

Protocol: Real-time PCR for verotoxin-producing bacteria

A. Introduction

This protocol uses real-time PCR with BHQ_{plus}™ probes to test for verotoxin-producing bacteria, including verotoxin-producing *Escherichia coli* (VTEC), as well as *Shigella dysenteriae*. The assay targets two verotoxin genes, VT1 and VT2, which are major pathogenicity factors for both VTEC and *Shigella*.

Approximate real-time PCR run time is 35 min.

B. Equipment

- Spartan DX™ instrument
- Microcentrifuge
- Vortex
- Ice bucket or cold block
- Pipettes

C. Materials

- 0.2 ml flat-cap PCR tubes (Fisher Scientific, Cat. No. 08-408-214)
- Filtered pipette tips
- PCR-grade Mineral Oil (Biotools, Cat. No. 20.032)
- QUANTIPROBES Reaction Mix (Biotools, Cat. No. 10.601)
- Sterile water (DNase-free)
- 1.5 ml microcentrifuge tubes
- Primers that recognize VT1 and VT2 (Integrated DNA Technologies)(Table 1)
- BHQ_{plus} probes (Biosearch Technologies) that recognize VT1 and VT2 (Table 1)

Primer/Probe	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
VT1 primers	ATg CAT CgC gAg TTg CCA gA	TTg CCC CCA gAg Tgg ATg AA	120
VT1 probe	6FAM-TTC CAT CTg CCg gAC A-BHQ _{plus}		
VT2 primers	ggg gAC CAC ATC ggT gTC Tg	gAT ggT CAA AAC gCg CCT gA	101
VT2 probe	6FAM-CAA AAT AAC TgC CCg gTg gg-BHQ _{plus}		

6-FAM = 6-carboxy-fluorescein, BHQ_{plus} = Black Hole Quencher *plus*

Table 1. Primer/probe sequences and amplicon sizes.

D. Preparation

1. Any commercially-available DNA purification kit may be used to extract the bacterial DNA .
2. Turn on Spartan DX instrument and let it warm up for a minimum of 10 min
3. Set up thermal cycling program as per Table 2

Step	Temperature	Time	Cycles
Initial denaturation	95.0°C	10 s	1
Denaturation	95.0°C	25 s	45
Annealing/extension	50.0°C	20 s	45

Table 2. Cycling parameters.

E. Protocol

1. Prepare a master mix for VT1/VT2 as per Table 3
2. Since the Spartan DX instrument holds 4 tubes, we recommend setting up reactions as follows:
 - Tube 1: 18 µl of VT1 master mix
 - Tube 2: 18 µl of VT2 master mix
 - Tube 3: 18 µl of VT1 master mix
 - Tube 4: 18 µl of VT2 master mix
3. In a separate lab area, add the following to each tube:
 - Tube 1: 2 µl of purified DNA (sample in question)
 - Tube 2: 2 µl of purified DNA (sample in question)
 - Tube 3: 2 µl of sterile water (VT1 negative control)
 - Tube 4: 2 µl of sterile water (VT2 negative control)
4. Mix and spin down reactions
5. Overlay reaction mixture with 15 µl of mineral oil
6. Spin down reaction tubes
7. Insert samples into Spartan DX instrument and start your run
8. Example of real-time graph is depicted in Figure 1

Reagent	VT1 Master Mix		VT2 Master Mix	
	Reaction Formulation	Volume	Reaction Formulation	Volume
QUANTIPROBES Reaction Mix (2X)	10 µl x (____+0.5*) Sample #		10 µl x (____+0.5*) Sample #	
Forward primer (10 µM)	1 µl x (____+0.5*) Sample #		2 µl x (____+0.5*) Sample #	
Reverse primer (10 µM)	1 µl x (____+0.5*) Sample #		2 µl x (____+0.5*) Sample #	
Probe (1 µM)	2 µl x (____+0.5*) Sample #		2 µl x (____+0.5*) Sample #	
Sterile water	4 µl x (____+0.5*) Sample #		4 µl x (____+0.5*) Sample #	
Total volume of master mix	18 µl/reaction	____ µl	18 µl/reaction	____ µl

* Recommended volume correction factor for pipetting error.

Table 3. Components of real-time PCR master mix for VT1 and VT2.

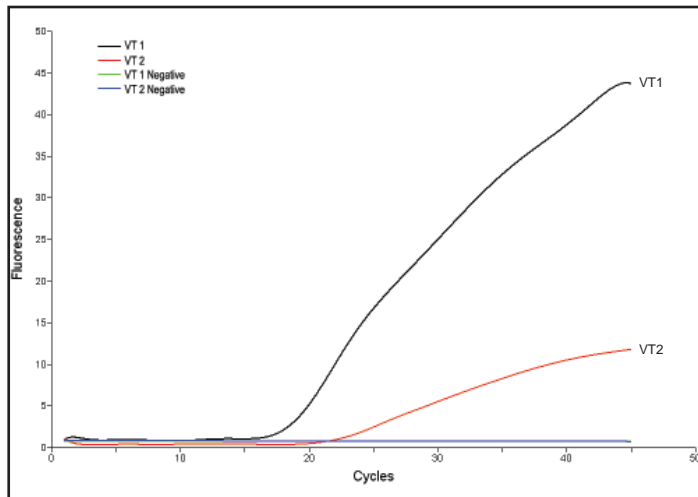


Figure 1. Real-time PCR result for verotoxin-producing *E. coli* (VTEC)

Disclaimer

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