VERSION 3

JUL 10, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.8epv5zrw4v1b/v3

Protocol Citation: Dilip Abraham, Nicola Elviss, Nicholas Feasey, Nick Grassly, Jacob John, Gagandeep Kang, John Scott Meschke, Venkata Raghava Mohan, Satheesh Nair, Jonathan Rigby, Rajan Srinivasan, Catherine Troman, Christopher Uzzell, Nicolette Zhou 2023. Extraction and qPCR of Environmental Surveillance samples for the detection of Salmonella Typhi.
protocols.io
https://dx.doi.org/10.17504/protocols.io.8epv5zrw4v1b/v3
Version created by Catherine Troman

MANUSCRIPT CITATION:
Uzzell C, Troman C, Rigby J, Mohan V, John J, Abraham D, Srinivasan R, Nair S, Meschke J, Elviss N, Kang G, Feasey N, Grassly N (2021) Environmental surveillance for *Salmonella* Typhi as a tool to estimate the incidence of typhoid fever in low-income populations. medRxiv, doi: 10.1101/2021.05.21.21257547

Extraction and qPCR of Environmental Surveillance samples for the detection of Salmonella Typhi V.3

Dilip Abraham[1], Nicola Elviss[2], Nicholas Feasey[3], Nick Grassly[4], Jacob John[1], John Scott Gagandeep Kang[1], Meschke[5], Venkata Raghava Mohan[1], Satheesh Nair[2], Jonathan Rigby[3], Rajan Srinivasan[1], Catherine Troman[4], Christopher Uzzell[4], Nicolette Zhou[5]

[1]Christian Medical College, Vellore, India; [2]UK Health Security Agency; [3]Malawi Liverpool Wellcome Trust Clinical Research Program, Blantyre, Malawi;
[4]Imperial College London; [5]University of Washington

Typhoid Environmental Surveillance

Catherine Troman

DISCLAIMER

Please note, the author list is in alphabetical order and does not reflect contribution.

Protocol status: Working
We use this protocol and it's working

Created: Jun 05, 2023

Last Modified: Jul 10, 2023

PROTOCOL integer ID: 82877

ABSTRACT

The following protocol is for use on environmental samples that have been collected by Moore swab or grab sampling methods and processed up to the stage of DNA extraction. The protocol includes DNA extraction and qPCR analysis of the samples to detect three gene targets for identifying Salmonella Typhi, a human faecal indicator (HF183), and a commercial spike-in control.

The qPCR uses fluorescence of Taqman probes for measuring the product. Care must be taken to ensure that the fluorescent dyes chosen for the probes are suitable for the machine being used or can be calibrated for use on the machine. If the dyes selected are different from those described in the protocol, make sure that their excitation/emission spectra do not overlap to avoid crossover between targets.

Generation of standard curves is mentioned in this protocol but is described in more detail in a separate protocol in the Typhoid ES workspace.

MATERIALS

QIAamp PowerFecal Pro DNA Kit **Qiagen Catalog #51804**

Water, nuclease free **Contributed by users**

qPCR DNA Extraction and Inhibition Control CY5-QXL670 **Eurogentec Catalog #RT-SPCC-Q02**

Takyon Low ROX Probe 2x MasterMix dTTP blue **Eurogentec Catalog #UF-LPMT B0701**

Primers and Probes (detailed in protocol)
gBlocks DNA fragments (detailed in protocol)

qPCR machine
96-well plates for qPCR and plate seals
Vortex with tube adapter
96-well plate spinner
Microcentrifuge
1.5ml tubes

DNA Extraction

1 For extraction from Moore swabs, empty the contents of a PowerBead tube provided in the extraction kit into the PowerBead tube used for storing the filter.

For the pellet from membrane filtration, resuspend the pellet in 800µl CD1 from the first step of the DNA extraction protocol, then transfer to the PowerBead tube for homogenisation.

For an extraction negative control, carry out the extraction protocol starting with just the CD1 (step2.1) and no sample.

2 Carry out the DNA extraction according to the manufacturer's protocol provided with the DNA extraction kit.
Note that x g and rcf are the same and may be named either way depending on your centrifuge.

In brief:

2.1 Add 800µl of Solution CD1 to the PowerBead tube and 1ul of validated SPC dilution from the Extraction and Inhibition control kit.

1) Before first use of the SPC, prepare a 1/10th and a 1/100th dilution of the control DNA in pure water and store them on ice

2) Evaluate the non-diluted and the 2 diluted control DNA solutions in separate extractions by adding 1µL of control DNA into your reference sample before parallel extractions. Perform the extraction and qPCR and select the dilution factor generating Ct values between 30 and 33

2.2 Homogenize using a vortex adapter at maximum speed for up to 10 minutes

2.3 Centrifuge the PowerBead tube at 15000 x g, 00:01:00 1m

2.4 Transfer the supernatant to a clean Microcentrifuge tube, add 200µl of Solution CD2 then vortex for 5s

2.5 Centrifuge at 15000 x g, 00:01:00 and transfer up to 700µl of supernatant to a clean 2ml Microcentrifuge tube, avoiding the pellet

2.6 Add 600µl of Solution CD3 and vortex for 5s

2.7 Load 650µl onto an MB Spin Column and centrifuge at 15000 x g, 00:01:00

2.8 Discard the flow-through and repeat until all the lysate has been passed through the spin column

2.9 Carefully place the spin column into a clean 2ml collection tube. Avoid splashing any flow-through onto the column

2.10 Add 500µl of Solution EA to the spin column and centrifuge at 15000 x g, 00:01:00

2.11 Discard the flow-through and add 500µl of Solution C5 to the spin column and centrifuge at 15000 x g, 00:01:00

2.12 Discard the flow through and place the column in a clean 2ml collection tube

2.13 Centrifuge at 16000 x g, 00:02:00 then place the column in a clean 1.5ml collection tube

2.14 Add 50µl of Solution C6 to the center of the filter membrane and let it sit for 1 minute

2.15 Centrifuge at 15000 x g, 00:01:00 and discard the spin column

2.16 The DNA is now ready for qPCR, or storage at -20°C

Preparation of Primers and Probes

3 S. Typhi genes being targeted are staG, tviB, and ttr. Primers and probes have also been included for HF183 human faecal indicator to use as a positive control for faecal contamination in the samples and sample sites.

Before ordering probes, please make sure the selected fluorophores are compatible with the machine you will be using (see guidance note).

	A	B
	Primer/Probe Name	Sequence (5' -3')
	staG_F	CGC GAA GTC AGA GTC GAC ATA G
	staG_R	AAG ACC TCA ACG CCG ATC AC
	staG_P	[Cy5] - CA TTT GTT CTG GAG CAG GCT GAC GG - [BHQ2]
	ttr_F	CTC ACC AGG AGA TTA CAA CAT GG
	ttr_R	AGC TCA GAC CAA AAG TGA CCA TC
	ttr_P	[FAM] - CA CCG ACG GCG AGA CCG ACT TT - [BHQ1]
	tviB_F	TGT GGT AAA GGA ACT CGG TAA A
	tviB_R	GAC TTC CGA TAC CGG GAT AAT G
	tviB_P	[JOE] - TG GAT GCC GAA GAG GTA AGA CGA GA - [BHQ1]
	HF183_F	ATC ATG AGT TCA CAT GTC CG
	HF183_R	CTT CCT CTC AGA ACC CCT ATC C
	HF183_P	[FAM] - CT AAT GGA ACG CAT CCC - [BHQ1]

Table1: Primer (F or R) and probe (P) sequences. Fluorescent dyes and quenchers are shown in

square brackets.

4 Resuspend the probes and primers in the appropriate volume of nuclease free water and make stock dilutions of each primer at 20µM and each probe at 5µM.

Preparation of Standards

5 Using gBlocks gene fragments (IDT) with the target amplicon sequences, a standard curve can be obtained by running a qPCR on a dilution series of the DNA fragments.

This allows determination of potential Ct cut off values to help with determining a positive result in the qPCR with real samples.

A separate protocol for carrying out this experiment is provided in the Typhoid ES workspace.

	A	B
	gene target	gBlocks sequence (5' - 3')
	staG	CGGCGCGAAGTCAGAGTCGACATAGGCATAGATTTTCAGGCCATACATTAATTTGCCAAGGTTGCTATAAACATTTGTTCTGGAGCAGGCTGACGGAAATTCCGTGAACTCGCTGGTGATCGGCGTTGAGGTCTTATC
	ttr	GAAACGCTGAACGGACTCACCAGGAGATTACAACATGGCTAATTTAACCCGTCGTCAGTGGCTAAAAGTCGGTCTCGCCGTCGGTGGGATGGTCACTTTTGGTCTGAGCTACCGTGATGTGGCGA
	tviB	CTTGATTTGACTTCCGATACCGGGATAATGCCATACTCTCGTCTTACCTCTTCGGCATCCACCCATGGATCAAAAATATCCACTTTACAACTATATTTACCGAGTTCCTTTACCACATCAATAAT
	HF183	GGGATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGGTC

Table 2: Sequences used for gBlocks gene fragments. Sequences for the *S.*Typhi targets were taken from the CT18 reference genome (AL513382.1) and the HF183 sequence was taken from a *Bacteroides dorei* 16S rRNA partial gene sequence (MT464394.1). 3 additional bases were added either side of the *staG* and HF183 amplicon sequences. 15 and 8 bases were added to either end of the *ttr* and *tviB* sequences respectively to reach the minimum length for the gBlocks fragment.

Quantitative PCR

6 Thaw qPCR reagents and samples on ice and briefly spin down.

7 Set up a master mix for the number of samples to be tested plus a negative control and one extra to allow for pipetting error.

If you wish to carry out single-plex reactions for any of the gene targets, input the same amount of the mastermix, target primer and probe, and substitute the remaining volume with nuclease free water.

	A	B	C
	Reagent	Volume per reaction (uL)	Final Concentration (uM)
	ttr_F	0.25	0.2
	ttr_R	0.25	0.2
	ttr_P	0.5	0.1
	tviB_F	0.5	0.4
	tviB_R	0.5	0.4
	tviB_P	1	0.2
	staG_F	0.5	0.4
	staG_R	0.5	0.4
	staG_P	1	0.2
	2x MasterMix with ROX	12.5	1x
	Nuclease free water	2.5	-

Table 3: Mastermix composition for the *S.*Typhi qPCR

8 Make up another master mix for the HF183 qPCR and internal control

	A	B	C
	Reagent	Volume per reaction (uL)	Final Concentration (uM)
	HF183_F	0.5	0.4

	A	B	C
	HF183_R	0.5	0.4
	HF183_P	1	0.2
	2x MasterMix with ROX	12.5	1x
	10x Control mix (Eurogentec)	2.5	1x
	Nuclease free water	3	-

Table 4: Mastermix composition for the HF183 and extraction control qPCR

9 Aliquot 20µl of mastermix to each required well in a 96-well plate.

10 Add 5µl of sample, or 5µl of nuclease free water for negative controls.

11 Seal the plate carefully with a plate seal the spin the plate down briefly to gather all reagents at the bottom of the well and remove bubbles.

12 Load the plate into the real-time PCR machine after setting it up appropriately and carry out cycling using the following conditions:

	A	B	C
	Cycle	Temperature (ºC)	Duration
	1	50	2 minutes
	1	95	2 minutes
	40	95	15 seconds
		60	30 seconds
		72	30 seconds

13 Once the run is complete assess the normalised Ct values for each gene target for each sample, determining positivity using the cut-off Ct values chosen from running the standards.

13.1 If all gene targets have Ct values higher than the cut-off, check the Ct values for the HF183 target and the spike-in control.

- If the spike-in control is negative or lower than expected this may indicate an issue with PCR inhibition or during DNA extraction
- If the HF183 is negative and spike-in control was positive, then this may mean that there was no or insufficient faecal contamination in the sample site

13.2 If all three targets are positive, then the sample is considered positive for *S.*Typhi.

If the sample is positive for *ttr* and only one of either *staG* or *tviB,* repeat the target that was negative in a singleplex reaction as this may result in a positive if the target is just very close to the limit of detection.

The following table gives expamples for interpretation of the qPCR results (after singleplex repeats):

	A	B	C
	qPCR result	Low Ct values	High Ct values
	ttr+ staG+ tviB+	S.Typhi	S.Typhi (or a more complex mixture but unlikely)
	ttr+ staG- tviB+	Mixture (NTS)	Mixture (NTS)
	ttr+ staG+ tviB-	Mixture (NTS)	Mixture or S.Typhi where Vi gene is lost