Exclusivity)

10.4.1. In-Slico Analytical Specificity

Primers and probes intended for a target sequence may also attach to similar sequences if they closely match or differ by only a few base pairs from the non-targeted sequence. To ensure specificity to the target amplicon sequence, it's essential to screen the primers and probe against the reference database transcript or genome database for the intended templates, as well as any databases containing potential contaminating templates.

Table 13. The results of On-Panel and Off-Panel organisms tested for cross-reactivity.

On-Panel/Off-Panel	Name of the organism	Cross Reactivity*		
		Forward	Probe	Reverse
On-Panel	Sapovirus (I, II, IV, and V)	None	None	None

On-Panel	Adenovirus F40/41	None	None	None
On-Panel	Norovirus GI/GII	None	None	None
On-Panel	Astrovirus	None	None	None
On-Panel	Rotavirus A	None	None	None
On-Panel	*Plesiomonas shigelloides*	None	None	None
On-Panel	*Vibrio vulnificus*	None	None	None
On-Panel	*Vibrio cholerae*	None	None	None
On-Panel	*Vibrio parahaemolyticus*	None	None	None
On-Panel	*Salmonella spp.*	None	None	None
On-Panel	*Campylobacter spp.*	None	None	None
On-Panel	*Enteroaggregative E. coli (EAEC)*	None	None	None
On-Panel	*Shiga toxin-producing E. coli (STEC)*	None	None	None
On-Panel	*Shigella/Enteroinvasive E. coli (EIEC)*	None	None	None
On-Panel	*Enteropathogenic E. coli (EPEC)*	None	None	None
On-Panel	*Enterotoxigenic E. coli (ETEC)*	None	None	None
On-Panel	*Yersinia enterocolitica*	None	None	None
On-Panel	*Clostridioides (Clostridium) difficile toxin A*	None	None	None
On-Panel	*Clostridioides (Clostridium) difficile toxin B*	None	None	None
On-Panel	*Clostridioides (Clostridium) difficile binary toxin A/B*	None	None	None
On-Panel	*Giardia lamblia*	None	None	None
On-Panel	*Entamoeba histolytica*	None	None	None
On-Panel	*Cryptosporidium spp.*	None	None	None
On-Panel	*Cyclospora cayetanensis*	None	None	None
Off-Panel	*Abiotrophia defectivia*	None	None	None
Off-Panel	*Acinetobacter baumannii*	None	None	None
Off-Panel	*Acinetobacter lwoffii*	None	None	None
Off-Panel	*Aeromonas hydrophila*	None	None	None
Off-Panel	*Alcaligenes faecalis*	None	None	None
Off-Panel	*Anaerococcus tetradius*	None	None	None
Off-Panel	*Arcobacter butzleri*	None	None	None

Off-Panel	*Arcobacter cryaerophilus*	None	None	None
Off-Panel	*Bacillus cereus*	None	None	None
Off-Panel	*Bacteroides fragilis*	None	None	None
Off-Panel	*Bacteroides thetaiotaomicron*	None	None	None
Off-Panel	*Bacteroides vulgatus*	None	None	None
Off-Panel	*Bifidobacterium adolescentisa*	None	None	None
Off-Panel	*Bifidobacterium bifiduma*	None	None	None
Off-Panel	*Bifidobacterium longuma*	None	None	None
Off-Panel	*Cedecea davisaeb*	None	None	None
Off-Panel	*Chlamydia trachomatis*	None	None	None
Off-Panel	*Citrobacter amalonaticus*	None	None	None
Off-Panel	*Citrobacter freundii*	None	None	None
Off-Panel	*Clostridium acetobutylicum*	None	None	None
Off-Panel	*Clostridium botulinum*	None	None	None
Off-Panel	*Clostridium difficile non-toxigenic*	None	None	None
Off-Panel	*Clostridium histolyticum*	None	None	None
Off-Panel	*Clostridium methylpentosum*	None	None	None
Off-Panel	*Clostridium novyi*	None	None	None
Off-Panel	*Clostridium perfringens*	None	None	None
Off-Panel	*Clostridium ramosum*	None	None	None
Off-Panel	*Clostridium septicum*	None	None	None
Off-Panel	*Clostridium sordellii*	None	None	None
Off-Panel	*Clostridium tetani*	None	None	None
Off-Panel	*Collinsella aerofaciens*	None	None	None
Off-Panel	*Corynebacterium genitalium*	None	None	None
Off-Panel	*Desulfovibrio piger*	None	None	None
Off-Panel	*Diffusely adherent E.coli*	None	None	None
Off-Panel	*Edwardsiella tarda*	None	None	None
Off-Panel	*Egglerthella lenta*	None	None	None
Off-Panel	*Enterobacter aerogenes*	None	None	None
Off-Panel	*Enterobacter cloacae*	None	None	None
Off-Panel	*Enterococcus faecalis*	None	None	None

Off-Panel	*Enterococcus faecium*	None	None	None
Off-Panel	*Escherichia blattae*	None	None	None
Off-Panel	*Escherichia fergusonii*	None	None	None
Off-Panel	*Escherichia hermannii*	None	None	None
Off-Panel	*Escherichia vulneris*	None	None	None
Off-Panel	*Eubacterium cylindroides*	None	None	None
Off-Panel	*Eubacterium rectale*	None	None	None
Off-Panel	*Faecalibacterium prausnitzii*	None	None	None
Off-Panel	*Fusobacterium varium*	None	None	None
Off-Panel	*Gardnerella vaginalis*	None	None	None
Off-Panel	*Gemella morbillorum*	None	None	None
Off-Panel	*Haemophilus influenzae*	None	None	None
Off-Panel	*Hafnia alveib*	None	None	None
Off-Panel	*Helicobacter fennelliae*	None	None	None
Off-Panel	*Helicobacter pylori*	None	None	None
Off-Panel	*Klebsiella oxytoca*	None	None	None
Off-Panel	*Klebsiella pneumoniae*	None	None	None
Off-Panel	*Lactobacillus acidophilus*	None	None	None
Off-Panel	*Lactobacillus reuteri*	None	None	None
Off-Panel	*Lactococcus lactis*	None	None	None
Off-Panel	*Leminorella grimontii*	None	None	None
Off-Panel	*Listeria monocytogenes*	None	None	None
Off-Panel	*Megamonas hypermegale*	None	None	None
Off-Panel	*Megasphaeara elsdenii*	None	None	None
Off-Panel	*Methanobrevibacter smithii*	None	None	None
Off-Panel	*Morganella morganii*	None	None	None
Off-Panel	*Peptoniphilus asaccharolyticus*	None	None	None
Off-Panel	*Peptostreptococcus anaerobius*	None	None	None
Off-Panel	*Photobacterium damselae*	None	None	None
Off-Panel	*Porphyromonas asaccharolytica*	None	None	None
Off-Panel	*Prevotella melaninogenica*	None	None	None
Off-Panel	*Proteus mirabilis*	None	None	None

Off-Panel	*Proteus penneri*	None	None	None
Off-Panel	*Proteus vulgaris*	None	None	None
Off-Panel	*Provedencia alcalifaciens*	None	None	None
Off-Panel	*Pseudomonas aeruginosa*	None	None	None
Off-Panel	*Ruminococcus bromiia*	None	None	None
Off-Panel	*Ruminococcus flavefaciensa*	None	None	None
Off-Panel	*Ruminococcus obeuma*	None	None	None
Off-Panel	*Selenomonas ruminantium*	None	None	None
Off-Panel	*Serratia liquefaciens*	None	None	None
Off-Panel	*Serratia marcescens*	None	None	None
Off-Panel	*Shewanella algae*	None	None	None
Off-Panel	*Staphylococcus aureus*	None	None	None
Off-Panel	*Staphylococcus epidermidis*	None	None	None
Off-Panel	*Stenotrophomonas maltophilia*	None	None	None
Off-Panel	*Streptococcus agalactiae*	None	None	None
Off-Panel	*Streptococcus intermedius*	None	None	None
Off-Panel	*Streptococcus pyogenes*	None	None	None
Off-Panel	*Streptococcus salivarius*	None	None	None
Off-Panel	*Streptococcus suis*	None	None	None
Off-Panel	*Trabulsiella guamensis*	None	None	None
Off-Panel	*Veillonella parvula*	None	None	None
Off-Panel	*Yersinia bercovieri*	None	None	None
Off-Panel	*Yersinia frederiksenii*	None	None	None
Off-Panel	*Yersinia intermedia*	None	None	None
Off-Panel	*Yersinia mollaretii*	None	None	None
Off-Panel	*Yersinia pseudotuberculosis*	None	None	None
Off-Panel	*Yersinia rohdei*	None	None	None
Off-Panel	*Ancylostoma duodenale*	None	None	None
Off-Panel	*Ascaris lumbricoides*	None	None	None
Off-Panel	*Aspergillus fumigatus*	None	None	None
Off-Panel	*Babesia microti*	None	None	None
Off-Panel	*Balantidium coli*	None	None	None

Off-Panel	*Blastocystis hominis*	None	None	None
Off-Panel	*Candida albicans*	None	None	None
Off-Panel	*Candida catenulate*	None	None	None
Off-Panel	*Chilomastix mesnili*	None	None	None
Off-Panel	*Conidiobolus lachnodes*	None	None	None
Off-Panel	*Conidiobolus lobatus*	None	None	None
Off-Panel	*Dientamoeba fragilis*	None	None	None
Off-Panel	*Encephalitozoon hellem*	None	None	None
Off-Panel	*Encephalitozoon intestinalis*	None	None	None
Off-Panel	*Endolimax nana*	None	None	None
Off-Panel	*Entamoeba coli*	None	None	None
Off-Panel	*Entamoeba gingivalis*	None	None	None
Off-Panel	*Entamoeba hartmanni*	None	None	None
Off-Panel	*Entamoeba moshkovskii*	None	None	None
Off-Panel	*Entamoeba polecki*	None	None	None
Off-Panel	*Enterobius vermicularis*	None	None	None
Off-Panel	*Enteromonas hominis*	None	None	None
Off-Panel	*Giardia muris*	None	None	None
Off-Panel	*Isospora belli*	None	None	None
Off-Panel	*Necator americanus*	None	None	None
Off-Panel	*Penicillium marneffei*	None	None	None
Off-Panel	*Pentatrichomonas hominis*	None	None	None
Off-Panel	*Saccharomyces boulardi*	None	None	None
Off-Panel	*Saccharomyces cerevisiae*	None	None	None
Off-Panel	*Schistosoma mansoni*	None	None	None
Off-Panel	*Toxoplasma gondii*	None	None	None
Off-Panel	*Trichomonas tenax*	None	None	None
Off-Panel	Adenovirus A	None	None	None
Off-Panel	Adenovirus B	None	None	None
Off-Panel	Adenovirus C	None	None	None
Off-Panel	Adenovirus D	None	None	None
Off-Panel	Adenovirus E	None	None	None

Off-Panel	Adenovirus G	None	None	None
Off-Panel	Astrovirus variant MLB	None	None	None
Off-Panel	Astrovirus variant VA1	None	None	None
Off-Panel	Bocavirus Type 1	None	None	None
Off-Panel	Cytomegalovirus	None	None	None
Off-Panel	Echovirus 6	None	None	None
Off-Panel	Enterovirus 68	None	None	None
Off-Panel	Hepatitis A	None	None	None

* Homology should be <80% between the cross-reactivity microorganisms and the test primers/ probe(s).

** In silico sequence analysis indicates the potential for cross-reactivity of Bordetella pertussis with certain strains of Bordetella bronchiseptica.

10.4.2. Wet-Test Analytical Specificity

The potential for non-specific amplification by assays for detection of analytes was evaluated by testing high concentrations of organisms or nucleic acids with the ***MarinaBiolab Gastrointestinal Panel PCR Kit***. On-panel organisms were tested to assess the potential for intra-panel cross-reactivity, and off-panel organisms were tested to evaluate panel specificity. Off-panel organisms included normal flora and pathogens that may be present in specimens as well as near-neighbors or species genetically related to the organisms detected by the ***MarinaBiolab Gastrointestinal Panel PCR Kit***. The concentration of organism tested (in triplicate) was at least 1.0E+06 CFU/mL for bacteria, fungi and parasite, and at least 1.0E+05 unit/mL for viruses. For the few organisms of interest that were not available for laboratory testing, results of in silico analysis of the organism's whole genome sequences are indicated. The on-panel and off-panel organisms tested are shown in Table 14 and Table 15.

Table 14. On-Panel organisms tested for evaluation of ***MarinaBiolab Gastrointestinal Panel PCR Kit*** analytical specificity.

Organism	Isolate ID/Source	Cross Reactivity Detected
Sapovirus (I, II, IV, and V)	ATCC VR-3237SD	None
Adenovirus F40/41	Zeptometrix 0810084CF	None
Norovirus GI	ATCC VR-3234SD	None
Norovirus GII	ATCC VR-3235SD	None
Astrovirus	ATCC VR-1936	None
Rotavirus A	Zeptometrix 0810041CF	None
Plesiomonas shigelloides	Zeptometrix 0801899	None
Vibrio vulnificus	Zeptometrix 0804349	None
Vibrio cholerae	Zeptometrix 0801789	None
Vibrio parahaemolyticus	Zeptometrix 0801903	None
Salmonella spp.	Zeptometrix 0804268	None

Campylobacter spp.	Zeptometrix 0804272	None
Enteroaggregative E. coli (EAEC)	Zeptometrix 0801919	None
Shiga toxin-producing E. coli (STEC)	Zeptometrix 0801793	None
Shigella/Enteroinvasive E. coli (EIEC)	Zeptometrix 0801747	None
Enteropathogenic E. coli (EPEC)	Zeptometrix 0801747	None
Enterotoxigenic E. coli (ETEC)	Zeptometrix 0801624DNA-10UG	None
Yersinia enterocolitica	Zeptometrix 0801734	None
Clostridioides (Clostridium) difficile toxin A	ATCC 9689	None
Clostridioides (Clostridium) difficile toxin B	ATCC 9689	None
Clostridioides (Clostridium) difficile binary toxin A/B	ATCC BAA-1870	None
Giardia lamblia	Zeptometrix 0801788	None
Entamoeba histolytica	Zeptometrix NATEHI(DS4)-GP	None
Cryptosporidium spp.	Zeptometrix 0801700	None
Cyclospora cayetanensis	ATCC PRA-3000SD	None

Table 15. Off-Panel organisms were tested for evaluation of ***MarinaBiolab Gastrointestinal Panel PCR Kit*** analytical specificity.

Organism	Isolate ID/Source	Cross Reactivity Detected
Abiotrophia defectivia	ATCC 700209	None
Acinetobacter baumannii	Zeptometrix 801597	None
Acinetobacter lwoffii	Zeptometrix 801909	None
Aeromonas hydrophila	Zeptometrix 804098	None
Alcaligenes faecalis	Zeptometrix 801995	None
Anaerococcus tetradius	ATCC 35098	None
Arcobacter butzleri	ATCC 49616	None
Arcobacter cryaerophilus	ATCC 43158	None
Bacillus cereus	Zeptometrix 801823	None
Bacteroides fragilis	Zeptometrix 801583	None
Bacteroides thetaiotaomicron	Zeptometrix 801743	None
Bifidobacterium adolescentisa	Zeptometrix 801998	None
Bifidobacterium longuma	Zeptometrix 804047	None
Cedecea davisaeb	ATCC 33431	None
Chlamydia trachomatis	Zeptometrix 804400	None

Citrobacter amalonaticus	Zeptometrix 801718	None
Citrobacter freundii	Zeptometrix 801563	None
Clostridium acetobutylicum	ATCC 824	None
Clostridium difficile non-toxigenic	Zeptometrix 804105	None
Clostridium histolyticum	Zeptometrix 804054	None
Clostridium methylpentosum	ATCC 43829	None
Clostridium novyi	Zeptometrix 804056	None
Clostridium perfringens	Zeptometrix 801585	None
Clostridium ramosum	Zeptometrix 804058	None
Clostridium septicum	Zeptometrix 801885	None
Clostridium sordellii	Zeptometrix 801587	None
Clostridium tetani	Zeptometrix 804063	None
Collinsella aerofaciens	Zeptometrix 804064	None
Corynebacterium genitalium	Zeptometrix 804108	None
Desulfovibrio piger	ATCC 29098	None
Edwardsiella tarda	Zeptometrix 804065	None
Egglerthella lenta	Zeptometrix 804066	None
Enterobacter cloacae	Zeptometrix 801830	None
Enterococcus faecalis	Zeptometrix 804216	None
Enterococcus faecium	Zeptometrix 804328	None
Escherichia fergusonii	Zeptometrix 804113	None
Escherichia hermannii	Zeptometrix 804068	None
Eubacterium cylindroides	ATCC 27805	None
Eubacterium rectale	ATCC 33656	None
Faecalibacterium prausnitzii	ATCC 27768	None
Fusobacterium varium	Zeptometrix 804069	None
Gardnerella vaginalis	Zeptometrix 801894	None
Gemella morbillorum	Zeptometrix 804253	None
Haemophilus influenzae	Zeptometrix 801680	None
Hafnia alveib	ATCC 13337	None
Helicobacter pylori	Zeptometrix 804383	None
Klebsiella oxytoca	Zeptometrix 801881	None

Klebsiella pneumoniae	Zeptometrix 804295	None
Lactobacillus acidophilus	Zeptometrix 801540	None
Lactobacillus reuteri	Zeptometrix 804322	None
Lactococcus lactis	Zeptometrix 804157	None
Leminorella grimontii	Zeptometrix 804070	None
Listeria monocytogenes	Zeptometrix 804339	None
Megamonas hypermegale	ATCC 25560	None
Megasphaeara elsdenii	ATCC 25940	None
Morganella morganii	Zeptometrix 804010	None
Peptoniphilus asaccharolyticus	Zeptometrix 804245	None
Peptostreptococcus anaerobius	Zeptometrix 804012	None
Photobacterium damselae	ATCC 33539	None
Porphyromonas asaccharolytica	ATCC 27908	None
Prevotella melaninogenica	Zeptometrix 804292	None
Proteus mirabilis	Zeptometrix 801544	None
Proteus penneri	Zeptometrix 804442	None
Proteus vulgaris	Zeptometrix 801898	None
Provedencia alcalifaciens	Zeptometrix 804079	None
Pseudomonas aeruginosa	Zeptometrix 801908	None
Ruminococcus bromiia	ATCC 27255	None
Selenomonas ruminantium	ATCC 12561	None
Serratia liquefaciens	Zeptometrix 804207	None
Serratia marcescens	Zeptometrix 801723	None
Shewanella algae	Zeptometrix 804207	None
Staphylococcus aureus	Zeptometrix 804275	None
Staphylococcus epidermidis	Zeptometrix 804281	None
Stenotrophomonas maltophilia	Zeptometrix 801569	None
Streptococcus agalactiae	Zeptometrix 801556	None
Streptococcus intermedius	Zeptometrix 801895	None
Streptococcus pyogenes	Zeptometrix 801512	None
Aspergillus fumigatus	Zeptometrix 801716	None

10.5. Interferences

The ability of endogenous or exogenous substances that could be present in research samples (or introduced during sample collection and handling) to interfere with accurate detection of analytes was evaluated with select direct testing on the ***MarinaBiolab Gastrointestinal Panel PCR Kit*** and extrapolated from the interference evaluation of the ***MarinaBiolab Gastrointestinal Panel PCR Kit***.

Potentially interfering substances were evaluated using contrived samples spiked with substance. Results from samples containing a substance were compared to results from control samples without substance. The substances tested included endogenous substances that may be found in sample at normal or elevated levels (e.g., blood, mucus/mucin, human genomic DNA), various commensal or infectious microorganisms, medications, washes or topical applications, various swabs and transport media for sample collection, and substances used to clean, decontaminate, or disinfect work areas. Each substance was added to contrived samples containing representative organisms at concentrations near (3x) LoD. The concentration of substance added to the samples was equal to or greater than the highest level expected to be in research sample and each sample was tested in triplicate.

None of the substances were shown to interfere with the ***MarinaBiolab Gastrointestinal Panel PCR Kit***.

Table 16. Evaluation of potentially interfering substances on the ***MarinaBiolab Gastrointestinal Panel PCR Kit***.

Substance Tested	Concentration Tested	Observed Interference
Endogenous Substances		
Human Blood	10% v/v	No Interference
Fatty Acids (Palmitic Acid)	10% v/v	No Interference
Human Urine	-	No Interference
Human Stool	-	No Interference
Competitive Microorganisms		
Sapovirus (I, II, IV, and V)	1.0E+05 unit/mL	No Interference
Adenovirus F40/41	1.0E+05 unit/mL	No Interference
Norovirus GI	1.0E+05 unit/mL	No Interference
Norovirus GII	1.0E+05 unit/mL	No Interference
Astrovirus	1.0E+05 unit/mL	No Interference
Rotavirus A	1.0E+05 unit/mL	No Interference
Plesiomonas shigelloides	1.0E+06 CFU/mL	No Interference
Vibrio vulnificus	1.0E+06 CFU/mL	No Interference
Vibrio cholerae	1.0E+06 CFU/mL	No Interference
Vibrio parahaemolyticus	1.0E+06 CFU/mL	No Interference
Salmonella spp.	1.0E+06 CFU/mL	No Interference
Campylobacter spp.	1.0E+06 CFU/mL	No Interference

Enteroaggregative E. coli (EAEC)	1.0E+06 CFU/mL	No Interference
Shiga toxin-producing E. coli (STEC)	1.0E+06 CFU/mL	No Interference
Shigella/Enteroinvasive E. coli (EIEC)	1.0E+06 CFU/mL	No Interference
Enteropathogenic E. coli (EPEC)	1.0E+06 CFU/mL	No Interference
Enterotoxigenic E. coli (ETEC)	1.0E+06 CFU/mL	No Interference
Yersinia enterocolitica	1.0E+06 CFU/mL	No Interference
Clostridioides (Clostridium) difficile toxin A	1.0E+06 CFU/mL	No Interference
Clostridioides (Clostridium) difficile toxin B	1.0E+06 CFU/mL	No Interference
Clostridioides (Clostridium) difficile binary toxin A/B	1.0E+06 CFU/mL	No Interference
Giardia lamblia	1.0E+06 CFU/mL	No Interference
Entamoeba histolytica	1.0E+06 CFU/mL	No Interference
Cryptosporidium spp.	1.0E+06 CFU/mL	No Interference
Cyclospora cayetanensis	1.0E+06 CFU/mL	No Interference
Exogenous Substances		
Bacitracin	1% w/v	No Interference
Nystatin	1% w/v	No Interference
Glycerin	1% w/v	No Interference
Magnesium hydroxide	1% w/v	No Interference

11. TROUBLESHOOTING

Problem	Cause	Solution
Target-specific and/or IC signals are detected in the Negative Control well.	Contamination from the environment, contamination of extraction and/or RT-qPCR reagents, or well-to-well cross contamination. The signal is not true target amplification, but background curves generated by the software of the qPCR instrument.	Repeat the RT-qPCR with new reagents. Follow the general rules of GLP in a PCR lab (e.g., Decontaminate all surfaces and instruments with sodium hypochlorite or ethanol. Ensure that filters tips are used during the procedure and changed between samples.). It is recommended to set up the RT-qPCR reactions in a separate area, where no RNA/DNA is handled and with equipment designated for pre-PCR activities. Ignore the Cq value of NTC if the amplification curve looks not real but background noise. If the problem persists, contact Technical Support.
No IC signal is detected, but target-specific signal is detected in sample wells.	A high copy number of target nucleic acid exists in samples, resulting in preferential amplification of the target-specific nucleic acid.	No action is required. The result is considered positive.
The Positive Control did not meet the criteria set for acceptable values of the kit. The assay is invalid.	Positive Control was not stored at the recommended conditions. Kit shelf-life expired.	Check the kit label for storage conditions and expiration date. Replace the Positive Control. Use a new kit if necessary.
High Cq values observed for repeated samples.	Frozen samples were not mixed properly after thawing. Degraded nucleic acids.	Make sure, thaw frozen samples with mild agitation to ensure thorough mixing. Ensure that samples are stored correctly and not subjected to multiple freeze-thaw cycles
Target-specific and/or IC signal detected after 38 Cycles in Positive Control.	Incorrect RT-qPCR set-up or the kit reagents may have been compromised (e.g., improper storage or more than 15 freeze-thaw cycles).	Replace the control. If the problem persists, contact Technical Support.
No target-specific and IC signal is detected in sample wells.	Sampling, extraction, or inhibition problem.	Dilute the nucleic acid isolate 1/10 and repeat the RT-qPCR. If the diluted sample does not give a positive result in the IC channel, request for a new sample and repeat the NA extraction. Repeat the NA extraction and the RT-qPCR. Request for a new sample, repeat the NA extraction and the RT-qPCR. If the problem persists, contact Technical Support.

12. EXPLANATION of SYMBOLS

Symbol	Title of Symbol	Symbol	Title of Symbol
	Research Use Only		Use-by date
	Manufacturer		Batch code
	Negative control		Non-sterile
	Positive control		Consult instructions for use or consult electronic instructions for use
	Control		Caution
	Temperature limit		Catalogue number
	Keep away from sunlight		Do not use if package is damaged and consult instructions for use
	Keep dry		Keep upright
	Contains sufficient for <n> tests		Protect from heat and radioactive sources

Custom care and technical support

Tel: +1 510 579-5802

e-mail customer care: accounting@marinabiolab.com

e-mail Technical Support: rd@marinabiolab.com

MarinaBiolab LTD.

Address: Hürriyet Mah. Erler So. No: 4/1A Nexus No: 13 Kartal/Istanbul/Turkey

For research use only (RUO)! Not for use in diagnostic procedures.