ViralSEQ™ Vesivirus Real-Time PCR Detection Kit

The ViralSEQ™ Vesivirus Real-Time PCR Detection Kit is a TaqMan®-based Real-Time PCR kit for highly sensitive detection of Vesivirus in cell culture samples. The kit contains TaqMan probe and primer mix, TaqMan Environmental Master Mix 2.0, negative control, and a positive control.

Procedure to set RT-PCR reactions

Prepare the sample

Refer to the *PrepSEQ Mycoplasma Nucleic Acid Extraction Kit Protocol* (PN 4401253) for details on sample preparation.

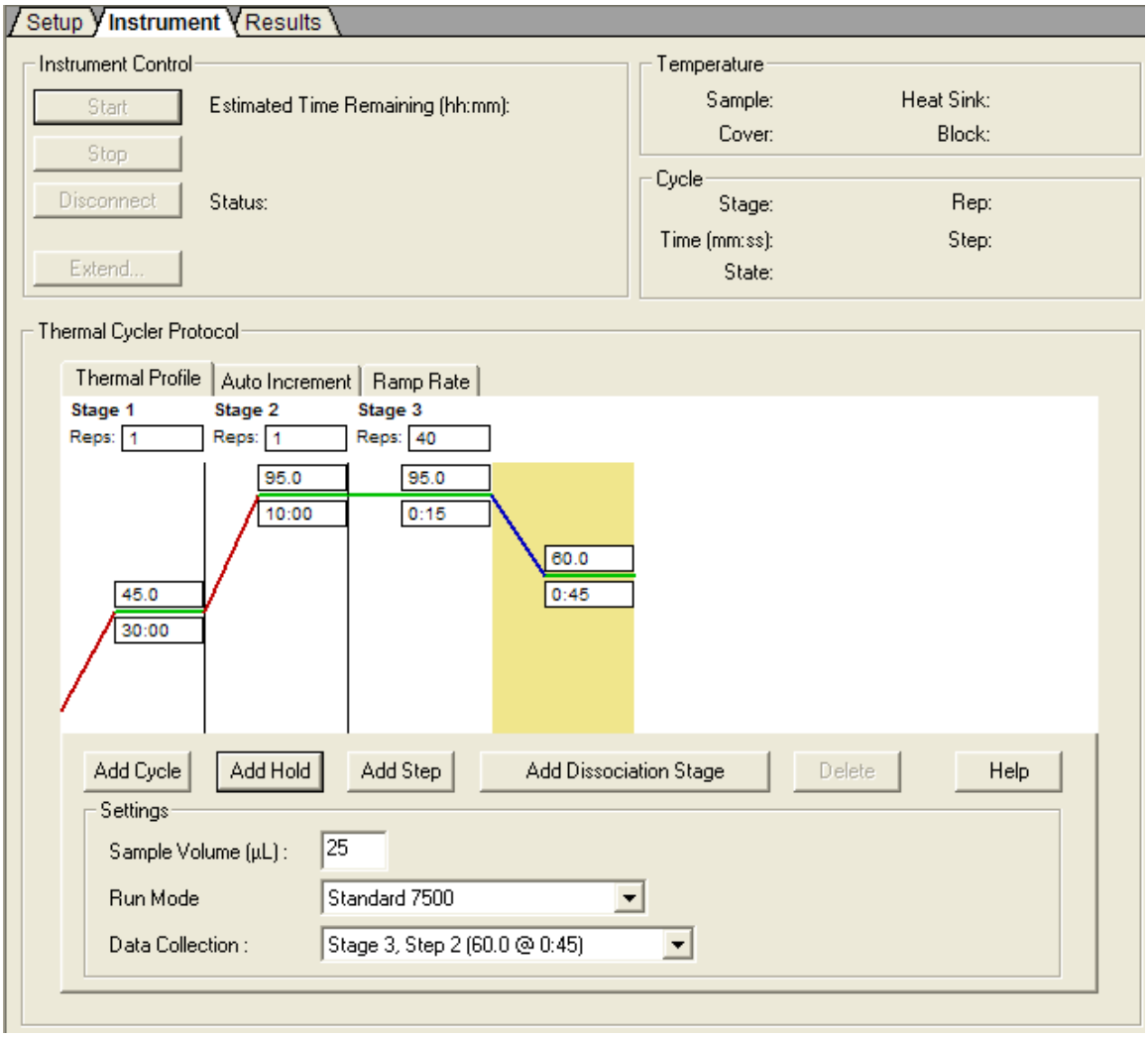
Prepare for PCR

Prepare the plate document

1. During setup of the plate document, in the Assay drop‑down list, select **Absolute Quantification**.
2. Select FAM™, VIC®, and NED™ detectors with:
   - Quencher Dye set to (**none**) or (**Non Fluorescent**)
   - Passive Reference set to **ROX™**
3. Set thermal‑cycling conditions as shown in the table and figure below. For details, refer to the *7300/7500/7500 Fast Real‑Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* (PN 4347825) or the *7900HT Fast Real‑Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* (PN 4364014).

Step	Reverse Transcription	AmpliTaq Gold® enzyme activation	PCR	
	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/extend
Temp	45 °C	95 °C	95 °C	60 °C
Time	30 min	1o min	15 sec	45 sec

- Set Sample Volume to **25 µL**.
- For the 7500 Fast system, set Run Mode to **Standard 7500**.
- Set Data Collection to **Stage 3, Step 2 (60.0 @ 0:45)**.



Prepare the kit reagents and premix solution

1. Thaw all kit reagents completely at 4 °C.
2. Vortex reagents (except for the 25x RT-PCR enzyme mix). Spin down all reagents.
3. Label a microcentrifuge tube for the premix solution, and for each sample and control reaction.
4. Prepare the Premix Solution according to the following table.

ⓘ IMPORTANT! Use a separate pipet tip for each component.

Component for premix solution	Volume for one 25-µL reaction (µL)	Volume for four 25-µL reactions (µL)†
2x RT-PCR Buffer	12.5	55.0
10x Assay mix	2.5	11.0
25x RT-PCR Enzyme Mix	1.0	4.4
Total Premix Solution Volume	16.0	70.4

† Includes 10% excess to compensate for pipetting errors.

5. Mix the Premix Solution by gently pipetting up and down, then cap the tube.

Prepare the RT-PCR reactions

Into the wells of a reaction plate, pipet the reagent volume specified in the following table.

To prepare…	Combine in each well
Negative-control reaction	• 16 µL of Premix Solution • 9 µL of Negative Control (water)
Your unknown sample reaction	• 16 µL of Premix Solution • 2 - 9 µL of unknown sample (To increase detection sensitivity, use 9 µL of sample) • Adjust the final reaction volume to 25 µL with Negative Control (water)
Positive-control reaction	• 16 µL of Premix Solution • 2 µL of Vesivirus RNA Positive Control • 7 µL of Negative Control (water)

1. Dispense 16 µL of Premix Solution into each well to be used, gently pipetting at the bottom of the well. For the:
• 7500 Fast system – Dispense into a Fast optical 96‑well plate (PN 4346906).
• 7500 and 7900HT Fast (standard block) systems – Dispense into a standard optical 96‑well plate (PN 4306737).
• 7900HT Fast system (Fast block) – Dispense into a Fast optical 96‑well plate (PN 4346906).

2. For each row of wells that you use, place in sequence from left to right the negative control, unknown sample, then positive control.

ⓘ IMPORTANT! Use at least one negative and one positive control per run.

ⓘ IMPORTANT! Mix each sample very gently by placing the pipet tip at the bottom of the tube and pipetting up and down to minimize aerosol formation and cross‑contamination.

ⓘ IMPORTANT! Use a new tip for each well, even when aliquoting the same solution.

Perform PCR

On an Applied Biosystems Real‑Time PCR System:
1. Open the plate document that corresponds to the reaction plate you created.
2. Load the reaction plate into the real‑time PCR system.
3. Start the run.

Analyze the results

For instructions on how to analyze your results, refer to the user guide of your real-time PCR instrument.

• View the amplification plots for the entire plate.

• Set the baseline and threshold values. For all reactions, use default Analysis Settings:
  – Select **Manual Baseline**.
  – Set Start (cycle) to **3**.
  – Set End (cycle) to **15**.
  – Set Threshold to **0.2**.
• Examine each sample for FAM™ dye (Vesivirus probe), VIC® dye (dual - purpose discriminatory positive
control probe), and NED™ dye (IPC).

• Use the following table as a basic guide for evaluating the results.

Result interpretation

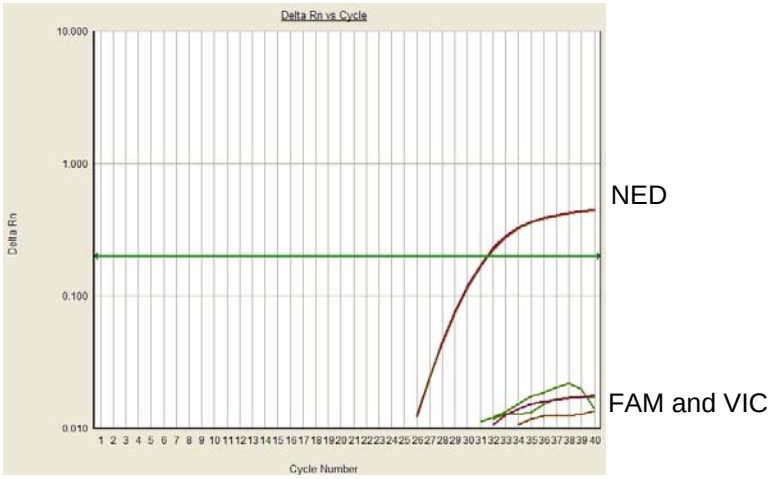
1. It is very important to set the Threshold at 0.2.

2. Vesivirus target is detected by FAM dye. Internal positive control (IPC) is detected by NED. The positive RNA control is detected by both FAM and VIC dye. (see 3 for examples of results)

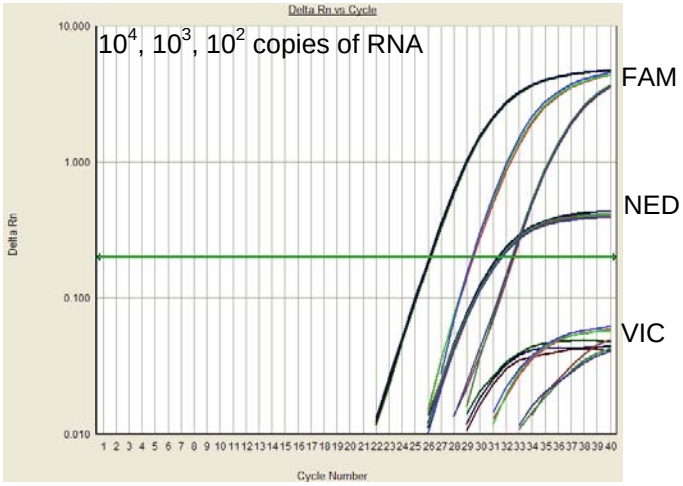
- NED should appear in every reaction. If the unknown sample has negative FAM, and the NED C_T (IPC) from unknown sample is higher than NED C_T of NTC reaction by 2, it is likely that the unknown reaction contains PCR inhibitor. Repeat sample preparation of the sample and RT-PCR reaction.
- VIC signal should appear only in the positive reaction. Appearance of VIC signal in the unknown sample or negative control reaction means contamination of RNA positive control. Reactions should be repeated.

3. Example of results
   Note: C_T values may vary depending on assay lots, operator, different experiments and PCR instrument.

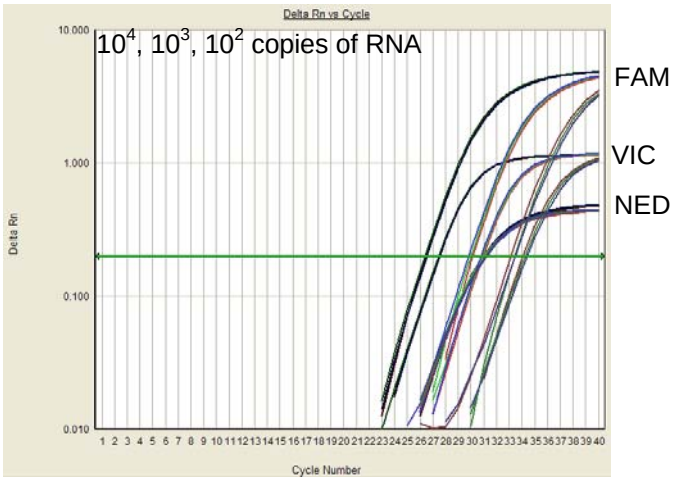
- Negative control reaction



- Positive Vesivirus RNA (FAM and NED have signals and VIC is negative)



- Vesivirus RNA positive control (FAM, NED, and VIC all positive)



4. Interpretation guides

Results				
Sample Type	**FAM Signal[1]**	**VIC Signal[2]**	**NED (IPC) Signal**	**Call**
Unknown	Ct ≤ 36	undetermined	Ct ≤ 32 to 34	Positive
Unknown	Ct ≥ 36	undetermined	Ct ≤ 32 to 34	Low level positive[3]
Unknown	undetermined	undetermined	Ct ≤ 32 to 34	Negative
Unknown	Ct ≤ 36	Ct ≤ 39	Ct ≤ 32 to 34	Invalid due to contamination[4]
Unknown	Ct ≥ 36	Ct ≤ 39	Ct ≤ 32 to 34	Invalid due to contamination[4]
Positive Control (1,000 copies/Rxn)	Ct ≤ 30	Ct ≤ 32	Ct ≤ 32 to 34	Positive

Negative Control	undetermined	undetermined	Ct ≤ 32 to 34	Negative
Negative Control	Ct ≤ 36	Ct ≤ 37	Ct ≤ 32 to 34	Invalid due to contamination[4]

1. The average FAM Ct for 10 copies of Vesivirus RNA is ~37.
2. The average VIC Ct for 10 copies of Vesivirus Positive Control is ~38
3. Samples resulting in a "Low Level Positive" call can be confirmed by repeating the sample preparation, using an elution volume of 30 - 50 µL, and repeating the assay. Or repeat sample preparation after one-more day of cell culture.
4. Reactions resulting in an "Invalid due to contamination" call should be confirmed by either repeating PCR reaction or sample preparation followed by a new PCR reaction.

5. Cross-talk between FAM and VIC

Cross-talk between FAM and VIC will give a false VIC signals and result in a wrong call of contamination of RNA positive control in the reaction. When cross-talk happens, parallel FAM and VIC curves are seen as shown in the following figure. After testing several instrument, it was found that VIC signal due to cross-talk with FAM never across the threshold of 0.2, if the instrument is properly calibrated.

Important! Make sure that the instrument is properly calibrated and select threshold to 0.2 before doing data analysis.