PCRassays.com

ASSAY NAME: GI-Diarrhea Panel for QuantStudio

Quantity: 100 x 20µL PCR reactions

14-plex (5 color) assay: detects 13 genes for toxins and virulence factors, and human RPP30 DNA

SKU #: BUN-GIDIAR-D-QS-100 (QuantStudio)

(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-GIDIAR-D-QS-100 on a QuantStudio™ 7 Pro Real-Time System. 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-Diarrhea assay are provided in Tube 1 as a 5X concentrated working solution that detects 13 genes for toxins and virulence factors plus a human extraction control.

Table of Dyes used in this assay:

Toxin genes / Virulence Factors	Dyes	Quencher	Refs.
aap, aata, aggR, Sta, STb, and/or LTa	**FAM**	BHQ-1	1, 2
Shiga toxins stxA1stxA2	**HEX**	BHQ-1	3
RPP30-DNA control	**TAMRA**	BHQ-2	4, 5
ipaH (EIEC)	**TEX615**	BHQ-2	6
rfbE and/or eae	**Cy5**	BHQ-2	7

IMPORTANT: See Table and DEFINITIONS on next page for proper interpretation of results.

The probes are designed as TaqMan[8] cleavage mechanism and the reaction requires a DNA polymerase with 5'-exonuclease activity.

ASSAY HANDLING

The GI-Diarrhea assay is shipped at ambient temperature, 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 13 genes for toxins and virulence factors, and hRPP30DNA.
Tube 2: **(Do NOT add to specimen unknowns)** Mixed positive control: 5000 copies/µl of synthetic 500 bp DNA fragments for 13 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
Molecular biology grade water	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.

A PCR protocol was used for verification on a QuantStudio™ 7 Pro Real-Time System, 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 22 seconds
4	Plate Read
5	Go to Step 2, repeat 44× more

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 QuantStudio™ 7 Pro with 96 well plate with 100 µL wells and 20 µL reaction volume, the average RFU was approximately 250,000 and we used a threshold of 25,000 for calling positives or "+" in the Table below.

NOTE: In the Table below, the *E. coli* (or *Shigella* spp.) pathotype is identified and differentiated based upon the one or two Dye channel positives. To simplify the presentation, a partial partial list of possible outcomes is provided. Other outcomes involving multiple dye positives would require expert analysis and perhaps additional testing. See DEFINITIONS after the Table.

aap aata aggR STa STb LTa FAM	stxA1 stxA2 HEX	RPP30 TAMRA	ipaH TEX 615	rfbE eae Cy5	Pathotype / Diarrhea Type Interpretation
–	–	–	–	–	The PCR reaction failed. Please repeat the experiment.
–	–	+	–	–	The sample contains human RPP30 DNA. The sample doesn't contain toxins or virulence factors detected by this assay.
+	–	+/–	–	–	The sample contains **ETEC** and/or **EAEC** DNA.
–	+	+/–	–	–	The sample contains **non-O157 STEC** DNA.
+	+	+/–	–	–	The sample may contain ***E. coli* O104:H4** or non-typical **EAEC**
–	–	+/–	+	–	The sample contains **EIEC / *Shigella* spp.** DNA.
–	–	+/–	–	+	The sample contains **EPEC** DNA.
–	+	+/–	–	+	The sample contains **STEC O157** 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

A serotype of STEC that carries **rfbE** (O157 antigen biosynthesis), **eae** (intimin adherence factor), and typically **stx1** and/or **stx2**. It is the classic cause of outbreaks of bloody diarrhea and HUS.

EIEC (Enteroinvasive *E. coli*)

Does **not** produce 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.

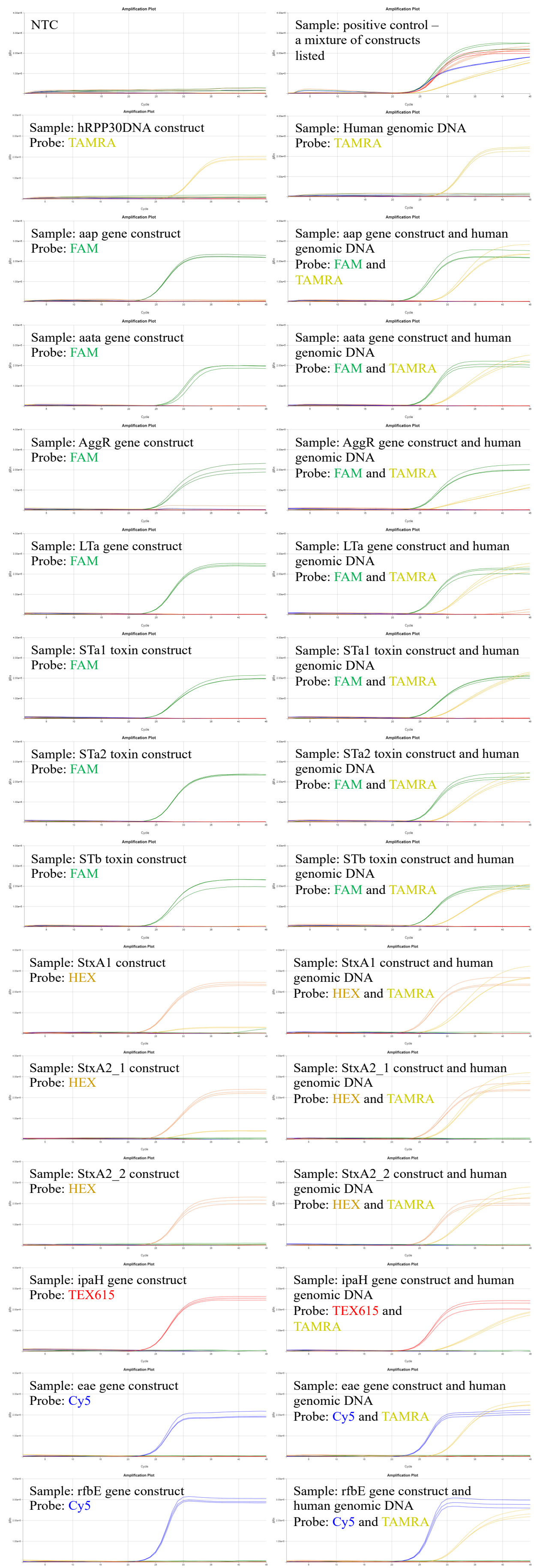

Figure 1 (RightPanel): Verification experiments with single and multiple targets (given in text boxes for each panel). All sets of probes and primers are present in every reaction, but positive signal is only observed when the target(s) is present, indicating that the amplification is specific.

VERIFICATION EXPERIMENTS

The GI-Diarrhea assay verification was carried out as a 14-plex assay, which simultaneously detects DNA from 13 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 14-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 14-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.

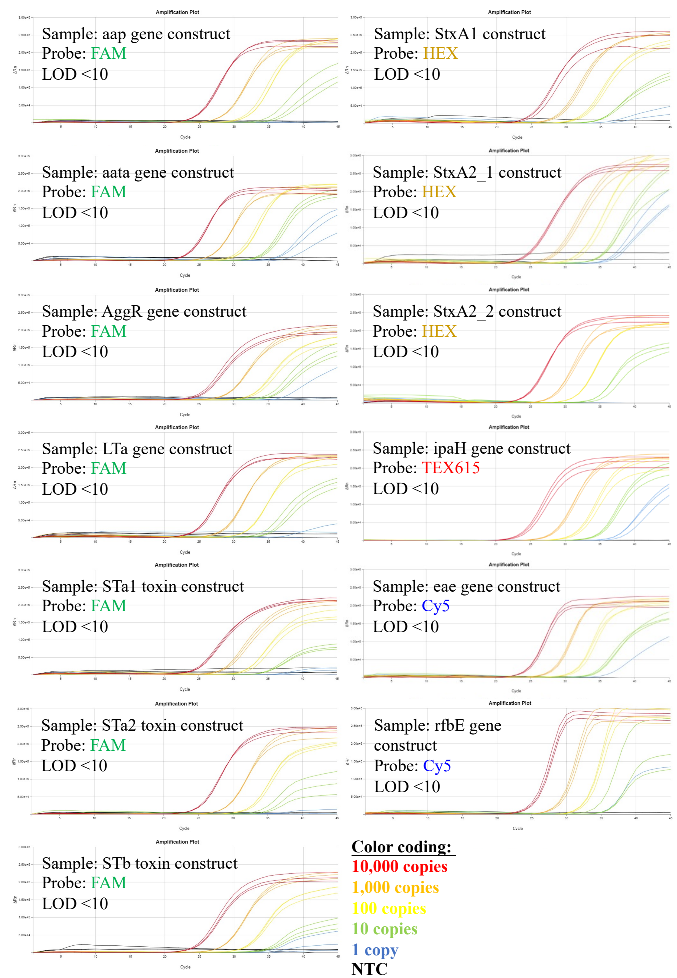

Figure 2: Serial dilution experiments show LOD <10 molecules for the synthetic DNA construct of each target.

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] TAMRA (Carboxyltetramethylrhodamine) is a trademark of Applera Cor.
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CONTACT US

For assistance, please contact DNA Software using the link: https://www.pcrassays.com/contact/

Address: Michigan Life Science and Innovation Center,
46701 Commerce Center Dr, Plymouth, MI 48170

Phone: (734) 222-9080