Title: Quantitative PCR (qPCR) Method for Detection of Replication Competent Lentivirus

SOP Number: 22209

Revision Number: 00

Supersedes: Revision N/A

Effective Date: **MAY 24 2019**

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

Table of Contents

1.0 Purpose

This SOP describes the materials and methods needed to detect replication-competent lentivirus (RCL) in a sample by quantitative PCR (qPCR).

2.0 Scope

This method relies upon the ability of the primer/probe sequences to bind to VSV-G sequence that would be present if a replication-competentlentivirus were present in a sample. **If** the VSV-G plasmid used in the generation of the lentivirus vector possesses a VSV-G sequence that differs from the primer/probe sequences used in this method, this SOP would not be appropriate for detecting replication competent lentivirus in that sample.

3.0 Background

Lentiviral vectors are engineered to be replication defective, however, replication competent lentiviruses could be generated through recombination events among the plasmids used in the lentivirus vector production. One of these plasmids used in lentivirus vector production is the envelope plasmid encoding VSV-G which is used for virus pseudotyping. The replication competent lentivirus qPCR assay targets the VSV-G sequence to determine whether replication competent lentivirus was generated.

4.0 Authority and Responsibility

4.1 The Director, Process Analytics/Quality Control (PA/QC), has the authority to define this procedure.

4.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA)

4.3 PA/QC personnel are responsible for the performance of this procedure.

4.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

4.5 BQA is responsible for quality oversight of this operation.

5.0 Equipment, Materials and Reagents

5.1 Clinical Center VSVG Forward Primer (BDP PN 31162)

Sequence: 5’ – TGCAAGGAAAGCATTGAACAA – 3’

5.2 Clinical Center VSVG Reverse Primer (BDP PN 31163)

Sequence: 5’ – GAGGAGTCACCTGGACAATCACT – 3’

5.3 Clinical Center VSVG Probe (BDP PN 31164)

Sequence: 5’ – FAM-AGGAACTTGGCTGAATCCAGGCTTCC-QSY – 3’

5.4 TaqMan Universal PCR Master Mix (2X) (BDP PN 30268)

5.5 MicroAmp Optical 96 Well Reaction Plate (BDP PN 21141 or BDP-approved equivalent)

5.6 MicroAmp Optical Adhesive Film (BDP PN 21142 or BDP-approved equivalent)

5.7 Distilled Water, DNase Free RNase Free (BDP PN 10189 or BDP-approved equivalent)

5.8 1X TE (BDP PN 30267 or BDP-approved equivalent)

5.9 1X PBS (BDP PN 30007 or BDP-approved equivalent)

5.10 VSV-G DNA Control (BDP PN 31238)

5.11 qPCR Instrument – QuantStudio 6, SDS 7900HT or BDP-approved equivalent

6.0 Procedure

6.1 Extract genomic DNA from each sample as described in **SOP 22212 - Purification of DNA Using the DNeasy Blood and Tissue Kit** or **SOP 22972 - Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions**.

6.1.1 Perform two or more replicate extractions for each transduced test sample.

6.1.2 If available, a non-transduced sample should be extracted in parallel with the transduced samples.

6.1.3 An Extraction Negative Control should be performed in which 200 µL of 1X PBS are extracted in parallel with the test samples.

6.1.4 An Extraction Positive Control should be performed in which 20 µL of VSV-G DNA Control at 1.00E+03 copies/µL is added to 180 µL of buffer blank or 1X PBS.

NOTE: The Extraction Positive Control should not be centrifuged prior to the addition of Proteinase K and Buffer AL.

6.2 Quantify the amount of DNA in each test sample using the NanoDrop ND1000 spectrophotometer according to **SOP 22946 - The Operation of the NanoDrop ND1000 Spectrophotometer.**

NOTE: It is not necessary to perform a spectrophotometry analysis of the Extraction Negative Control and Extraction Positive Control.

6.3 Dilute an aliquot of each sample to 8 ng/µL with 1X TE in a total volume of 100 µL (total DNA is 800 ng).

6.4 Replication Competent Lentivirus qPCR Master Mix Preparation

6.4.1 Record all reagent part numbers, lot numbers and expiration dates as well as any additional comments on **Form 22209-01.**

6.4.2 Vortex the reagents.

6.4.3 Prepare a qPCR master mix as described below:

Reagent	Volume for 1 Reaction	Final Concentration
TaqMan Universal PCR Master Mix (2X)	25.0	**1X**
Clinical Center VSVG Forward Primer (10 **µM)**	1.0	200 nM
Clinical Center VSVG Reverse Primer (10 µM)	1.0	200 nM
Clinical Center VSVG Probe **(FAM/QSY)** (10 µM)	0.5	100 nM
Water	10	**N/A**

6.4.4 Prepare a sufficient volume of master mix to account for all test samples, standard curve points and controls with 10% excess to account for losses due to repeated pipetting.

6.4.5 Record the volumes used on **Form 22209-02.**

6.4.6 Mix the master mix by pipetting up and down.

6.4.7 Add 37.5 µL of master mix to each well of the 96-well PCR plate that will be used.

6.4.8 Add a 12.5 µL aliquot of 1X TE to each of three No Template Control (NTC) wells.

6.4.9 Seal the plate with adhesive film. The plate may be stored at 2-8°C for up to 1 hour while samples are prepared for addition to the plate.

6.5 Sample addition to the PCR plate

6.5.1 Test Sample: Add 12.5 µL of each extracted sample (5 replicate wells), Extraction Negative Control and Extraction Positive Control to the appropriate wells of the PCR plate.

6.5.2 Inhibition-control Reaction: Add 12.5 µL of non-transducedcontrol sample+ 2 µL of VSV-G DNA Control at 1.00E+03 copies/µL.

NOTE: If a non-transducedcontrol sample is unavailable, add 2 µL of VSV-G DNA Control at 1.00E+03 copies/µL to 12.5 µL of transduced sample.

NOTE : The PCR inhibition spike control samples will contain a final volume greater than 50 µL due to the extra spike volume.

6.5.3 Dilute an aliquot of VSV-G DNA Control in 1X TE over a range of concentrations from 1.00E+06 to 1.00E+01 copies/µL. Add 12.5 µL of each VSV-G DNA Control standard curve point to the appropriate wells of the plate.

6.6 Seal the plate with adhesive film. The plate may be kept at roomtemperature for up to 30 minutesprior to amplification on a qPCR instrument.

6.7 Place the sealed plate into the qPCR instrument.

6.8 Program the qPCR instrument to detect a FAM reporter and Non-fluorescent quencher with the following amplificationthermal profile:

Temperature	Time (Min:Sec)	Repetition
50°C	02:00	N/A
95°c	10:00	N/A
95°c	00:15	40
60°C	01:00	

6.9 Upon completion of the qPCR run, unload and discard the plate. **Do not remove the adhesive cover to prevent laboratory contamination with the target amplicon.** Retain and reuse the compression pad.

6.10 Save, analyze and print the run results using a manual Rn thresholdof 0.2.

NOTE: Use of other threshold values may be reported with PA Supervisor approval.

7.0 Validity of the Assay

7.1 Record the assay control results on **Form 22209-03**.

7.2 For the assay to be valid, the following criteria must be met:

7.2.1 The slope of the standard curve must be ≥ -3.7 and ≤ -3.1.

7.2.2 The R2 for the standard curve must be ≥ 0.990.

7.2.3 For the Extraction Positive Control, ≥10% of the spiked DNA must be recovered.

7.2.4 For the Inhibition Control, ≥10% of the inhibition spike must be detected.

7.3 If the run is valid, proceed to Step 7.1 to determine whether the sample is positive or negative for replication competent lentivirus.

7.4 If the run is invalid, the assay may be repeated with supervisor approval and indicate in the comments section of **Form 22209-03**.

8.0 Analysis of Results

8.1 Assay results are reported as either “Positive” or “Negative” for the presence of replication competent lentivirus on **Form 22209-04**.

8.2 Record the qPCR data on **Form 22209-05.**

8.3 A sample replicate is reported RCL-positive if at least three out of five wells are positive; if less than three wells are positive, the sample is reported RCL-negative.

8.4 If both sample replicates are positive, the sample is reported RCL-positive.

8.5 If both sample replicates are negative, the sample is reported RCL-negative.

8.6 If one replicate is positive and one replicate is negative, the assay must be repeated (indicate in the comments section of **Form 22209-04)**.

8.6.1 If both replicates in the repeat assay are negative, the sample is reported RCL-negative.

8.6.2 If one or both replicates in the repeat assay are positive, the sample is reported RCL-positive.

9.0 Documentation

Record all reagent part numbers, lot numbers and expiration dates on **Form 22209-01** (Attachment 1). Record the 96-well plate layout and master mix preparation volumes on **Form 22209-02** (Attachment 2). Record control results on **Form 22209-03** (Attachment 3). Record sample analysis results on **Form 22209-04** (Attachment 4). Record RCL-qPCR data on **Form 22209-05** (Attachment 5).

10.0 References and Related Documents

10.1 **SOP 22212** *Purification of DNA Using the DNeasy Blood and Tissue Kit*

10.2 **SOP 22972** *Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions*

10.3 **SOP 22946** *The Operation of the NanoDrop ND1000 Spectrophotometer*

11.0 Attachments

11.1	**Attachment 1**	Form 22209-01, Quantitative PCR Reagents
11.2	**Attachment 2**	Form 22209-02, 96-Well qPCR Plate Key, Master Mix Preparation and Dilution of Standard
11.3	**Attachment 3**	Form 22209-03, RCL-qPCR Assay Validity Criteria Analysis
11.4	**Attachment 4**	Form 22209-04, RCL-qPCR Assay Sample Analysis
11.5	**Attachment 5**	Form 22209-05, RCL-qPCR Data

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 1
Form 22209-1, Quantitative PCR Reagents

FNLCR, BDP
Form No.: 22209-01
SOP No.: 22209
Revision 00: MAY 24 2019

Quantitative PCR Reagents

Reagent	Part Number	Lot #	Expiration
TaqMan Universal PCR Master Mix (2X)			
Clinical Center VSVG Forward Primer			
Clinical Center VSVG Reverse Primer			
CC VSVG Probe			
96-Well Optical Plates			
Optical Adhesive Covers			
Water/Buffer (type)			
Standard Control DNA			
1X TE			

Instrument	MEF Number	Calibration/PM Due Date
qPCR Instrument		

Comments:

_____

_____

Form Completed By:_____**Date:** _____

Reviewed and Approved By:_____**Date:**_____

QC Test Request #_____
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Attachment 2
Form 22209-2, 96-Well qPCR Plate Key

FNLCR, BDP
Form No.: 22209-02
SOP No.: 22209
Revision 00: MAY 24 2019

96-Well qPCR Plate Key

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTE: unlabeled wells were not used

Master Mix Preparation		
Reagent	Volume for 1 Reaction (µl)	Volume for __ (Number) Reactions (µl)
Water	10	
TaqMan Universal PCR Master Mix (2X)	25	
Clinical Center VSVG Forward Primer (10 µM)	1	
Clinical Center VSVG Reverse Primer (10 µM)	1	
Clinical Center VSVG Probe (FAM/QSY) (10 µM)	0.5	
Template	12.5	
Total Volume	50	

Dilution Series of Standard				
Dilution	Starting Concentration of Standard	Volume of Standard (µl)	Volume of Diluent (µl)	Final Concentration of Standard
1				
2				
3				
4				
5				1.00E+06
6				1.00E+05
7				1.00E+04
8				1.00E+03
9				1.00E+02
10				1.00E+01

Pipette(s) Used:	MEF#/Serial#	Calibration Due Date

Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

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Attachment 3
Form 22209-3, RCL-qPCR Assay Validity Criteria Analysis

FNLCR, BDP
Form No.: 22209-03
SOP No.: 22209
Revision 00: MAY 24 2019

RCL-qPCR Assay Validity Criteria Analysis

qPCR Instrument Rn value used:	
If Rn other than 0.2 used, provide supervisor signature/Date:	
Comments:	

Standard Curve	
Slope of Standard Curve	
NTC Result (Pass/Fail):	

Standard curve result is Pass if the value is ≥ -3.7 and ≤ -3.1.

R^2 Value	
R^2 Value	
Extraction Negative Control Result (Pass/Fail):	

R^2 value result is Pass if the value is ≥ 0.990.

PCR Inhibition Spike Results	
(Post-Extraction Spike)	
Spike copy number used	
Copy number recovered	
Average recovery %:	
PCR Inhibition Result (Pass/Fail):	

PCR Inhibition result is pass if the amount recovered is ≥10%.

Extraction Positive Control Results	
(Pre-Extraction Spike)	
Spike copy number used	
Copy number recovered	
Average recovery %:	
Extraction Positive Control Result (Pass/Fail):	

Extraction Positive Control result is pass if the amount recovered is ≥10%.

RCL-qPCR Assay Validity (Pass/Fail):	

Comments:

QC Analyst Signature / Date:

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Attachment 4
Form 22209-4, RCL-qPCR Assay Sample Analysis

FNLCR, BDP
Form No.: 22209-04
SOP No.: 22209
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RCL-qPCR Assay Sample Analysis

Replicate Analysis

Result	# Positive Wells per 5 Total Wells
Positive	≥3
Negative	<3

Sample Analysis

Result	# Positive Replicates
Positive	2
Negative	0
Repeat*	1

*If 1 out of 2 replicates is positive in the repeated run, the sample is Positive.

Is this a repeated run (Yes/No)? _____

Sample ID	QC Test Request #	Replicate #	# of Positive Wells	Replicate Result (Positive/Negative)	Sample Result (Positive/Negative)

Comments:

QC Analyst Signature / Date:

QC Test Request #_____
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Attachment 5
Form 22209-5, RCL-qPCR Data

FNLCR, BDP
Form No.: 22209-05
SOP No.: 22209
Revision 00: MAY 24 2019

RCL-qPCR Data

Sample ID: _____

Paste qPCR Data Below:

Comments:
_____

_____

Form Completed By: _____ **Date:** _____

Reviewed and Approved By: _____ **Date:** _____

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