



**Title: Determination of Lentivirus Transduction Efficiency with the Protein
L Flow Cytometry Assay**

SOP Number: 22207

Revision Number: 01

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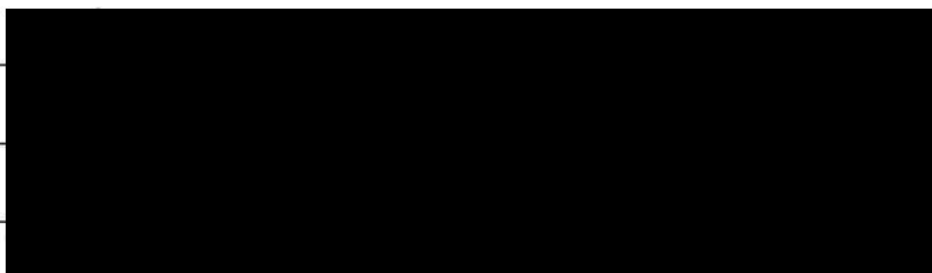


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1.0 Purpose

This SOP describes the materials and methods needed to measure transduction efficiency for recombinant lentiviruses that direct the expression of chimeric antigen receptors (CAR). The assay uses the detection of CARs by Protein L interaction in a flow cytometry format.

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



2.0 Scope

This SOP applies to BDP personnel performing the Protein L flow cytometry assay. The ability to detect lentivirus-transduced cells that express chimeric antigen receptors by Protein L depends upon the ability of the Protein L to access and bind to the kappa light chain (sub-types VkI, VkIII, and VkIV) in the ScFv region of the chimeric antigen receptor. If that interaction is sterically- hindered or otherwise compromised, the Protein L assay may not be used to determine transduction efficiency. The utility of the Protein L flow cytometry assay must be determined empirically for each new CAR construct or sequence modification.

3.0 Authority and Responsibility

- 3.1 The Director, Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.
- 3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA)
- 3.3 PA/QC personnel are responsible for the performance of this procedure.
- 3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.
- 3.5 BQA is responsible for quality oversight of this operation.

4.0 Equipment, Materials and Reagents

- 4.1 MACSQuant Analyzer 10 Flow Cytometer, Miltenyi Biotec
- 4.2 Biosafety Cabinet
- 4.3 Refrigerator
- 4.4 Eppendorf Centrifuge 5417C, or equivalent
- 4.5 FACS Tubes with Cell Strainer (BDP PN 31168 or BDP-approved equivalent)
- 4.6 MACSQuant Running Buffer (BDP PN 31172)
- 4.7 MACSQuant Calibration Beads (BDP PN 31171)
- 4.8 MACS Comp Bead Kit, anti-REA (BDP PN 31176)
- 4.9 Hanks' Balanced Salt Solution (HBSS) (BDP PN 30656)
- 4.10 Recombinant Protein L (BDP PN 31169)
- 4.11 Anti-Protein L-FITC (BDP PN 31170)
- 4.12 Albumin, Human, 10% Solution (BDP PN 30652 or BDP-approved equivalent)
- 4.13 Anti-CD14-FITC, mouse, clone REA934 (BDP PN 31208)
- 4.14 Anti-CD33-PE, human, clone REA775 (BDP PN 31212)
- 4.15 Anti-CD3-PE-Vio 770, human, clone REA613 (BDP PN 31199)
- 4.16 7-AAD Staining Solution (BDP PN 31213 or BDP-approved equivalent)

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4.17 15 mL Centrifuge Tube (BDP PN 20006-2) or 50 mL Centrifuge Tube (BDP PN 20140)

4.18 Anti-CD45-VioGreen, human, clone REA747 (BDP PN 31215)

5.0 Procedure

5.1 Record reagents and equipment used on Form 22207-01.

5.2 Record the completion of each step on Form 22207-02.

5.3 Turn on the MACSQuant Analyzer 10 and allow the optical bench to warm up for at least 30 minutes, as described in **SOP 23131, Operation of the MACSQuant Analyzer 10 Flow Cytometer**.

5.4 Perform a PMT calibration as described in **SOP 23131, Operation of the MACSQuant Analyzer 10 Flow Cytometer** and document the calibration on Form 23131-01.

5.5 Adjust the FSC gain to approximately 250 on the X-axis of the FSC vs. SSC plot using a 50 µL aliquot of the sample to be analyzed as described in **SOP 23131, Operation of the MACSQuant Analyzer 10 Flow Cytometer**.

5.6 Perform a spectral compensation as described in **SOP 23131, Operation of the MACSQuant Analyzer 10 Flow Cytometer** using the MACS Comp Bead Kit, anti-REA and 4 µL (1:50 dilutions) of each antibody used in the compensation. Document the compensation incubation on Form 23131-01.

5.6.1 Channels compensated: VioGreen, FITC, PE, PE-Vio770, PI, BLANK

5.7 Prepare Tscm staining solution (TSS) in a 15 mL or 50 mL centrifuge tube by diluting 10% human serum albumin 1:10 (1% final concentration) in Hanks' Balanced Salt Solution (HBSS) and keep cold in the refrigerator (2-8°C) throughout the procedure (Record reagent volumes on Form 22207-03). The TSS should be prepared fresh for each experiment. Label the centrifuge tube with TSS, the analyst's initials and the date of preparation.

5.8 Store an aliquot of HBSS in the refrigerator (2-8°C) throughout the procedure.

5.9 Place an aliquot of cells (up to 1E7 cells) in a microcentrifuge tube and record the input sample volume on Form 22207-03.

5.10 Dilute the sample 1:2 by adding an equivalent volume of cold HBSS to the aliquot of cells.

5.11 Centrifuge at 2,000 rpm (425 xg) for 5 minutes with a slow stop.

5.12 Remove the supernatant and wash the cells 2 times with 250 µL of cold HBSS and a 2,000 rpm (425 xg) spin.

5.13 Resuspend each cell pellet in 50 µL of cold TSS and add 20 µL of 50 µg/mL Protein L.

5.14 Incubate the samples in the refrigerator (2-8°C) for 20 minutes ± 1 minute. Record the incubation start and end times on Form 22207-03.

5.15 Centrifuge the samples at 2,000 rpm (425 xg) for 5 minutes with a slow stop.

- 5.16 Remove the supernatant and wash each pellet 2 times with 250 μ L of cold HBSS and a spin at 2,000 rpm (425 xg) for 5 minutes with a slow stop.
- 5.17 Discard the supernatant from the second wash and resuspend each pellet in 87 μ L of cold TSS.
- 5.18 Dilute the anti-Protein L-FITC antibody 1:5 in cold HBSS (Record the reagent volumes on Form 22207-03).
- 5.19 Add 5 μ L of diluted anti-Protein L-FITC antibody, 2 μ L anti-CD45-VioGreen, 2 μ L anti-CD33-PE, 2 μ L 7-AAD and 2 μ L anti-CD3-PE-Vio 770.
- 5.20 Vortex briefly to mix the contents of each tube.
- 5.21 Incubate each sample in the refrigerator (2-8°C) for 20 minutes \pm 1 minute. Record the incubation start and end times on Form 22207-03.
- 5.22 Centrifuge the samples at 2,000 rpm (425 xg) for 5 minutes with a slow stop.
- 5.23 Remove the supernatant and wash each pellet 2 times with 250 μ L of cold HBSS and a spin at 2,000 rpm (425 xg) for 5 minutes with a slow stop.
- 5.24 Remove the supernatant and resuspend each cell pellet in an appropriate volume of cold HBSS (typically 200 μ L to 1000 μ L) depending upon the number of cells stained. Record the resuspension volume on Form 22207-03 as "Post-Staining Sample Volume (μ L)".
- 5.25 Analyze each sample directly following resuspension using a MACSQuant Analyzer 10 flow cytometer as described below (Section 6).

6.0 Sample Acquisition

- 6.1 Click on File and select New Workspace.
- 6.2 Click on File→ Open...and select Instrument setting on the left side of the Open box.
- 6.3 Browse through the Public, Private or External locations to find the compensation instrument setting prepared in step 5.6.
- 6.4 Select the correct instrument setting and click on Open.
- 6.5 Select the Channels Tab on the sidebar and click on Advanced.
- 6.6 Make sure that the Height box has been selected and press OK.
- 6.7 Click on File→ Open...and select Analysis on the left side of the Open box.
- 6.8 Browse through the Public, Private or External locations to find the proper analysis template.
- 6.9 Select the "Protein L" Analysis template and click on Open.
- 6.10 A warning box will appear indicating that "All existing regions and windows will be removed! Proceed?". Click on "OK."
- 6.11 Select the "Experiment" tab on the side toolbar.
- 6.12 Samples may be analyzed one tube at a time by selecting the "Single tube rack" or up to 24 at a time by selecting the "Chill 5 rack" in the Rack drop down menu.

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6.13 Single tube rack

6.13.1 Enter QC Test Request number or a Project name in the Project box.

6.13.2 Enter sample information in the Sample ID and Description boxes.

6.13.3 Select a flow rate and record the flow rate used on Form 22207-02 as "Flow Rate Used".

NOTE: Use a flow rate that is appropriate for the sample density (Low flow rate for samples that have a high cell density ($>1E7$), High flow rate for low cell densities ($<1E6$), Med flow rate for samples with an intermediate cell density).

6.13.4 Select Mix gentle in the Mix sample drop-down menu.

6.13.5 Select Extended in the Mode drop-down menu.

6.13.6 Enter an appropriate volume for analysis in the Uptake volume box (Record volume on Form 22207-03) and enter the total sample volume (Post-Staining Sample Volume from Form 22207-03) in the Sample volume box.

6.13.7 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel V2 = CD45-VioGreen, Channel B1 = Protein L-FITC, Channel B2 = CD33-PE, Channel B3 = 7-AAD-PerCP-Vio700, Channel B4 = CD3-PEVio770

6.13.8 Place the tube in the single tube rack and start acquisition by clicking on the



Start Measurement button in the instrument status bar.

6.14 Chill 5 Rack

6.14.1 Enter a Project name or QC Test Request number in the Project box.

6.14.2 Select the wells to be used on the rack window.

6.14.3 Click on Group.

6.14.4 Select a flow rate and record the flow rate used on Form 22207-03 as "Flow Rate Used".

6.14.5 Select Mix gentle in the Mix sample drop-down menu.

6.14.6 Select Extended in the Mode drop-down menu.

6.14.7 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel V2 = CD45-VioGreen, Channel B1 = Protein L-FITC, Channel B2 = CD33-PE, Channel B3 = 7-AAD-PerCP-Vio700, Channel B4 = CD3-PEVio770

6.14.8 Select Ungroup.

6.14.9 For each tube, enter sample information in the Sample ID and Description boxes for each sample in the Chill 5 rack.

- 6.14.10 For each tube, enter an appropriate volume for analysis in the Uptake volume box (Record volume on Form 22207-03) and enter the total sample volume (Post-Staining Sample Volume from Form 22207-03) in the Sample volume box.
- 6.14.11 Place the tubes in the Chill 5 rack in the correct locations and start the sample acquisition by clicking on the Start Measurement button in the instrument



status bar.

7.0 Data Analysis

- 7.1 The data may be analyzed on the MACSQuant Analyzer 10 instrument or may be copied to an external drive (Scientific Data network location or USB drive) and analyzed on an alternative computer (see **SOP 23131, Operation of the MACSQuant Analyzer 10 Flow Cytometer** for details on how to copy files to an external USB drive).
- 7.2 Click on File and select New Workspace.
- 7.3 Click on the Sample tab.
- 7.4 Right-click and select “Add...”
- 7.5 In the Select Data files to add window, browse through the folders to find the data file to be analyzed, select the file and click on Open.
- NOTE:** MACSQuant Analyzer 10 data files have a *.mqd extension.
- 7.6 The flow data may be visualized using the “Protein L” analysis template in either of two ways:
- 7.6.1 Since the “Protein L” analysis template was selected prior to data acquisition (Step 6.9), that analysis template may be applied to the data by going to the Samples tab, right-clicking on the sample name and selecting Apply analysis template.
- 7.6.2 Alternatively, the “Protein L” analysis template may be applied to the data file by clicking on File → Open. Select Analysis and browse through the folders to find the “Protein L” analysis template and click on Open.
- 7.7 Double-click on each plot to enlarge it and check to ensure that the targeted population of cells is included in the gate.
- 7.7.1 Use the gate edit points to adjust the size and shape of the gate.
- 7.7.2 Click and drag a gate to move it to a different area of the plot.
- 7.8 Record the percent of CD45⁺ cells that also express CD3 on Form 22207-04, as indicated by %-# on the CD3/CD45 flow plot.
- 7.9 For the Protein L histogram plot, double-click on the plot to enlarge it and adjust the interval gate as needed to include the entire population of Protein L-positive cells as depicted in Attachment 4.

- 7.10 Record the Transduction Efficiency Percentage, as indicated by %# on the histogram, on Form 22207-04.
- 7.11 Click on Edit and select "Copy plot".
- 7.12 Paste the histogram into the area indicated on Form 22207-04.

8.0 Documentation

Record all reagent part numbers, lot numbers and expiration dates on Form 22207-01 (Attachment 1). Record the completion of each step on the procedural checklist on Form 22207-02 (Attachment 2). Record the calibration and compensation details on Form 23131-01. Record sample preparation details on Form 22207-03 (Attachment 3). Record the results of the assay on Form 22207-04 (Attachment 5).

9.0 References and Related Documents

- 9.1 **SOP 23131** *Operation of the MACSQuant Analyzer 10 Flow Cytometer*

10.0 Attachments

- 10.1 **Attachment 1** Form 22207-01, Protein L Flow Cytometry Assay Reagents
- 10.2 **Attachment 2** Form 22207-02, Protein L Flow Cytometry Procedural Checklist
- 10.3 **Attachment 3** Form 22207-03, Protein L Sample Information
- 10.4 **Attachment 4** Representative Protein L Histogram Plot
- 10.5 **Attachment 5** Form 22207-04, Protein L Flow Cytometry Results

Attachment 1

Form 22207-01, Protein L Flow Cytometry Assay Reagents

FNLCR, BDP
Form No.: 22207-01
SOP No.: 22207
Revision 01: JUN 20 2019

QC Test Request # _____
Page _____ of _____
Initial / Date _____

Protein L Flow Cytometry Assay Reagents

Reagent	Part Number	Lot #	Expiration
MACSQuant Calibration Beads			
FACS Tubes			
MACSQuant Running Buffer			
Albumin, Human, 10% Solution			
Hanks' Balanced Salt Solution			
MACS Comp Bead Kit, anti-REA			
FITC-labeled Antibody (Compensation)			
Anti-CD45-VioGreen Antibody			
Anti-CD33-PE Antibody			
Anti-CD3-PE-Vio 770 Antibody			
Recombinant Protein L			
Anti-Protein L-FITC Antibody			
7-AAD Staining Solution			

Equipment	MEF Number	Calibration Due	Equipment	MEF Number	Calibration Due
MACSQuant Analyzer 10 Flow Cytometer		N/A	Biosafety Cabinet		
Eppendorf Centrifuge 5417C			Refrigerator		

Comments:

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

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

Attachment 2**Form 22207-02, Rev 00 Protein L Flow Cytometry Procedural Checklist**

FNLCR, BDP
Form No.: 22207-02
SOP No.: 22207
Revision 01: JUN 20 2019

Protein L Flow Cytometry Assay Procedural Checklist

QC Test Request Number: _____

Section	Step	Step Completed*
Pre-labeling	Centrifugation of Sample Aliquots	
	Pre-labeling HBSS Wash #1	
	Pre-labeling HBSS Wash #2	
	Resuspension of Cell Pellets in 50 μ L of TSS	
Protein L Labeling	Addition of 20 μ L of 50 μ g/mL Protein L	
	20 Minute Incubation with Protein L	
	Protein L Labeling HBSS Wash #1	
	Protein L Labeling HBSS Wash #2	
	Resuspension in 89 μ L of TSS	
Antibody Detection	Addition of Detection Antibodies	
	20 Minute Incubation with Detection Antibodies	
	Antibody Detection HBSS Wash #1	
	Antibody Detection HBSS Wash #2	
	Resuspension in HBSS for Analysis	

*Indicate when each step has been completed by adding a checkmark. If step is completed by a second analyst, second analyst must initial and date each step that they have completed instead of using a checkmark.

Comments:

Form Completed By: _____ Date: _____

Reviewed and Approved By: _____ Date: _____

QC Test Request # _____

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Initial / Date _____

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Attachment 3

Form 22207-03, Rev 00 Protein L Sample Information

FNLCR, BDP
Form No.: 22207-03
SOP No.: 22207
Revision 01: JUN 20 2019

Protein L Sample Information

QC# _____

Preparation of Tscm staining solution (1% HSA in HBSS) - Volume of 10% HSA: _____, Volume of HBSS: _____

Preparation of anti-Protein L antibody dilution - Volume of anti-Protein L antibody: _____, Volume of HBSS: _____

Sample Information

Sample Name/Number	Volume of Sample Aliquot Used for Staining (µL)	Protein L Incubation Start Time	Protein L Incubation End Time	Anti-Protein L Incubation Start Time	Anti-Protein L Incubation End Time	Post-Staining Sample Volume (µL)	Flow Rate Used	Uptake Volume (µL)

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

Form Completed By: _____ Date: _____

Reviewed and Approved By: _____ Date: _____

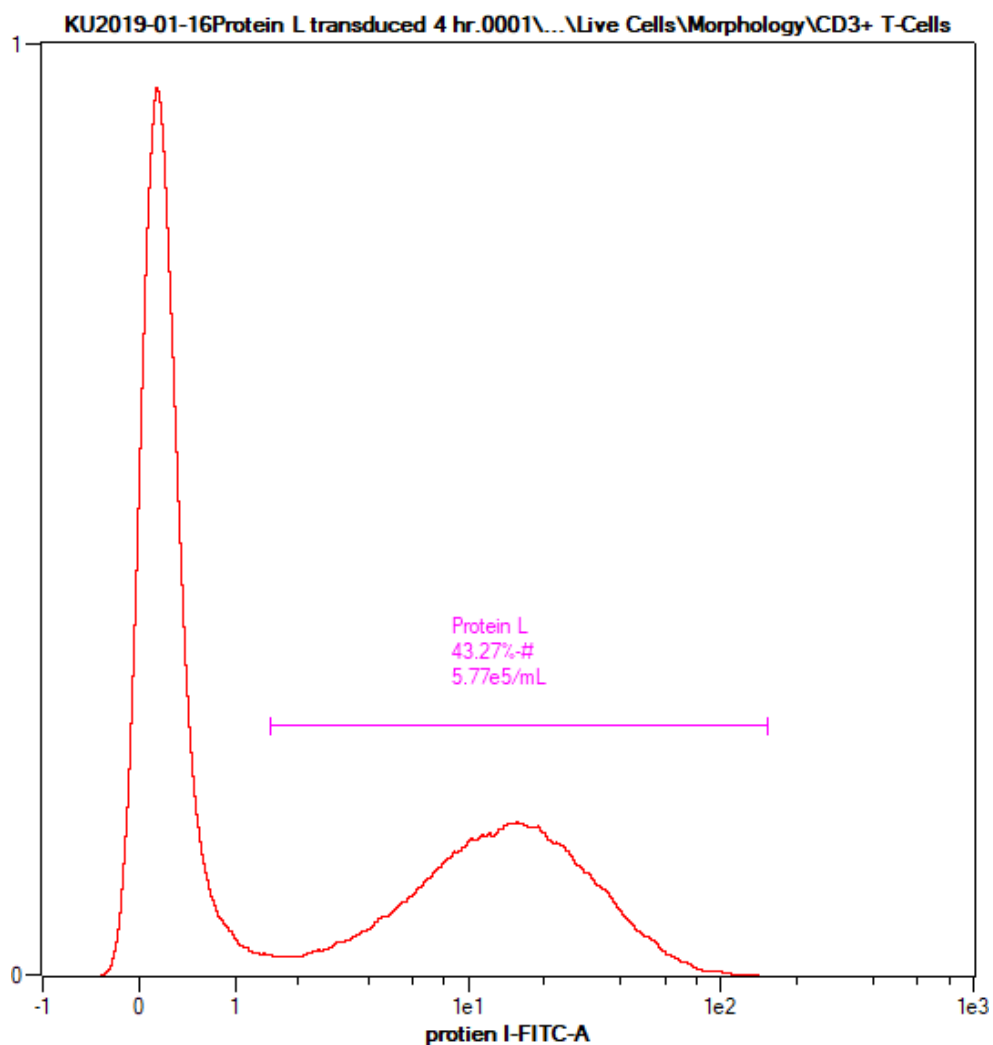
QC Test Request # _____

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Attachment 4

Representative Protein L Histogram Plot



Attachment 5

Form 22207-04, Rev 00 Protein L Results

FNLCR, BDP
Form No.: 22207-04
SOP No.: 22207
Revision 01: JUN 20 2019

Protein L Flow Cytometry Results

Sample Name: _____

Percent CD3⁺ of CD45⁺ cells: _____

Transduction Efficiency Percentage by Protein L Flow Assay: _____

Paste Protein L Histogram Below:

Comments:

Form Completed By: _____ Date: _____

Reviewed and Approved By: _____ Date: _____

QC Test Request # _____

Page _____ of _____

Initial / Date _____

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