Feb 02, 2026 Version 3

Generating Ct cut-off values using gBlocks gene fragments V.3

DOI
https://dx.doi.org/10.17504/protocols.io.n2bvj8kr5gk5/v3

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Typhoid Environmental ...

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Protocol Citation: Jonathan Rigby, Catherine Troman, Jaspreet Mahindroo, Alex Shaw, Dilip Abraham, Nick Grassly 2026. Generating Ct cut-off values using gBlocks gene fragments. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj8kr5gk5/v3Version created by **Jonathan Rigby**

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

Created: December 16, 2025

Last Modified: February 02, 2026

Protocol Integer ID: 235158

Keywords: Salmonella, Typhi, Paratyphi, wastewater, environmental surveillance, qpcr of gblocks gene fragment, gblocks gene fragment, using gblocks gene, gblocks gene, rrna gene, generating ct, stranded dna oligo, gblock, dna oligo, triplex qpcr, stranded dna, dna, qpcr, hf183 bacteroides rrna gene, hf183 bacteroide

Funders Acknowledgements:
Bill and Melinda Gates Foundation

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*, C6) and the HF183 *Bacteroides* rRNA gene.

Materials

TE Buffer

gBlocks gene fragments (see protocol)

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

Nuclease free water

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

gBlocks 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, tviB* and C6 in *S.* Typhi, and HF183 in *Bacteroides*.

	A	B	C
	Gene target	Size (bp)	gBlock sequence (5' - 3')
	ttr	125	GAAACGCTGAACGGACTCACCAGGAGATTACAACATGGCTAATTTAACCCGTCGTCAGTGGCTAAAAGTCGGTCTCGCCGTCGGTGGGATGGTCACTTTTGGTCTGAGCTACCGTGATGTGGCGA
	staG	138	CGGCGCGAAGTCAGAGTCGACATAGGCATAGATTTTCAGGCCATACATTAATTTGCCAAGGTTGCTATAAACATTTGTTCTGGAGCAGGCTGACGGAAATTCCGTGAACTCGCTGGTGATCGGCGTTGAGGTCTTATC
	tviB	125	CTTGATTTGACTTCCGATACCGGGATAATGCCATACTCTCGTCTTACCTCTTCGGCATCCACCCATGGATCAAAAATATCCACTTTACAACTATATTTACCGAGTTCCTTTACCACATCAATAAT
	C6	125	AGTCCGATACGCGATGTAATTTATCTCACGGAAATAGGTACGTTTATCGCGCGGCGCATCAATCAGGCTTTCTCGCGGGATGCCAGTTCCGTCAAATATCGCGATGAATCTCACTTTGCAGATTC
	HF183	132	GGGATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGGTC

Table 1: Sequences for gBlocks gene fragments for S. Typhi gene targets and HF183.

1.1 IDT's gBlocks must be a minimum of 125 bases long. Other brands may have different length requirements.

Resuspending and diluting the gBlocks

2 gBlocks are supplied as a lyophilised pellet and must be resuspended. To ensure the best recovery of product, the following steps are recommended from the manufacturer: Link: (**Tips for working with IDT Gene Fragments**)

2.1 For this protocol, a solution of [M] 10 ng/µL is required.

Information on the ng provided lyophilised, 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:
Link: (**IDT Resuspension Calculator**)
Link: (**IDT Oligo Analyzer**)

2.2 Centrifuge the Lyophilised gBlock for 3-5 seconds before opening

2.3 Resuspend in molecular-grade nuclease-free water or 1x TE Buffer to reach the concentration of [M] 10 ng/µL

- Example, if the gBlock states 250 ng, add 25 µL of solvent

2.4 Vortex for 3-5 seconds

2.5 Incubate at 50 °C for 15-20 minutes

2.6 Vortex for 3-5 seconds and then centrifuge for 3-5 seconds, allowing to cool before opening the tube.

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

3.1 If possible, dilute to [M] 10 ng/µL for storage; if below this already, normalise to [M] 1 ng/µL, the first concentration for the serial dilution below.

- Note - gBlocks stored at a concentration of [M] <1 ng/µL, loss of material may occur over time due to adherence to the plastic tube
- To minimise loss, add tRNA or Poly A to storage stock, at a final concentration of [M] 0.1 mg/mL to [M] 1 mg/mL in the dilution.

Note - gBlock stored at a concentration of

4 Create serial dilutions of your stock solution adding 2 µL of stock into 18 µL of nuclease-free water. We recommend carrying out 12 dilutions to create a series of 12 concentrations starting at [M] 1 ng/µL.

- Each dilution will have a final volume of 18 µL, this is sufficient for 3 replicates with some room for pipetting error.
- We recommend at least 10 replicates split over at least two days. The more replicates, the more accurate the Probit calculation will be for Ct value cut-offs.
- Aliquot the dilution stocks into usable quantities, so they may be stored for use without needing to freeze-thaw larger volumes.

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.

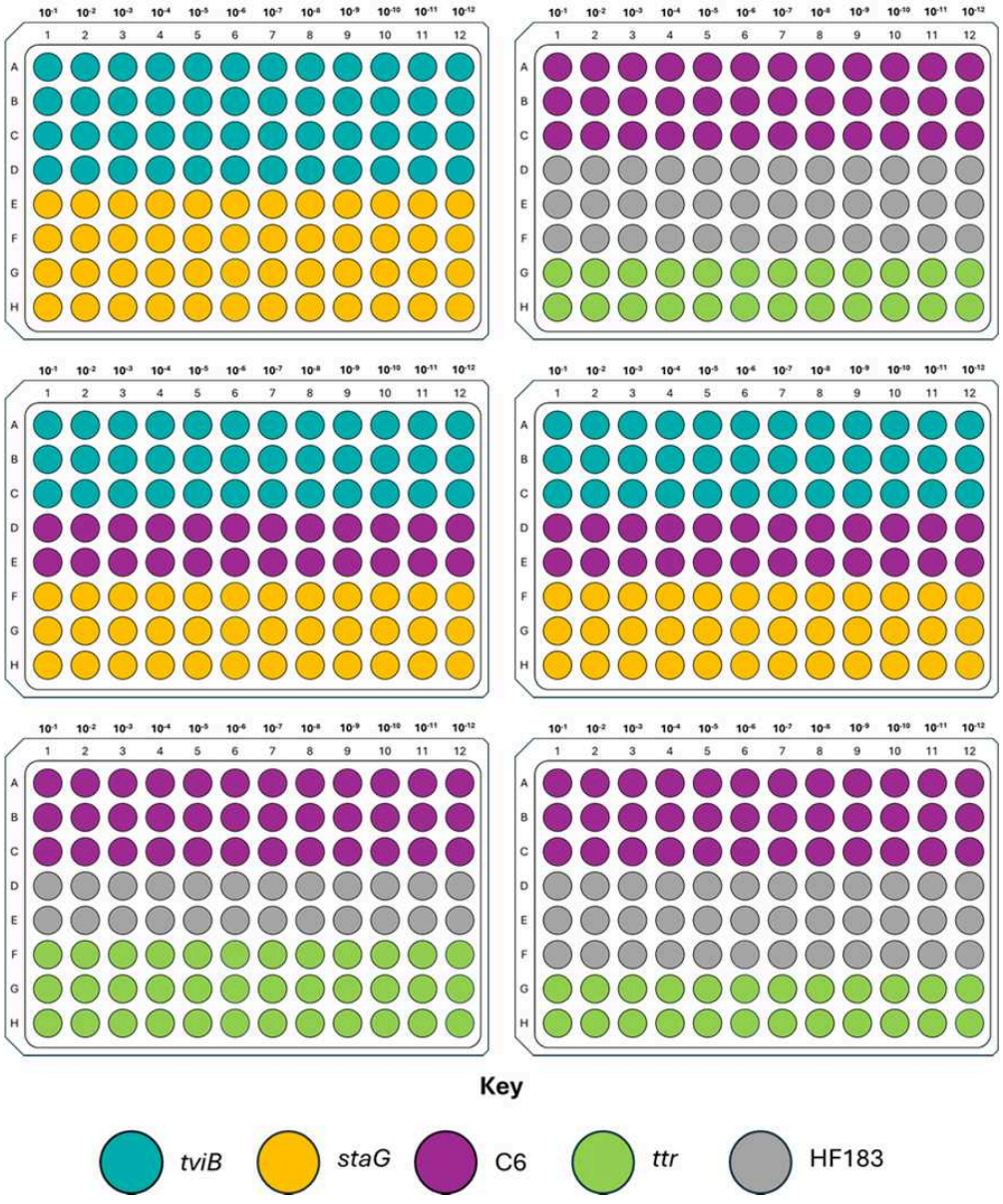

Figure 1: **Example** plate map for evaluation qPCR set up for Ct Value Cut-Off calculations.
Note: in this configuration ttr *and HF183 will only have 9 replicates.*

qPCR and generating a standard curve

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

	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 2: Mastermix composition for the S. Typhi Triplex qPCR

	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
	HF183_F	0.5	0.4

	A	B	C
	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 3: Mastermix composition for the Control Triplex qPCR

6 Aliquot 20 µL of master mix for each reaction in a 96-well plate. Add 5 µL 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/µL ([M] - GC/µL) 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: FILE LOD calculation Version 3.xlsm 35.4KB). 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/µL from the qPCR Ct values of actual samples you can use the equation:

log GC/µL = (Ct – intercept) / slope

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