Enhanced sensitivity detection of replication competent lentivirus by qPCR

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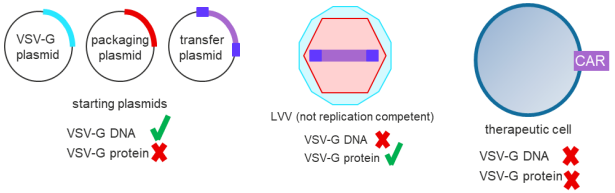
Introduction

Chimeric antigen receptor T (CAR T) cells require a retrovirus or lentivirus to introduce the chimeric antigen receptor gene into the activated T cells. A safety concern with using a lentivirus for this treatment is the potential for replication competent lentiviruses (RCL) to arise. Recent efforts in gene therapy have improved the lentiviral vector design to reduce the likelihood of generating RCLs during the manufacture of the CAR T cells by using a split plasmid design, where the vector genome, envelope, and packaging components are on separate plasmids. In addition, these lentiviral vectors have been modified by removing genes encoding essential regulatory functions. The FDA has posed safety concerns with the possibility of RCLs being created and requires monitoring of both CAR T products as well as patients undergoing therapy. We have developed a robust and sensitive qPCR test targeting the Indiana vesiculovirus G glycoprotein (VSV-G) suitable for detection of RCL in DNA isolated from peripheral blood collected from patients enrolled in relevant clinical trials.

VSV-G use in Lentiviral Vector Construction

Figure 1. CAR-T Production using LVV



- Multiple plasmids encoding separate pieces of the viral vector are co-transfected into packaging cells to generate LVV
- Separating viral genes onto 3+ plasmids decreases the risk of generating RCL
- Functional LVV contain VSV-G protein on the virion surface but should not contain any VSV-G DNA
- Similarly therapeutic cell or clinical drug product (CDP) should not contain VSV-G DNA

Method and Test Overview

The RCL Test is a qualitative real-time polymerase chain reaction test that provides sensitive and specific detection of VSV-G sequence in a matrix containing human genomic DNA on the ABI 7500 Fast Dx instrument. The RCL test is composed of two component assays in simplex, a VSV-G FAM assay and a Ribonuclease P RNA Component H1 (RPPH1) VIC assay to assess sample quality, each in triplicate wells. Positive and negative controls are analyzed for both VSV-G and RPPH1 in every run simultaneously with patient samples.

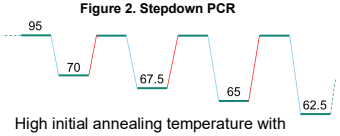
VSV-G negative peripheral blood (PB) was collected in EDTA sample tubes and extracted using the Qiagen QiaAMP DNA Midi Kit for the limit of blank study. Commercial plasmid containing the VSV-G gene (Sigma-Aldrich) was our source of target sequence in contrived validation samples and the positive control. Plasmid was linearized with HindIII (NEB) and quantified by Nanodrop 2000C (ThermoFisher) using the plasmid's molecular weight. In validation all samples and controls were tested at a standard input of 200 ng DNA per well (1.2 µg total). Commercial mixed human genomic DNA (Promega) was our source of VSV-G negative DNA for sample and control creation.

Stepdown PCR Reduces Spurious Mispriming

Table 1. Thermal Profile

Step	Temperature	Time	Cycles
Initial Denature	95°C	10 min	1
Denature	95°C	15 sec	4
Anneal/ Extend	70°C -> 62.5°C	1 min	
Denature	95°C	15 sec	36
Anneal/ Extend	60°C	1 min	

Figure 2. Stepdown PCR



High initial annealing temperature with incremental temperature decreases confers increased PCR stringency

Limit of Blank

DNA from 60 healthy donors was extracted from EDTA whole blood and run on the assay at the standard input (200 ng DNA per well, 1.2 µg total). No VSV-G signal was detected in any well in any of the 60 samples (**Figure 3**, **Table 2**). The limit of blank was determined to be zero. The average Ct for the RPPH1 reference assay was 24.33 (**Figure 4**, **Table 2**).

Figure 3. VSV-G Assay

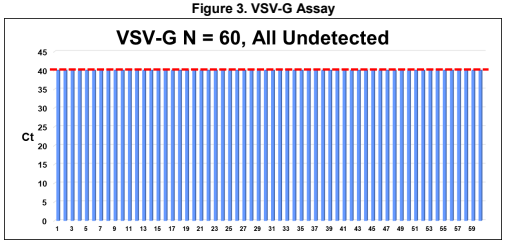


Figure 4. RPPH1 Assay

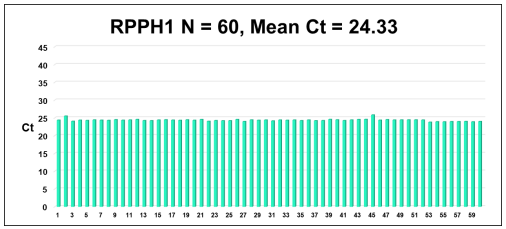


Table 2. Summary of LoB results for 200ng/well input

	VSV-G Ct	RPPH1 Ct
Min	Not Detected	23.78
Max	Not Detected	25.82
Average	Not Detected	24.33
SD	NA	0.32

Linearity/Inter-operator Precision

Samples spanning 5.4 log10s of VSV-G linearized plasmid in a matrix of 200 ng/well genomic DNA were evaluated on 4 replicate plates by 2 operators. VSV-G input was determined by Nanodrop 2000C using the plasmid's molecular weight. The VSV-G assay was shown to be linear across this input range with an R^2 of 1.00 and an average PCR efficiency of 98.7% (**Figure 5**). The data were highly concordant between the four runs demonstrating high precision (**Table 3**).

Figure 5. VSV-G PCR efficiency

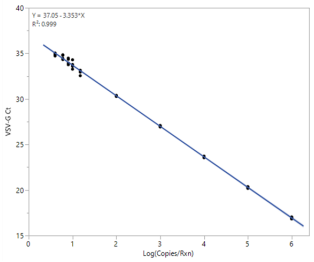


Table 3. Summary of linearity results for 200 ng/well input

Sample Name	VSV-G Copies/Rxn	VSV-G Ct	SD	RPPH1 Ct	SD
Level 1	1000000	16.92	0.09	23.72	0.21
Level 2	100000	20.28	0.07	23.79	0.20
Level 3	10000	23.66	0.08	23.75	0.16
Level 4	1000	27.03	0.07	23.75	0.15
Level 5	100	30.32	0.04	23.75	0.20
Level 6	15	32.95	0.25	23.91	0.16
Level 7	10	33.74	0.37	23.78	0.23
Level 8	8	34.13	0.29	23.80	0.18
Level 9	6	34.60	0.22	23.76	0.21
Level 10	4	34.87	0.12	23.73	0.17

Limit of Detection

20 replicates of each of the three lowest input levels from the linearity study were analyzed to determine the assay LoD. The limit of detection was experimentally determined to be less than 4 copies per reaction.

Table 4. Summary of LoD results for 200ng/well input

		VSV-G (FAM)			RPPH1 (VIC)		
Sample Name	Copies/Rxn	% Detected	Mean Ct	SD	% Detected	Mean Ct	SD
Level 8	8	100	34.08	0.40	100	23.85	0.19
Level 9*	6	100*	34.55	0.58	100	23.86	0.20
Level 10	4	100	34.91	0.41	100	23.81	0.22

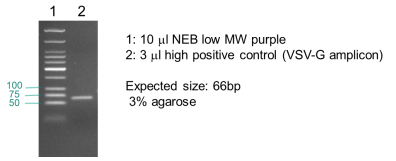
*sample was detected in two of three wells in one instance and Ct for undetected well was set to 40 for calculations

Samples with an average VSV-G CT > 34.9 are considered negative. Samples with an average VSV-G CT ≤ 34.9 are considered positive.

Verification of PCR product

3 µL from a single well (20 µL reaction volume) for a single positive contrived sample (linearized plasmid in genomic DNA at standard input) was loaded onto a 3% agarose gel. The assay produces a single band of the expected size (**Figure 6**).

Figure 6. DNA Gel Analysis



The VSV-G assay PCR product was TOPO cloned and submitted for bidirectional Sanger sequencing. The results were 100% concordant with the expected sequence (**Table 5**).

Table 5. Summary of Sanger Sequencing Results

Sample	Assay	# of Bases	# of Mismatches	Concordance
High Positive Control	VSV-G	66	0	100%

Overall Accuracy

Zero false postives and zero false negatives were detected during validation demonstrating excellent specificity and sensitivity. Samples and controls containing VSV-G linearized plasmid at any input were categorized as predicted positive. Samples and controls without VSV-G plasmid were categorized as predicted negative.

Table 6. Overall Accuracy Summary

		Actual	
		Positive	Negative
Predicted	Positive	True Positive 100% (n=122)	False Positive 0%
	Negative	False Negative 0%	True Negative 100% (n=70)

Assay Summary

We have developed a qualitative RCL qPCR assay with an LoD of <4 copies per reaction and a 0% false positive rate in EDTA whole blood and contrived samples. The assay is specific to the target sequence and has an LoB of 0. The assay meets or exceeds all validation acceptance criteria. This highly sensitive, accurate, and precise test is critical for monitoring patients undergoing treatment with LVV based CAR-T cells that employ the VSV-G envelope in the vector.