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Extraction and qPCR of Environmental Surveillance samples for the detection of *Salmonella* Typhi V.4

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Jonathan Rigby[1,2,3], Catherine Troman[4,1], Dilip Abraham[5], Jaspreet Mahindroo[1], Christopher Uzzell[1], Jacob John[5], Venkata Raghava Mohan[5], Rajan Srinivasan[5], Nicolette Zhou[6], Satheesh Nair[7], Alex Shaw[1], Nicola Elviss[7], Nicholas Feasey[8], John Scott Meschke[6], Gagandeep Kang[5], Nick Grassly[1]

[1]Imperial College London; [2]Malawi Liverpool Wellcome Trust; [3]Liverpool School of Tropical Medicine; [4]UiT The Arctic University of Norway; [5]Christian Medical College, Vellore, India; [6]University of Washington; [7]UK Health Security Agency; [8]Malawi Liverpool Wellcome Trust Clinical Research Program, Blantyre, Malawi

Typhoid Environmental ...

Jonathan Rigby
Imperial College London

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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

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

For extraction from Moore swabs, add 800 µL of CD1 to the membrane strips already stored in PowerBead tubes.

1.1 If stored in another microcentrifuge tube, pour the zirconia beads from a PowerBead tube included in the Qiagen PowerFaecal Pro kit into the tube and add 800 µL of CD1.

1.2 For an extraction negative control, carry out the extraction protocol starting with just the CD1 and no sample.

1.3 A positive control should also be extracted if a stock of Positive DNA has not been made previously.

Not to be confused with the Sample Processing Control (SPC) added in Step 2.1; this should be an isolate of an *S*. Typhi Ty21a, ATCC/NCTC strain, or a confirmed wild type isolate.

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 To the previously added 800 µL of Solution CD1 to the PowerBead tube and 1 µL 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 both diluted control DNA solutions in separate extractions by adding 1 µL 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 00:10:00 minutes

2.3 Centrifuge the PowerBead tube at 15000 x g, Room temperature, 00:01:00

2.4 Transfer the supernatant to a clean Microcentrifuge tube, add 200 µL of Solution CD2 then vortex for 00:00:05 seconds

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

2.6 Add 600 µL of Solution CD3 and vortex for 00:00:05 seconds

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

2.8 Discard the flow-through and repeat go to step #2.7 until all the lysate has been passed through the spin column

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

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

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

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

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

2.14 Add 50 µL of Solution C6 to the center of the filter membrane and let it sit for 00:01:00 minute

2.15 Centrifuge at 15000 x g, Room temperature, 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 For this assay, six qPCR primer/probe targets are utilised.

For *S*. Typhi, primers and probes targeting the *staG* and *tviB* genes and an intergenic region, currently named C6, are used.

The control triplex targets *ttr*, a pan-salmonella gene; HF183, a *Bacteroides dorei* 16s human faecal indicator and the Sample Processing Control (SPC) from Eurogentec.

Before ordering probes, please make sure you select the best fluorophores for the channels of your particular model of qPCR 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_Pr	[Fluorophore 1] - CA TTT GTT CTG GAG CAG GCT GAC GG - [Quencher]
	tviB_F	TGT GGT AAA GGA ACT CGG TAA A
	tviB_R	GAC TTC CGA TAC CGG GAT AAT G

	A	B
	tviB_Pr	[Fluorophore 2] - TG GAT GCC GAA GAG GTA AGA CGA GA - [Quencher]
	C6_F	GCG ATG TAA TTT ATC TCA CGG AAA
	C6_R	AAG TGA GAT TCA TCG CGA TAT TTG
	C6_Pr	[Fluorophore 3] - TGG CAT CCC GCG AGA AAG CC - [Quencher]

Table 1: Primer (F or R) and probe (P) sequences for the *S*. Typhi Triplex

	A	B
	ttr_F	CTC ACC AGG AGA TTA CAA CAT GG
	ttr_R	AGC TCA GAC CAA AAG TGA CCA TC
	ttr_Pr	[Fluorophore 1] - CA CCG ACG GCG AGA CCG ACT TT - [Quencher]
	HF183_F	ATC ATG AGT TCA CAT GTC CG
	HF183_R	CTT CCT CTC AGA ACC CCT ATC C
	HF183_Pr	[Fluorophore 2] - CT AAT GGA ACG CAT CCC - [Quencher
	SPC	[Fluorophore 3] - N/A - [Quencher]

Table 2: Primer (F or R) and probe (P) sequences for the Assay Control Triplex

Guidance Note:

SPC is sold with either Yakima Yellow or Cy5 fluorophores.
For the Applied Biosystems QuantStudio series of machines, in particular the QS7∗ Flex, we recommend the following combination of fluorophores and Quenchers for the probes:

	A	B	C
		Dye	Quencher
	Fluorophore 1	FAM	BHQ 1 or QSY
	Fluorophore 2	VIC	BHQ 1 or QSY
	Fluorophore 3	Cy5	BHQ 3 or QSY 2

Table 3: Suggested Fluorophore Dye and Quencher combinations for Tables 1 and 2

*Thermofisher also provide a multiplex option for QuantStudio systems using: FAM, VIC, ABY, JUN, Cy5 and Cy5.5 with their QSY Quenchers, however JUN is not compatible with the ROX in the Takyon Mastermix, and Passive Reference Dye would need to be set to none

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

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.

Protocol

NAME

Generating Ct cut-off values using gBlocks gene fragments

CREATED BY

Jonathan Rigby

Preview

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 positive control, extraction control and qPCR 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)**
	tviB_F	0.5	0.4
	tviB_R	0.5	0.4
	tviB_Pr	1	0.2
	staG_F	0.5	0.4
	staG_R	0.5	0.4
	staG_Pr	1	0.2
	C6_F	0.5	0.4
	C6_R	0.5	0.4
	C6_Pr	1	0.2
	2x MasterMix with ROX	12.5	1x
	Nuclease free water	1.5	-

Table 4: Mastermix composition for the *S.* Typhi qPCR

8 Make up another master mix for the Control Triplex

	A	B	C
	Reagent	**Volume per reaction (uL)**	**Final Concentration (uM)**
	ttr_F	0.5	0.4
	ttr_R	0.5	0.4
	ttr_Pr	1	0.2

	A	B	C
	HF183_F	0.5	0.4
	HF183_R	0.5	0.4
	HF183_Pr	1	0.2
	2x MasterMix with ROX	12.5	1x
	10x Control mix (Eurogentec)	2.5	1x
	Nuclease free water	1	-

Table 5: Mastermix composition for the HF183 and extraction control qPCR

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

10 Add 5 µL of sample, positive control or extraction control; or 5 µL of nuclease free water for negative controls.

11

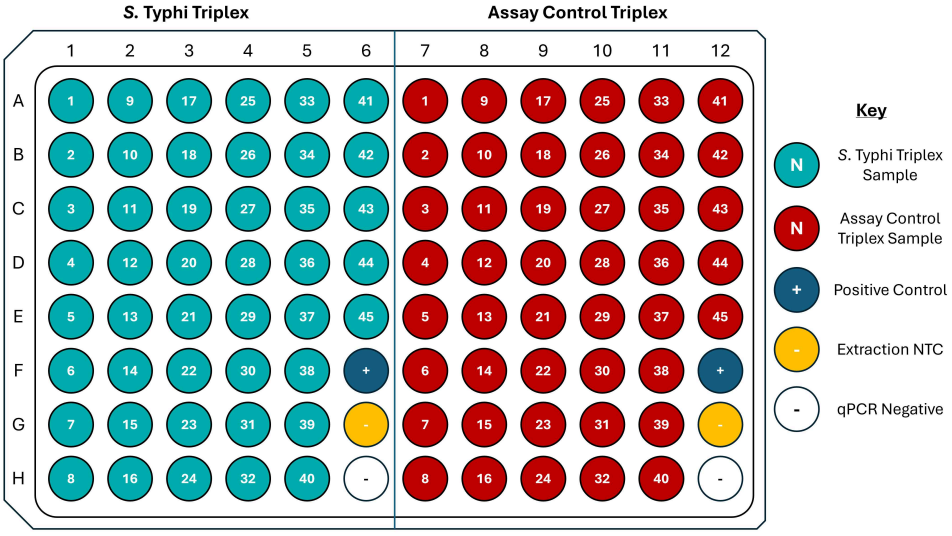

Figure 1: Example of a plate map for the qPCR split between both triplexes in use. Positive *S.* Typhi control, Extraction control (including SPC) and qPCR Negative control are all present.

12 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.

13 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	D
	Cycle	**Temperature (ºC)**	**Duration**	Step
	1	50	2 minutes	Carry-over prevention
	1	95	2 minutes	Takyon® activation
	40	95	15 seconds	Denaturation
		60	30 seconds	Annealing
		72	30 seconds	Extension

Table 6: Cycling conditions for the qPCR. If not automatically set by the software or hardware, the data collection function should be set at the Extension step.

14 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.

14.1 First, screen the controls in both halves of the Assay to ensure the quality control passes:

1) The positive control should amplify *ttr*, *tviB*, *staG* and C6 - if any of these have not amplified, then there may be an issue with qPCR set up, please refer to the QA/QC Guidance document.

2) The Extraction NTC should have no amplification for all targets except for the SPC - ensure this spike in control is at the appropriate Ct value, as deviance from this may indicate inhibition or loss of amplification efficiency. Amplification of any other target may indicate cross-contamination or non-target amplification. Please refer to the QA/QC Guidance document.

3) qPCR Negative should have no amplification for all targets, including the SPC. Any deviation from this may indicate cross-contamination or non-target amplification. Please refer to the QA/QC Guidance document.

14.2 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

14.3 If all three targets, *staG*, *tviB* and C6, are positive, then the sample is considered positive for *S*. Typhi. If only *staG* and *tviB*, or C6 are positive, then it may be suspected positive for *S*. Typhi and progress to Amplicon Sequencing.

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

The following table gives examples for interpretation of the qPCR results (after monoplex repeats):

	A	B	C
	qPCR result	**Low Ct values**	**High Ct values**
	ttr+ staG+ tviB+ C6+	S. Typhi	S. Typhi (inhibitors or low concentration)
	ttr+ staG+/- tviB+/- C6+	Suspect S. Typhi	Mixture with low concentration or NTS
	ttr+ staG+ tviB+ C6-	Suspect S. Typhi	Mixture with low concentration or NTS
	ttr+ staG- tviB+ C6-	Mixture (NTS)	Mixture with low concentration or NTS
	ttr+ staG+ tviB- C6-	Mixture (NTS)	Mixture or S. Typhi where Vi gene is lost
	ttr+ staG- tviB- C6-	NTS	NTS

Table 7: Result interpretation