

Protocol: Real-time PCR for VRE from bacterial colonies

A. Introduction

This protocol uses real-time PCR with BHQ_{plus}™ probes for Vancomycin-resistant *Enterococci* (VRE). This assay identifies target sequences from the *Van A*, *Van B*, and *Van C* genes for Vancomycin resistance. *Van A* and *Van B* (*Van B1* and *Van B2/3* variants) are detected using one probe each. For *Van C*, the *Van C1*, *Van C2*, and *Van C3* variants are detected using two probes.

Approximate time for DNA extraction from bacterial colonies is 10 min. Real-time PCR run time is 70 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)
- Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Cat. No. 11730-017)
- Sterile water (DNase-free)
- 1.5 ml microcentrifuge tubes
- Primers which recognize *Van A*, *Van B*, and *Van C* genes (Biosearch Technologies) (Table 1)
- BHQ_{plus} probes for *Van A*, *Van B*, and *Van C* genes (Biosearch Technologies) (Table 1)

Primer/Probe	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
<i>Van A</i> primers	TAT gAT ggC CgC TgC Agg TA	Cgg TgA AAT TAT CCC AAg Tgg C	163
<i>Van A</i> probe	6-FAM-TgC ACT TCC CgA ACT g-BHQ _{plus}		
<i>Van B</i> primers	gCC ATg CAA AAC Cgg gAA Ag	CAA gCg ATT TCg ggC TgT gA	192
<i>Van B</i> probe	6-FAM-TgA gCC ACg gTA TCT TC-BHQ _{plus}		
<i>Van C</i> primers	ggg AAg ATg gCA gTA TCC AAg g	gCT TgA TgC AgC AgC CAT TT	109
<i>Van C</i> probe 1	6-FAM-CCT TAT gTT ggT TgC CA-BHQ1 _{plus}		
<i>Van C</i> probe 2	6-FAM-TgC CTT ATg TAg gCT gC-BHQ1 _{plus}		

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

Table 1. Primer/probe sequences and amplicon sizes.

D. Preparation

1. With a sterile transfer device, pick 4 bacterial colonies from a culture plate
2. Resuspend the colonies in 200 µl of sterile water
3. Boil samples for 10 min
4. Turn on Spartan DX instrument and let it warm up for a minimum of 10 min
5. Set up cycling program as per Table 2

Step	Temperature	Time	Cycles
Initial denaturation	95°C	10 s	1
Denaturation	95°C	30 s	50
Annealing/extension	50°C	55 s	50

Table 2. Cycling parameters.

E. Protocol

1. Prepare separate *Van A*, *Van B*, and *Van C* master mixes, as per Tables 3 and 4
2. Since the Spartan DX instrument holds 4 tubes, we recommend setting up reactions as follows:
 - Tube 1: 18 µl of *Van A* master mix
 - Tube 2: 18 µl of *Van B* master mix
 - Tube 3: 18 µl of *Van C* master mix
 - Tube 4: 18 µl of *Van A* master mix
3. In a separate lab area, add the following to each tube:
 - Tube 1: 2 µl of extracted DNA (sample in question)
 - Tube 2: 2 µl of extracted DNA (sample in question)
 - Tube 3: 2 µl of extracted DNA (sample in question)
 - Tube 4: 2 µl of sterile water (*Van A* negative control)
4. Mix and spin down reaction tubes
5. Overlay reaction mixtures with 15 µl of mineral oil
6. Spin down reaction tubes
7. Insert tubes into Spartan DX instrument and start your run
8. Representative real-time PCR results are shown in Figures 1, 2 and 3.

Reagent	Van A Master Mix		Van B Master Mix	
	Reaction Formulation	Volume	Reaction Formulation	Volume
Platinum Quantitative PCR Super-Mix-UDG Reaction Mix (2X)	10 µl x (____+0.5*) Sample #		10 µl x (____+0.5*) Sample #	
Forward primer (10 µM)	2 µl x (____+0.5*) Sample #		2 µl x (____+0.5*) Sample #	
Reverse primer (10 µM)	2 µl x (____+0.5*) Sample #		2 µl x (____+0.5*) Sample #	
Probe (1 µM)	0.2 µl x (____+0.5*) Sample #		0.2 µl x (____+0.5*) Sample #	
Sterile water	3.8 µl x (____+0.5*) Sample #		3.8 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 mixes for *Van A* and *Van B*.

Reagent	Van C Master Mix	
	Reaction Formulation	Volume
Platinum Quantitative PCR Super-Mix-UDG Reaction Mix (2X)	10 µl x (____+0.5*) Sample #	
Forward primer (10 µM)	2 µl x (____+0.5*) Sample #	
Reverse primer (10 µM)	2 µl x (____+0.5*) Sample #	
Probes 1 & 2 (1 µM/each)	0.4 µl x (____+0.5*) Sample #	
Sterile water	3.2 µl x (____+0.5*) Sample #	
Total volume of master mix	18 µl/reaction	____ µl

* Recommended volume correction factor for pipetting error.

Table 4. Components of real-time PCR master mix for *Van C*.

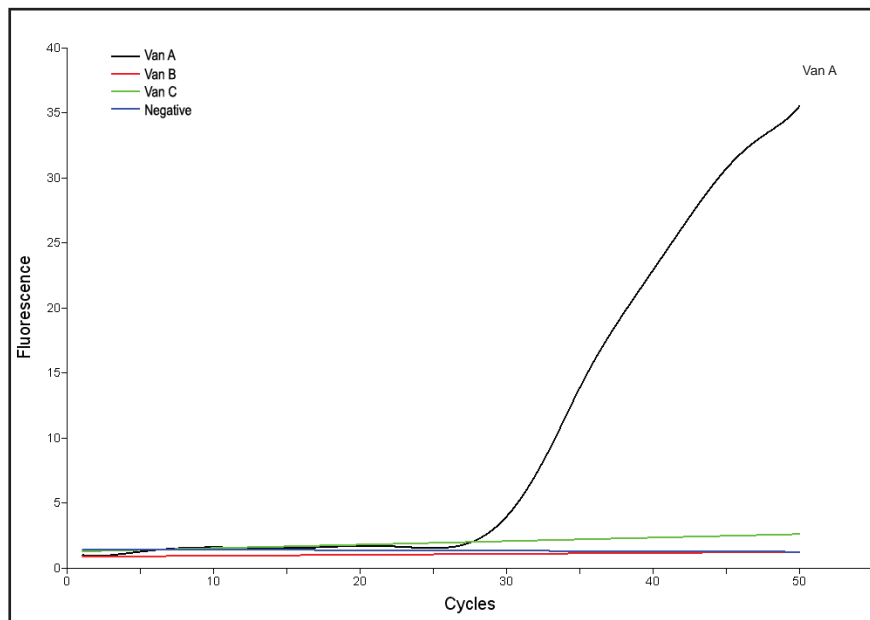


Figure 1. Real-time graph of *Van A* positive sample.

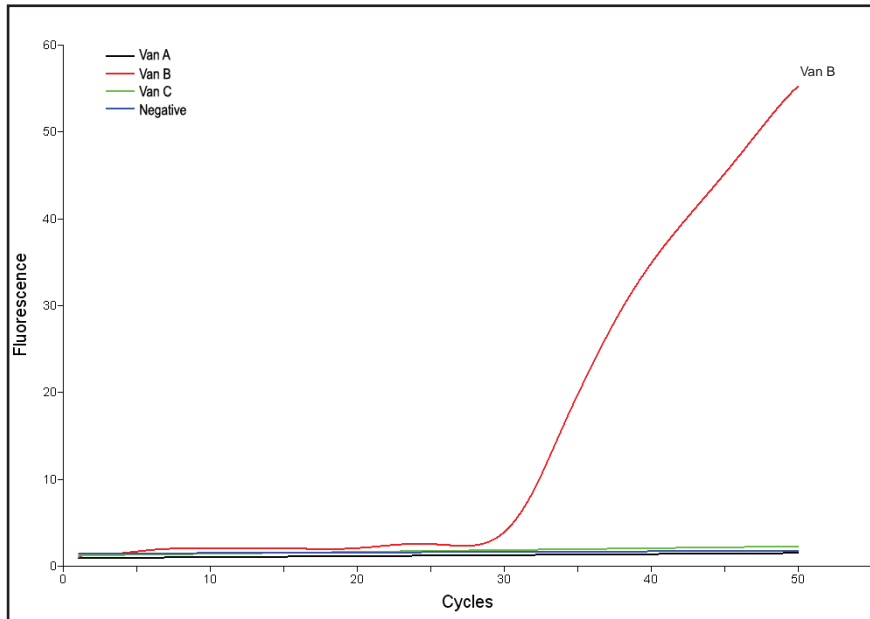


Figure 2. Real-time graph of *Van B* positive sample.

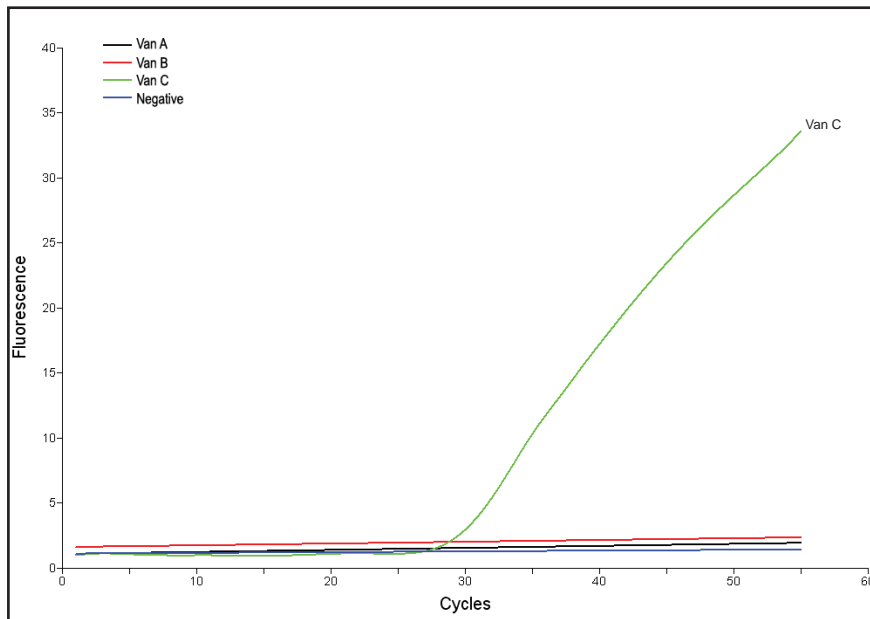


Figure 3. Real-time graph of *Van C* positive sample.

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