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qPCR based multipathogen detection for Salmonella Paratyphi "A" and Vibrio cholerae from wastewater samples

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We use this protocol and it's working

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Abstract

The following protocol is optimized for the detection of *Salmonella* Paratyphi A and *Vibrio cholerae* using multiplex qPCR assays from TNA extracted from environmental samples (wastewater samples). Total Nucleic Acid (TNA) extracted from the pellet particles is used as template in a real-time PCR assay which uses primer-probes specific for *Salmonella* Paratyphi A or *Vibrio cholerae* gene targets. Three different multiplex PCR assays were designed following standardization and validation of the primer probe combinations.

Guidelines

This protocol describes qPCR based detection of *Salmonella* Paratyphi "A" and *Vibrio cholerae* gene targets in three PCR panels from TNA extraction of processed environmental samples that were spiked with internal controls (SPC EGT control DNA - referenced below) . Additionally, HF183 which serves as a marker of fecal contamination in environmental samples, is included in the qPCR assays. Due to the similarity in cycling conditions, these assays can be conducted simultaneously for a sample as three distinct panels.

The positive controls (PCs) used here are linear oligos (gBlocks) that have been used in generating standard curves. Ideally the concentration of PCs used should yield Ct values that fall in the linear phase of the amplification curve.

Protocol

NAME

Extraction of Total Nucleic Acid from Environmental Samples for the Detection of Bacterial and Viral Targets

CREATED BY

Dilip Abraham

PREVIEW

Materials

1. QuantStudio7 Flex machine
2. Platinum™ Quantitative PCR SuperMix-UDG **Thermo Fisher Catalog #**11730025
3. qPCR DNA Extraction and Inhibition Control CY5-QXL670 **Eurogentec Catalog #**RT-SPCC-Q02
4. Nuclease-free water **Ambion Catalog #**AM9932
5. Primers and Probes (Sigma/IDT)(detailed in protocol)
6. Positive Controls: gBlocks gene fragments (IDT) (detailed in protocol)
7. MicroAmp™ Optical 96-Well Reaction Plate with Barcode Thermofisher Catalog #4306737
8. MicroAmp™ Optical Adhesive Film Thermofisher Catalog # 4311971
9. 1.7 mL MaxyClear Snaplock Microcentrifuge Tube Axygen Catalog #MCT-175-C
10. Finnpipette F1 100 to 1000 µL Thermo Fisher Catalog #4641100N
11. Finnpipette F1 20 to 200 µL Thermo Fisher Catalog #4641080N
12. Finnpipette F1 2 to 20 µL Thermo Fisher Catalog #4641060N
13. Finnpipette F1 0.2 to 2 µL Thermo Fisher Catalog #4641010N
14. ART Barrier Specialty Pipette tips 1000 µL Thermo Fisher Catalog #2279-05PK
15. ART Barrier Specialty Pipette tips 200 µL Thermo Fisher Catalog #2069-05PK
16. ART Barrier Specialty Pipette tips 20 µL Thermo Fisher Catalog #2149P-05PK
17. ART Barrier Specialty Pipette tips 10 µL Thermo Fisher Catalog #2139-05PK
18. Microplate Centrifuge, PCR Plate Spinner VWR® Catalog #VWRU89184-610

Before start

Ensure that the fluorescent dye used for TaqMan probes is compatible with the machine being used and properly calibrated for it.

Primer-Probe panel

1 The following primers and probes are employed for the detect *Salmonella* Paratyphi A and *Vibrio cholerae* specific gene targets.

1.1 PCR 1: *Salmonella* Paratyphi A (SPA2308) and *V. cholerae* ctxA PCR panel

	A	B	C
	TARGET	PRIMERS	SEQUENCES 5' TO 3'
	ctxA	Forward	TCCGGAGCATAGAGCTTGGA
		Reverse	TCGATGATCTTGGAGCATTCC
		Probe	[CY5] - CCGTGGATTCATCATGCACCGC - [BHQ2]
	SPA2308	Forward	ACGATGATGACTGATTTATCGAAC
		Reverse	TGAAAAGATATCTCTCAGAGCTGG
		Probe	[FAM] - CCCATACAATTTCATTCTTATTGAGAATGCGC - [BHQ1]

Table1: SPA and ctxA Primer and probe sequences. Fluorescent dyes and quenchers are shown in square brackets.

1.2 PCR 2: *Vibrio cholerae*_1 PCR panel

	A	B	C
	TARGET	PRIMERS	SEQUENCES 5' TO 3'
	HF183	Forward	ATCATGAGTTCACA GTCCG
		Reverse	CTTCCTCTCAGAACCCCTATCC
		Probe	[FAM] - CTAATGGAACGCATCCC - [BHQ1]
	wbfO139	Forward	AGAAGCCAGTCGCAGTAAAG
		Reverse	TCGCCATCTTCCAGCATAAA
		Probe	[TAMRA] - TGGTGGTACAGCTTAGCCGCATTA - [BHQ2]
	tcpA classic	Forward	GCGTAATGCAGCAGCTAATAAA
		Reverse	TATGGGAACATATCACCGACAC
		Probe	[JOE] - ATGGTCTGACACAGGCTCAATGCA - [BHQ1]

Table2: *Vibrio cholerae*_1 PCR Primer and probe sequences. Fluorescent dyes and quenchers are shown in square brackets.

1.3 PCR 3: *Vibrio cholerae*_2 PCR panel

	A	B	C
	TARGET	PRIMERS	SEQUENCES 5' TO 3'
	OmpW	Forward	TCAATGATAGCTGGTTCCTCAAC
		Reverse	CGATGATAAATACCCAAGCATTGA
		Probe	[JOE]_TGGTATGCCAATATTGAAACAACG_[BHQ1]
	wbeO1	Forward	GTTGAGAAGGGCGGTCTAATAA
		Reverse	TGTCTGGTACTTGAGTTGGTAAG
		Probe	[FAM] - TGCCTCAGCAATGGA - [BHQ1]
	tcpA Eltor	Forward	ATCCTTTCACTGGTACAGCTATG
		Reverse	GTCAAGCCACCGACTGTAAT
		Probe	[TAMRA]- ACGAAACTCTGCAGCGAATAAAGC-[BHQ2]

Table3: *Vibrio cholerae*_2 PCR Primer and probe sequences. Fluorescent dyes and quenchers are shown in square brackets.

Primer-probe reconstitution

2 To reconstitute the lyophilized primers/probes, use the nmole information on the specification sheet received with primers/probes.

2.1 Multiply nmol value by 10 to get the required volume of Nuclease Free Water (NFW) needed to reconstitute the lyophilized primer/probes.
Ex: For a primer with 30 nmoles, to make [M] 100 micromolar (µM) stock solution:
30 nmol x 10 = 300 µL of NFW to make [M] 100 micromolar (µM) stock solution.

2.2 Add the required volume of nuclease free water, pulse vortex and spin down. This is the primer/probe stock with [M] 100 micromolar (µM) concentration.

2.3 Store at -20 °C for long term storage.

Primer-probe dilution

3 Prepare a working stock from [M] 100 micromolar (µM) stock solution.

3.1 In a fresh tube add 10 µL of [M] 100 micromolar (µM) primer/probe stock and 90 µL of nuclease free water to give 100 µL of [M] 10 micromolar (µM) working primer/probe.

3.2 Store at 4 °C for frequent usage or -20 °C for long-term storage.

qPCR controls

4 **Positive control:** gBlocks gene fragments corresponding to each gene target is included in qPCR assay to use as positive control.

	A	B	C	D
	gBlock gene	Sequence(5'-3')	Accession No:	bp size
	SPA2308	ACGATGATGACTGATTTATCGAACAACGACTCTCCCATACAATTTCATTCTTATTGAGAATGCGCTTATGTAATTTATACCCCAGCTCTGAGAGATATCTTTTCA	FM200053.1	105
	ctxA	TCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATTCATCATGCACCGCCGGGTTGTGGGAATGCTCCAAGATCATCGA	AF463401.1	80
	HF183	GGGATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGAT GGGGATGCGTTCCATTAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACG ATGGATAGGGGTTCTGAGAGGAAGGTC	MT464394.1	132
	wbfO139	AGAAGCCAGTCGCAGTAAAGCACTAGGGCGCATGGTGGTACAGCTTAGCCGCATTATGCGAGATGAGCCGGGTGCGGATTTTATGCTGGAAGATGGCGA	AB012956.1	99
	tcpA classic	GCGTAATGCAGCAGCTAATAAAGCATTTGCAATTTCAGTGGATGGTCTGACACAGGCTCAATGCAAGACACTTATTACCAGTGTCGGTGATATGTTCCCATA	M33514.1	102
	OmpW	TCAATGATAGCTGGTTCCTCAACGCTTCTGTGTGGTATGCCAATATTGAAACAACGGCAACCTACAAAGCAGGTGCAGATGCCAAATCCACGGATGTTGAAATCAATCCTTGGGTATTTATGATCG	X51948 modified	126
	wbeO1	GTTGAGAAGGGCGGTCTAATAACACCTAAAGAGTTTGCAGAGAAGCTTGCCTCAGCAATGGATAAGGCTCTTGTACGCTTACCAACTCAAGTACCAGACA	KC152957.1	100
	tcpA El-Tor	ATCCTTTCACTGGTACAGCTATGGGGATTTTCTCATTTCCACGAAACTCTGCAGCGAATAAAGCATTCGCAATTACAGTCGGTGGCTTGAC	KP187623.1	91

Table4: Sequences used for gBlocks gene fragments

5 **Negative control:** 3 µL of extraction blank of each batch of extraction.
NTC: Master mix alone used for no template control.

Quantitative PCR

6 Thaw qPCR reagents and samples on ice and briefly spin it down.
Prepare master mix for each of the 3 primer-probe panels.

7 **[PCR 1] *Salmonella* Paratyphi A (SPA2308) and ctxA**

7.1 Prepare the master mix as follows for the number of samples, positive and negative controls, NTC and one extra reaction to account for any pipetting error.

	A	B
	REAGENT	VOLUME FOR 1 REACTION (µL)
	UDG Mix	12.5
	MgCl2	1
	Rox dye	0.05
	ctxA F Primer (10 µM)	0.5
	ctxA R Primer (10 µM)	0.5
	ctxA Probe (10 µM)	0.25
	SPA2308 F (10 µM)	0.5
	SPA2308 R (10 µM)	0.5
	SPA2308 Probe (10 µM)	0.25
	NFW	5.95

Table5: Mastermix composition for SPA2308 and ctxA gene targets

8 **[PCR 2] *Vibrio cholerae*_1 PCR**

8.1 Prepare the master mix as follows for the number of samples, positive and negative controls, NTC and one extra reaction to account for any pipetting error.

	A	B
	REAGENT	VOLUME FOR 1 REACTION (µL)
	UDG Mix	12.5
	MgCl2	1
	ROX dye	0.05
	HF183 F Primer (10 µM)	0.5
	HF183 R Primer (10 µM)	0.5
	HF183 Probe (10 µM)	0.25
	wbfO139 F Primer (10 µM)	0.5
	wbfO139 R Primer (10 µM)	0.5
	wbfO139 Probe (10 µM)	0.25
	tcpA classic F Primer (10 µM)	0.5
	tcpA classic R Primer (10 µM)	0.5
	tcpA classic Probe (10 µM)	0.25
	SPC (10 X EGT Control Mix)	2.5
	NFW	2.2

Table6: Mastermix composition for *Vibrio cholerae*_1 PCR gene targets

Note

SPC is Sample Processing Control, an optimized TaqMan control designed to be used as qPCR DNA extraction and inhibition control. It is detected by Cy5-labelled probe (Cy5-QXL670 Probe) which comes as ready to use primer probe mix.

9 [PCR 3] *Vibrio cholerae*_2 PCR

9.1 Prepare the master mix as follows for the number of samples, positive and negative controls, NTC and one extra reaction to account for any pipetting error.

	A	B
	REAGENT	VOLUME FOR 1 REACTION (µL)
	UDG Mix	12.5

	A	B
	MgCl2	1
	ROX dye	0.05
	OmpW F Primer (10 µM)	0.5
	OmpW R Primer (10 µM)	0.5
	OmpW Probe (10 µM)	0.25
	wbeO1 F Primer (10 µM)	0.5
	wbeO1 R Primer (10 µM)	0.5
	wbeO1 Probe (10 µM)	0.25
	tcpA El-Tor F Primer (10 µM)	0.5
	tcpA El-Tor R Primer(10µM)	0.5
	tcpA El-Tor Probe (10 µM)	0.25
	NFW	4.7

Table7: Mastermix composition for *Vibrio cholerae*_2 PCR gene targets

10 For each PCR, aliquot 22 µl of master mix to each required well in a 96-well plate.
Add 3µl of sample, or 3 µl of nuclease free water for negative controls.

11 Seal the plate with a roller sealer and then centrifuge the plate for 1 min at 2,000g.

12 Load the plate into Quantstudio7 flex instrument after proper initiation of the instrument. Open QS7 software, then select "New Experiment set up".

13 Set up the experiment properties with 96-well block, TaqMan reagents, 0.2 ml PCR plate and standard run. Define sample ID and define the targets as described for respective PCR panels. Assign targets and sample ID to each well.

14 Set up the PCR cycling method as described below.
Cycling conditions remain the same for all 3 qPCRs.

	A	B	C
	Step (Hold Stage)	Temperature	Time
	Reverse Transcription	50°C	2 MIN
	PCR initial heat activation	95°C	2 MIN
	2-step cycling (40 cycles)		
	Denaturation	95°C	15 SEC

	A	B	C
	Combined annealing/extension	60°C (data collection step)	30 SEC

Start the qPCR by clicking “Run.”

15 Once the run is complete, adjust the thresholds and baseline if any abnormal baseline at the start or at the end is observed, which may lead to a false-positive curve. Verify if the PC is within the range using the cut-off Ct values chosen from running the standards.

16 Export the result to excel/csv file and upload both run and csv files.

17 The threshold for each target can be set such that the PC for that target falls within the pre-defined range obtained with the standard curves.

The sample is considered positive if the amplification curve is appropriate and the Ct value falls below the defined cut-off thresholds for each target.

A separate protocol, provided in the Typhoid ES workspace, serves as an example and can be followed to generate Ct cut-off values:

Protocol

NAME

Generating Ct cut-off values using gBlocks gene fragments

CREATED BY

Catherine Troman

PREVIEW

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