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qPCR based multipathogen detection for SARS-CoV-2, CrAssphage and Hepatitis E virus from wastewater samples.

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We use this protocol and it's working

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Abstract

This protocol outlines the steps for detecting multiple viral gene targets for the pathogens SARS-CoV-2, CrAssphage and HEV using a qPCR assay. Total Nucleic Acid (TNA) extracted from environmental samples (wastewater) is used for testing.

Key Steps and Considerations:

1. **qPCR Amplification Cycles:**
- Follow the outlined cycling conditions for optimal amplification of target viral RNA.
2. **Target Detection:**
- Detect SARS-CoV-2 and HEV using specific primers and probes designed for these targets.

Guidelines

This protocol describes qPCR based detection of SARS-CoV-2 and HEV gene targets. Additionally, CrAssphage, an indicator of human fecal contamination, and MS2, serving as an external control, are included in the qPCR assays. Due to the similarity in cycling conditions, these assays can be conducted simultaneously for a sample as two 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.

Materials

1. QuantStudio™ 7 Flex Real-Time PCR System OR QuantStudio™ 12K Flex Real-Time PCR System
2. AgPath-ID™ One-Step RT-PCR Reagents **Thermo Fisher Catalog #**4387391
3. RNA MS2 from Bacteriophage MS2 **Roche Catalog #**10165948001
4. Nuclease-free water **Ambion Catalog #**AM9932
5. gBlock gene fragments **IDT** (sequence described in protocol)
6. Primers and Probes (Sigma/Thermo Fisher) (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

Fluorescent Dye Selection:

- Ensure the chosen fluorescent dyes are compatible with the qPCR machine being used.
- If using different dyes than those specified in the protocol, verify their excitation/emission spectra to prevent overlap and avoid crossover between targets.
- Calibration of the qPCR machine may be necessary if using alternative fluorescent dyes to ensure accurate detection and measurement.

Primer-Probe Panel

1 The following primers and probes are employed for the detection of SARS-CoV-2, CrAssphage and HEV gene targets.

1.1 The following set of primer-probes are used for the detection of SARS-CoV2 and CrAssphage gene targets.

	A	B	C
	TARGET	PRIMERS	SEQUENCES 5' TO 3'
	SARS-CoV-2 N1	Forward	GACCCCAAAATCAGCGAAAT
		Reverse	TCTGGTTACTGCCAGTTGAATCTG
		Probe	[JOE]-ACCCCGCATTACGTTTGGTGGACC-[BHQ1]
	SARS-CoV-2 -N2	Forward	TTACAAACATTGGCCGCAAA
		Reverse	GCGCGACATTCCGAAGAA
		Probe	[TAMRA]-ACAATTTGCCCCCAGCGCTTCAG-[BHQ2]
	CrAssphage	Forward	CAGAAGTACAAACTCCTAAAAAACGTAGAG
		Reverse	GATGACCAATAAACAAGCCATTAGC
		Probe	[FAM]-AATAACGATTTACGTGATGTAAC-[MGB]

Table 1: Primers and Probes for SARS-CoV-2 and CrAssphage. Fluorescent dyes and quenchers are shown in square brackets.

1.2 The following set of primer-probes are used for the detection of HEV and MS2 targets.

	A	B	C
	TARGET	PRIMERS	SEQUENCES 5' TO 3'
	HEV	Forward	GGTGGTTTCTGGGGTGAC
		Reverse	AGGGGTTGGTTGGATGAA
		Probe	[FAM]-TGATTCTCAGCCCTTCGC-[MGB]
	MS2	Forward	TGGCACTACCCCTCTCCGTATTCAC
		Reverse	GTACGGGCGACCCCACGATGAC
		Probe	[TAMRA]-CACATCGATAGATCAAGGTGCC-[BHQ2]

Table 2: Primers and Probes for HEV and MS2. 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 the primers and probes.

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

2.2 Add the required volume of NFW, 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 Using the [M] 100 micromolar (µM) stock, prepare a [M] 20 micromolar (µM) working stock for each primer/probe.

3.1 In a fresh tube add 20 µL of [M] 100 micromolar (µM) primer stock and 80 µL of NFW to make 100 µL of [M] 20 micromolar (µM) working primer/probe.

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

qPCR Controls

4 Controls to be used when performing qPCR assay.

4.1 **Positive control:** gBlocks gene fragments corresponding to each gene target is included in PCR panels to use as positive control in pre-defined concentrations.

	gBLOCK Gene	Sequence	bp size
	SARS-CoV-2 N 1	TTCATCTAAACGAACAAACTAAAATGTCTGATAATGGACC CCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGG ACCCTCAGATTCAACTGGCAGTAACCAGAATGGAGAACG CAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTT TACCCAATAATACT	171
	SARS CoV2 N 2	TGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAA ACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTC AGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCAC ACCTTCGGGAACGTGGTTGACCT ACACAGGTGCCATCA AATTG	161
	HEV	GGTGGTTTCTGGGGTGACCGGGTTGATTCTCAGCCCTTC GCAATCCCCTATATTCATCCAACCAACCCCT	70
	CrAssphage	CAGAAGTACAAACTCCTAAAAAACGTAGAGGTAGAGGTA TTAATAACGATTTACGTGATGTAACTCGTAAAAAGTTTGA TGAACGTACTGATTGTAATAAAGCTAATGGCTTGTTTATT GGTCATC	126

Table 3: Sequences used for gBlocks gene fragments. Sequences for the targets SARS-CoV-2 N1 and N2 is taken from SARS-CoV-2 reference genome NC_045512.2) and the HEV sequence is taken from the HEV virus complete genome sequence(MN401238.1). CrAssphage reference genome (MK415410.1).

4.2 **Negative control:** 3 µL of extraction blank of each batch of extraction.

NTC: Master mix alone used for no template control.

Preparation of PCR reaction mix

5 Thaw qPCR reagents and samples on ice and briefly spin it down. Do not vortex the AgPath master mix, mix by flicking or pipetting.

5.1 **SARS-CoV-2/CrAssphage panel**

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.

	Reagents	volume (x1) ul
	Ag Path master mix	10
	25x Enzyme mix	0.8
	N1 F primer	0.25
	N2 R primer	0.25
	N1 probe	0.125

	Reagents	volume (x1) ul
	N2 F primer	0.25
	N2 R primer	0.25
	N2 probe	0.125
	CPQ F primer	0.25
	CPQ R primer	0.25
	CPQ probe	0.125
	NFW	4.325

Table 4: SARS-CoV-2/CrAssphage - PCR reaction mix

5.2 **Hepatitis-E Virus (HEV) and MS2 bacteriophage panel**

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.

	Reagents	volume (x1) ul
	Ag Path master mix	10
	25x Enzyme mix	0.8
	HEV F primer	0.25
	HEV R primer	0.25
	HEV probe	0.125
	MS2 F primer	0.25
	MS2 R primer	0.25
	MS2 probe	0.125
	NFW	4.95

Table 5: HEV/MS2 PCR reaction mix

HEV and MS2 PCR- Total volume of master mix to add will be 17 µL

5.3 Dispense 17 µL of master mix per reaction into the wells of a standard 96-well PCR plate on ice.
Add 3 µL of sample TNA (Total Nucleic Acid). Mix well my pipetting.
Seal the plate with a roller sealer and then centrifuge the plate for 1 min at 2000g.

6 Load the plate into the Quantstudio7 flex instrument after properly starting it up. Open QS7 software, then select - "New experiment set up".

7 Set up the experiment properties with 96-well block, TaqMan reagents,0.2ml 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.

Thermocycler conditions/ program

8 Set up the real time PCR conditions as follows:

	Step	Temperature in Celsius	Time
	Reverse Transcription	45° C	20 min
	PCR initial heat activation	95° C	10 min
	2-step cycling (40 cycles)		
	Denaturation	95° C	15 sec
	Combined annealing/extension	55° C (data collection step)	1 min

8.1 Click - "Run" to start the qPCR.

8.2 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 defined Ct values chosen from running the standards.

8.3 Export the result to excel/csv file. Upload both run and csv files to Dropbox/OneDrive for backup.

9 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

Protocol references

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