Generating Ct cut-off values using gBlocks gene fragments V.2

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Typhoid Environmental Surveillance

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

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ABSTRACT

The following protocol describes the resuspension, dilution, and qPCR of gBlocks gene fragments. gBlocks gene fragments are synthesised double stranded DNA oligos, which can be used for standardisation.

In this protocol, the standard curves generated by the gBlocks in a triplex qPCR are used to determine a Ct cut-off value for the S.Typhi gene targets (*ttr, tviB, staG*) and the HF183 bacteroides rRNA gene.

MATERIALS

TE Buffer **Contributed by users**

gBlocks gene fragments (see protocol)

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

Nuclease free water **Contributed by users**

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

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g-blocks details

1 gBlocks gene fragments are synthesised double stranded DNA fragments which contain the sequence for the amplicon of interest, in this case for *ttr*, *staG* and *tviB* in S.Typhi, and HF183 bacteroides.

	A	B	C
	Gene target	Size (bp)	gblock sequence (5' - 3')
	ttr	125	GAAACGCTGAACGGACTCACCAGGAGATTACAACATGGCTAATTTAACCC GTCGTCAGTGGCTAAAAGTCGGTCTCGCCGTCGGTGGGATGGTCACTTTT GGTCTGAGCTACCGTGATGTGGCGA
	staG	138	CGGCGCGAAGTCAGAGTCGACATAGGCATAGATTTTCAGGCCATACATTAA TTTGCCAAGGTTGCTATAAACATTTGTTCTGGAGCAGGCTGACGGAAATTC CGTGAACTCGCTGGTGATCGGCGTTGAGGTCTTATC
	tviB	125	CTTGATTTGACTTCCGATACCGGGATAATGCCATACTCTCGTCTTACCTCTT CGGCATCCACCCATGGATCAAAAATATCCACTTTACAACTATATTTACCGAG TTCCTTTACCACATCAATAAT
	HF183	132	GGGATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACG ATGGGGATGCGTTCCATTAGATAGTAGGCGGGGTAACGGCCCACCTAGTCA ACGATGGATAGGGGTTCTGAGAGGAAGGTC

Table1: Sequences for gBlocks gene fragments for S.Typhi gene targets and HF183.

Resuspending and diluting the gblocks

2 gBlocks are supplied as a lyophilised pellet. Resuspend in TE buffer to achieve a stock of 10ng/uL. Information on the ng provided, OD260, and molecular weight are given on the spec sheet provided with the gblocks.

The following online tools can be used to assist in these calculations:
Integrated DNA Technologies - Resuspension Calculator (idtdna.com)
Oligo Analyzer (idtdna.com)

3 Once resuspended, check the concentration via Qubit or Nanodrop.

3.1 If the concentration is not 10ng/ul, carry out your first dilution in step4 to make it 1ng/ul

4 Create serial dilutions of your stock solution adding 2uL of stock into 18uL of nuclease free water. We recommend carrying out 12 dilutions to create a series of 12 concentrations. We recommend at least 10 replicates split over at least two days.

4.1 For example, performing four replicates of 12 dilutions three times on three separate days. This would be two plates each day to include all targets.

qPCR and generating a standard curve

5 Prepare the triplex qPCR mastermix described below (or singleplex for HF183)

	A	B
	Reagent	Volume per reaction (uL)
	ttr_F (20uM)	0.25
	ttr_R (20uM)	0.25
	ttr_P (5uM)	0.5
	tviB_F (20uM)	0.5
	tviB_R (20uM)	0.5
	tviB_P (5uM)	1
	staG_F (20uM)	0.5
	staG_R (20uM)	0.5
	staG_P (5uM)	1
	2x Mastermix with ROX	12.5
	Nuclease free water	2.5

Table2: Mastermix composition for triplex S.Typhi qPCR. Primer and probe sequences are provided in the qPCR protocol in the TyphoidES workspace.

	A	B
	Reagent	Volume per reaction (uL)
	HF183_F	0.5
	HF183_R	0.5
	HF183_P	1
	10x Control Mix (Eurogentec)	2.5
	2xMastermix with ROX	12.5
	Nuclease free water	3

Table3: Mastermix composition for the singleplex HF183 reaction. Primer and probe sequences are provided in the qPCR protocol in the TyphoidES workspace.

6 Aliquot 20uL of master mix for each reaction in a 96-well plate. Add 5uL of gBlock dilution to each reaction.

Ensure that although the reaction is designed as a triplex, you only put one target gBlock in each reaction.

7 Seal the plate carefully then spin down briefly to gather all reagents at the bottom of the wells and remove bubbles.

8 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 (C)	Duration
	1	50	2 minutes
	1	95	2 minutes
	40	95	15 seconds
		60	30 seconds
		72	30 seconds

Table4: Cycling conditions for all qPCR reactions.

Analysis - determining Ct cut-off

9 The limit of detection (LOD_95) is the genome copy number/uL and associated Ct value at which a qPCR amplification would be observed 95% of the time. This can be calculated from the results of the dilution series using PROBIT analysis.

9.1 We have provided an Excel file to calculate the LOD_95 for you from your data (resource: LOD calculation update 250124.xlsm 35KB). Make sure you allow macros to be run. You will also need to enable the Microsoft Solver add-in. Instructions for doing so are here.

Alternatively you can use the statistical programming language R to fit the PROBIT curve. Example code:

```
#fit the profit curve
mod=glm(Ct_bin ~ log_conc, data=subset(gblocks, target=="ttr"),
family=binomial(link="probit"))
summary(mod)

#calculate the LOD_95 in log concentration (GC/uL)
LOD_est_ttr=(qnorm(0.95)-mod$coefficients[1])/mod$coefficients[2]

#predict the LOD_95 Ct and range
LOD_Ct_ttr=predict(lm(Ct ~ log_conc, data=subset(gblocks,
target=="ttr")), newdata=list(log_conc=LOD_est_ttr),
interval="prediction")
```

9.2 To calculate the GC/uL from the qPCR Ct values of actual samples you can use the equation:

log GC/uL = (Ct – intercept) / slope

where slope and intercept are from the linear regression of the Ct value on log GC/uL generated from the standard curves (e.g. as given in the Excel spreadsheet or from the linear model (lm) fit in R).