PCRassays.com

ASSAY NAME: GIDIAR1_BR384 (GI Diarrhea Panel for Bio-Rad 384 or 96)

Quantity: 100 x 20µL PCR reactions

6-plex (4 color) assay: detects 5 genes for toxins and virulence factors, and human RPP30 DNA

SKU #: BUN-GIDIAR1-D-BR384-100 (Bio-Rad)

(RUO). Research Use Only. Not for use in Diagnostic Procedures.

SCOPE OF THIS PRODUCT INFORMATION SHEET (PIS):

The oligonucleotide recipes are optimized for each instrument (QuantStudio, BioRad). The verification data presented in this PIS were performed with BUN-GIDIAR1-D-BR384-100 on a Bio-Rad CFX-96 Real-Time System using white-bottomed plates. This kit is compatible with both CFX-96 and CFX-384 instruments. The performance of the other SKUs on their corresponding instrument should be similar. Contact PCRassays.com if you are planning to use a different instrument.

CONTENTS

The primers and probes in the GI-Diarrhea1 assay are provided in Tube 1 as a 5X concentrated working solution that detects 5 genes for toxins and virulence factors and a human extraction control.

Table of Dyes used in this assay:

Pathogen/Target	Dyes	Quencher	Refs.
aap, aata, aggR	FAM	BHQ-1	1, 2
RPP30-DNA control	HEX	BHQ-1	3
ipaH	TEX615	BHQ-2	4,5
eae	Cy5	BHQ-2	6

The probes are designed as TaqMan[7] cleavage mechanism and thus the reaction requires a DNA polymerase with 5'-exonuclease activity.

ASSAY HANDLING

The GI-Diarrhea1 bundle is shipped on ice, and should be stored at -20 °C. The tubes should be kept on ice once thawed. Do not subject the enzyme to multiple freeze-thaw cycles.

Contamination should be avoided by using appropriate personal protective equipment (PPE), powder free gloves, aerosol barrier pipette tips, and a clean hood.

ASSAY CONTENTS:

Tube 1: 5X Primer/Probe mix for 5 genes for toxins and virulence factors, and hRPP30DNA.

Tube 2: (Do NOT add to specimen unknowns) Mixed positive control: Synthetic 500 bp DNA fragments for 5 genes for toxins and virulence factors, and hRPP30DNA.

Tube 3: InhibiTaq qPCR enzyme Mastermix (enough for 100 rxns. with 20 µL total volume). This is a custom formulation from Fortis Life Sciences to the specifications of PCRassays.com.

EXPERIMENTAL

Perform nucleic acid extraction/purification (recommended). It is important to use an extraction procedure with an appropriate cell wall lysis agent.

Set up your PCR reaction (20 µL) as follows on ice:

Component	Volume (µL)
InhibiTaq enzyme mastermix (2X)	10
Primer/Probe mix (5X)	4
Sample or positive control	2
Water (Molecular Biology Grade)	4

Notes: To improve assay sensitivity, up to 6 µL of sample can be added (water volume adjusted accordingly) for a total reaction volume of 20 µL.
For positive control rxns., add 2 µL of the solution from Tube 2.
For 10 µL reactions, divide all of the amounts above by a factor of 2.

A PCR protocol was used for verification on a Bio-Rad CFX96 System using white-bottomed plates, with the following program:

Step	Thermocycling Protocol:
1	Incubate @ 95 °C for 2 minutes
2	Incubate @ 95 °C for 3 seconds
3	Incubate @ 55 °C for 20 seconds*
4	Plate Read
5	Go to Step 2, repeat 44× more

*A 15 second extension time may be used for CFX96 instruments.

RESULT INTERPRETATION

After running the qPCR reaction, use the instrument software to determine the quantification cycle, C_q (or use C_T if your instrument does not have the capability to compute a C_q). Fluorescence channels with a C_q < 38 cycles, and final RFU >Threshold is considered "positive" or "+" in the Table below. The "Threshold" value for calling a PCR positive is dependent on the instrument model, well size, and sample volume; thus the user must determine the threshold that is appropriate for their method. For our Bio-Rad CFX96 with 200 µL wells and 20 µL reaction volume, the average RFU was approximately 4,600 and we used a threshold of 460 for calling positives or "+" in the Table below. **Note that the interpretation of results requires running BOTH the GIDIAR1 and GIDIAR2 assays.**

aap, aatA, aggR (GIDIAR1) FAM™	ipaH (GIDIAR1) TEX615™	eae (GIDIAR1) Cy5™	STa, STb, LTa (GIDIAR2) FAM™	stxA1, stxA2 (GIDIAR2) TEX615™	rfbE (GIDIAR2) Cy5™	RPP30 HEX™	Recommended Interpretation (Any combination not listed: Multiple targets detected — consistent with mixed infection or a hybrid strain. Culture and isolate characterization required to resolve.)
–	–	–	–	–	–	–	The PCR reaction failed. Please repeat the experiment.
–	–	–	–	–	–	+	The sample contains human RPP30 DNA. The sample doesn't contain target DNA.
+	–	–	–	–	–	–	The sample contains EAEC DNA. The sample may not contain human RPP30 DNA.
+	–	–	–	–	–	+	The sample contains EAEC DNA and human RPP30 DNA.
–	–	–	+	–	–	–	The sample contains ETEC DNA. The sample may not contain human RPP30 DNA.
–	–	–	+	–	–	+	The sample contains ETEC DNA and human RPP30 DNA.
–	+	–	–	–	–	–	The sample contains EIEC/Shigella spp. DNA. The sample may not contain human RPP30 DNA.
–	+	–	–	–	–	+	The sample contains EIEC/Shigella spp. DNA and human RPP30 DNA.
–	–	–	–	+	–	–	The sample contains STEC without O157 antigen DNA. The sample may not contain human RPP30 DNA.
–	–	–	–	+	–	+	The sample contains STEC without O157 antigen DNA and human RPP30 DNA.
–	–	+	–	–	–	–	The sample contains EPEC DNA. The sample may not contain human RPP30 DNA.
–	–	+	–	–	–	+	The sample contains EPEC DNA and human RPP30 DNA.
–	–	–	–	–	+	–	The sample contains non-STEC O157 (likely non-pathogenic) DNA. The sample may not contain human RPP30 DNA.
–	–	–	–	–	+	+	The sample contains non-STEC O157 (likely non-pathogenic) DNA and human RPP30 DNA.
–	–	–	–	+	+	–	The sample may contain O157 STEC, LEE-negative DNA. The sample may not contain human RPP30 DNA.
–	–	–	–	+	+	+	The sample may contain O157 STEC, LEE-negative DNA and human RPP30 DNA.
–	–	+	–	–	+	–	The sample may contain O157 EPEC or stx-loss O157 DNA. The sample may not contain human RPP30 DNA.
–	–	+	–	–	+	+	The sample may contain O157 EPEC or stx-loss O157 DNA and human RPP30 DNA.
–	–	+	–	+	+	–	The sample may contain O157 STEC/EHEC (classic O157:H7/NM pattern) DNA. The sample may not contain human RPP30 DNA.
–	–	+	–	+	+	+	The sample may contain O157 STEC/EHEC (classic O157:H7/NM pattern) DNA and human RPP30 DNA.
–	–	+	–	+	–	–	The sample may contain LEE-positive non-O157 STEC/EHEC DNA. The sample may not contain human RPP30 DNA.
–	–	+	–	+	–	+	The sample may contain LEE-positive non-O157 STEC/EHEC DNA and human RPP30 DNA.
+	–	–	–	+	–	–	The sample may contain STEC/EAEC hybrid (O104:H4 like) DNA. The sample may not contain human RPP30 DNA.
+	–	–	–	+	–	+	The sample may contain STEC/EAEC hybrid (O104:H4 like) DNA and human RPP30 DNA.
–	+	–	–	+	–	–	The sample may contain EIEC/Shigella with stx DNA. The sample may not contain human RPP30 DNA.
–	+	–	–	+	–	+	The sample may contain EIEC/Shigella with stx DNA and human RPP30 DNA.
+	–	–	+	–	–	–	The sample may contain ETEC/EAEC hybrid DNA. The sample may not contain human RPP30 DNA.
+	–	–	+	–	–	+	The sample may contain ETEC/EAEC hybrid DNA and human RPP30 DNA.
–	–	–	+	+	–	–	The sample may contain ETEC/STEC hybrid DNA. The sample may not contain human RPP30 DNA.
–	–	–	+	+	–	+	The sample may contain ETEC/STEC hybrid DNA and human RPP30 DNA.

DEFINITIONS for types of E. coli that produce either enterotoxins or virulence factors:

STEC (Shiga toxin–producing E. coli): Produces one or both of the *Shiga toxins* encoded by **stx1** and/or **stx2** genes. These toxins inhibit protein synthesis and can cause hemorrhagic colitis or hemolytic uremic syndrome (HUS).

***E. coli* O157:** *rfbE* identifies the O157 serogroup independent of toxin status. O157:H7 typically carries *eae* together with *stx1* and/or *stx2*. STEC O157 is defined by co-detection of *rfbE* with *stx1* and/or *stx2*.

EIEC (Enteroinvasive E. coli): Does **not** produce heat-stable or heat-labile enterotoxins, but carries virulence genes such as **ipaH**, which are also found in *Shigella* species. EIEC multiplies within intestinal epithelial cells, causing dysentery-like illness.

ETEC (Enterotoxigenic E. coli): Produces one or more **enterotoxins**, including heat-stable toxins (**STa1, STa2, STb**) and/or heat-labile toxin (**LTa**). These toxins induce watery diarrhea similar to cholera.

EAEC (Enteroaggregative E. coli): Characterized by the presence of **aap**, **aata**, and **aggR** (a transcriptional activator of virulence genes). EAEC typically does **not** produce *Shiga toxins* but can occasionally acquire them (e.g., O104:H4 strain).

EPEC (Enteropathogenic *E. coli*): Carries the **eae** gene (encoding intimin) but lacks **stx1** and **stx2**. EPEC causes characteristic "attaching and effacing" lesions on intestinal epithelial cells, leading to diarrhea, especially in infants.

VERIFICATION EXPERIMENTS

The GI-Diarrhea1 assay verification was carried out as a 6-plex assay, which simultaneously detects DNA from 5 genes for toxins and virulence factors, and human RPP30 DNA, which serves as a positive extraction-control assay.

Experiments were performed in triplicate using the experimental procedure given above, but with different samples added to each reaction. The samples used for the verification experiments contained 1×10^4 copies/reaction of 500 bp synthetic DNA constructs (from Twist Biosciences) harboring the regions of interest from the pathogen genomes, human RPP30 DNA gene, and human genomic DNA. **Figure 1** shows the results of these experiments, which indicate that the 6-plex specifically detects the different toxin (and/or virulence factor) producing *E. coli* in the human genomic DNA matrix.

The limit of detection (LOD) was estimated by performing serial dilution experiments in triplicate (**Figure 2**). For each dilution series only one target construct was added. The results show a limit of detection (LOD) <10 copies/reaction.

Conclusion: The data in **Figure 1** indicate that the 6-plex primers and probes specifically detect and differentiate the pathogens and are also compatible with RPP30_DNA positive control primers. Human genomic DNA doesn't interfere with the detection of the pathogens.

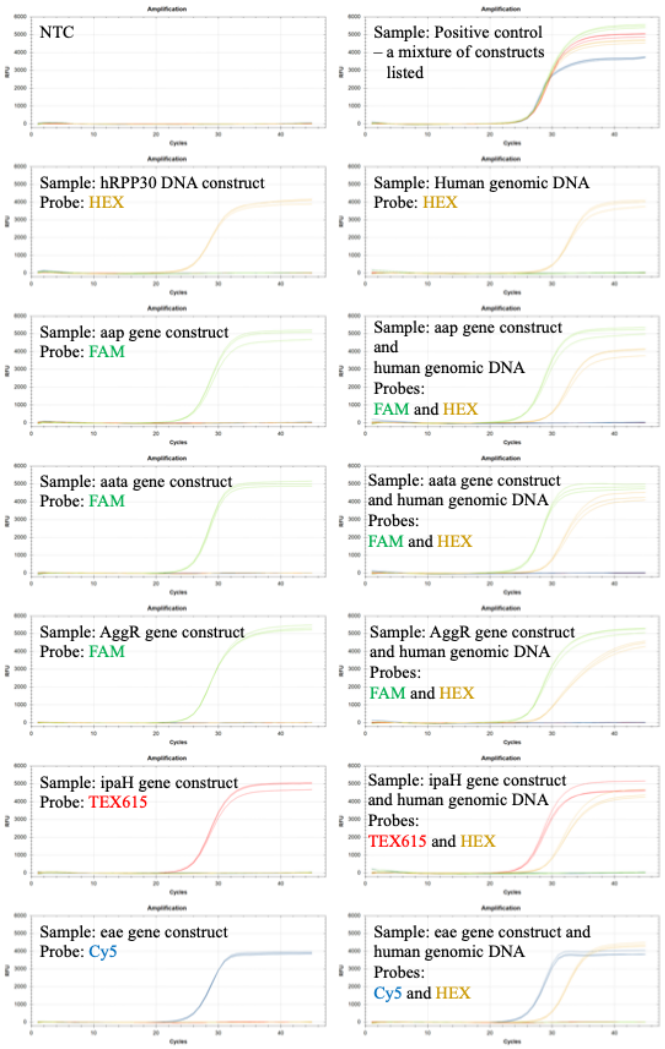

Figure 1: Verification experiments with single and multiple targets (given in text boxes for each panel). All sets of probes and primers are present in every reaction, but positive signal is only observed when the target(s) is present, indicating that the amplification is specific.

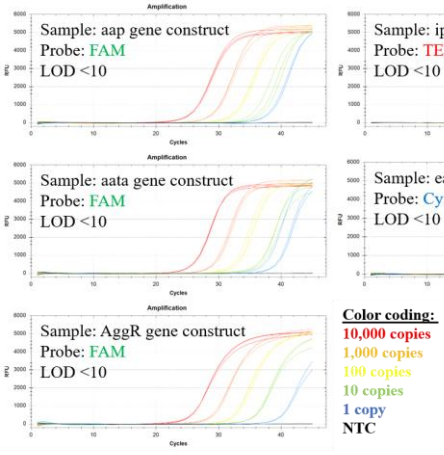

Figure 2: Serial dilution experiments show LOD <10 molecules for the synthetic DNA construct of each target.

NOTES

[1] FAM™ (Carboxyfluorescein) is a trademark of Life Technologies, Inc
[2] BHQ-1™ (Black Hole Quencher) is a trademark of Biosearch Technologies, Inc.
[3] HEX™ (Hexachloro-fluorescein) is a trademark of Applera Corp
[4] TEX615™ is a trademark of Thermo Fisher Scientific.
[5] BHQ-2™ (Black Hole Quencher) is a trademark of Biosearch Technologies, Inc.
[6] Cy5™ is a trademark of GE Healthcare
[7] "TaqMan" is a trademark of Roche Molecular Systems, Inc.

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