SOP Title:	**Detection of Truncated Human EGFR Expressing Chimeric Antigen Receptor (CAR) Cells by Flow Cytometry**
SOP Number:	**22980**
Revision:	**00**

TABLE OF CONTENTS

1. PURPOSE

This SOP describes the materials and methods needed to perform the truncated human EGFR flow cytometry assay. This assay detects the presence of the truncated human EGFR on the surface of cells via the binding of a PE-labeled anti-human EGFR antibody.

2. SCOPE

This SOP applies to Biopharmaceutical Development Program (BDP) personnel performing the EGFR flow cytometry assay.

3. BACKGROUND

This assay is intended for use with CAR vectors that direct the expression of a truncated EGFR for determining transduction efficiency. This assay would not be expected to be a transduction efficiency measurement for CARs that do not express truncated human EGFR protein. The use of this assay to determine the transduction efficiency for each new truncated EGFR-expressing CAR design must be evaluated.

4. RESPONSIBILITIES

4.1 Director, Process Analytics/Quality Control (PA/QC)

- Defines this procedure.

4.2 PA/QC

- Trains laboratory personnel.
- Performs this procedure.
- Reviews the data and documentation of the results of this procedure

4.3 Quality Assurance

- Provides quality oversight.

5. MATERIALS AND REAGENTS

Part Number	Description	BDP Approved Substitution Permitted?
31168 / 31334	FACS Tubes with Cell Strainer	☒ YES ☐ NO
31172	MACSQuant Running Buffer	☒ YES ☐ NO
31183	AutoMACS Rinsing Solution	☒ YES ☐ NO
31171	MACSQuant Calibration Beads	☒ YES ☐ NO
31176	MACS Comp Bead Kit, anti-REA	☒ YES ☐ NO
31183	MACS BSA Stock Solution	☒ YES ☐ NO
31332	Human EGFR PE-Conjugated Antibody	☒ YES ☐ NO
31215	Anti-CD45-VioGreen, human, clone REA747	☒ YES ☐ NO
31210	Anti-CD4-VioBright 667, human, clone REA623	☒ YES ☐ NO
31199	Anti-CD3-PE-Vio 770, human, clone REA613	☒ YES ☐ NO
31211	Anti-CD8-APC-Vio 770, human	☒ YES ☐ NO

Part Number	Description	BDP Approved Substitution Permitted?
31177	Anti-CD8-PE, human, clone REA734	☒ YES ☐ NO
31213	7-AAD Staining Solution	☒ YES ☐ NO
20006	15 mL Centrifuge Tube	☒ YES ☐ NO
20140	50 mL Centrifuge Tube	☒ YES ☐ NO

6. EQUIPMENT

- MACSQuant Analyzer 10 Flow Cytometer, Miltenyi Biotec
- Biosafety cabinet
- Refrigerator
- Eppendorf Centrifuge 5417C, or equivalent

7. PROCEDURE

7.1 Record reagents and equipment used on **Form 22980-01.**

7.2 Record the completion of each step on **Form 22980-02.**

7.3 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**.

7.4 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**.

7.5 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**.

7.6 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 on **Form 23131-01**.

Channels compensated: VioGreen, PE, PE-Vio770, VioBright 667, APC-Vio 770, PI, BLANK

7.7 Prepare a Miltenyi Staining Solution (MSS) in a 15 mL or 50 mL centrifuge tube by diluting MACS BSA Stock Solution 1:20 with AutoMACS Rinsing Solution. This gives a solution with 1X PBS, pH 7.2, 0.5% BSA and 2mM EDTA. Keep cold in the refrigerator (2-8°C) throughout the procedure (Record reagent volumes on **Form 22980-03**). The MSS should be prepared fresh for each experiment. Label the centrifuge tube with MSS, the analyst's initials, and the date of preparation.

7.8 Place an aliquot of cells (up to 1E7 cells) in a microcentrifuge tube and record the input sample volume on **Form 22980-03** as "Volume of Sample Aliquot Used for Staining (µL)".

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

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

7.11 Remove the supernatant and wash the cells one time with 250 µL of cold MSS and a 2,000 rpm (425 xg) spin.

7.12 Resuspend each cell pellet in 80 µL of cold MSS and add 20 µL hEGFR-PE Antibody Cocktail containing 10 µL EGFR-PE, 2 µL anti-CD45-VioGreen, 2 µL anti-CD4-VioBright 667, 2 µL anti- CD8-APC-Vio 770, 2 µL 7-AAD, and 2 µL anti-CD3-PE-Vio 770 to each sample tube (see below).

Reagent	Volume per sample (µL) in each tube
EGFR-PE-labeled Ab	10.0
CD3-PE-Vio770-labeled Ab	2.0
CD8-APC-Vio 770-labeled Ab	2.0
CD45-VioGreen-labeled Ab	2.0
CD4-VioBright 667-labeled Ab	2.0
7-AAD	2.0

7.13 Vortex briefly to mix the contents of each tube.

7.14 Incubate each sample at room temperature in the dark for 30 minutes. Record the incubation start and end times on **Form 22980-03**.

7.15 Add 250 µL cold MSS and centrifuge the samples at 2,000 rpm (425 xg) for 5 minutes with a slow stop.

7.16 Remove the supernatant and wash each pellet one time with 250 µL of cold MSS and a spin at 2,000 rpm (425 xg) for 5 minutes with a slow stop.

7.17 Remove the supernatant and resuspend each cell pellet in an appropriate volume of cold MSS (typically 200 µL to 1000µL) depending upon the number of cells stained. Record the resuspension volume on **Form 22980-03** as "Post-Staining Sample Volume (µL)".

7.18 Acquire and analyze each sample directly following resuspension using a MACSQuant Analyzer 10 flow cytometer as described below (Sections 6 and 7).

8. SAMPLE ACQUISITION

8.1 Click on File and select New Workspace.

8.2 Click on File→ Open…and select Instrument setting on the left side of the Open box.

8.3 Browse through the Public, Private, or External locations to find the compensation instrument setting prepared in section 7.6.

8.4 Select the correct instrument setting and click on Open.

8.5 Select the Channels Tab on the sidebar and click on Advanced.

8.6 Make sure that the Height box has been selected and press OK.

8.7 Click on File→ Open…and select Analysis on the left side of the Open box.

8.8 Browse through the Public, Private, or External locations to find the proper analysis template.

8.9 Select the "EGFR" Analysis template and click on Open.

8.10 A warning box will appear indicating that "All existing regions and windows will be removed! Proceed?". Click on "OK."

8.11 Select the "Experiment" tab on the side toolbar.

8.12 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.

8.13 Single tube rack

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

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

8.13.3 Select a flow rate and record the flow rate used on **Form 22980-03** as "Flow Rate Used".

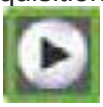
NOTE: Use a flow rate that is appropriate for the sample density (Low flow rate for samples that have a high cell density (>1E7), High flow rate for low cell densities (<1E6), Med flow rate for samples with an intermediate cell density).

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

8.15 Select Extended in the Mode drop-down menu.

8.16 Enter an appropriate volume for analysis in the Uptake volume box (Record volume on **Form 22980-03**) and enter the total sample volume (Post-Staining Sample Volume from **Form 22980-03**) in the Sample volume box.

8.17 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel V2= CD45-VioGreen, Channel B2 = EGFR-PE, Channel B3 = 7-AAD-PerCP-Vio700, Channel B4 = CD3-PEVio770, Channel R1= CD4-VioBright 667, Channel R2= CD8-APC- Vio770.

8.18 Place the tube in the single tube rack and start acquisition by clicking on the Start Measurement button in the instrument status bar. 

8.18.1 Chill 5 Rack

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

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

8.18.4 Click on Group.

8.18.5 Select a flow rate and record the flow rate used on **Form 22980-03** as "Flow Rate Used".

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

8.18.7 Select Extended in the Mode drop-down menu.

8.18.8 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel V2= CD45-VioGreen, Channel B2 = EGFR-PE, Channel B3 = 7-AAD-PerCP-Vio700, Channel B4 = CD3-PEVio770, Channel R1= CD4-VioBright 667, Channel R2= CD8-APC-Vio770.

8.18.9 Select Ungroup.

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

8.18.11 For each tube, enter an appropriate volume for analysis in the Uptake volume box (Record volume on **Form 22980-03**) and enter the total sample volume (Post-Staining Sample Volume from **Form 22980-03**) in the Sample volume box.

8.18.12 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. 

9. DATA ANALYSIS

9.1 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 or BDP network.

9.2 Click on File and select New Workspace.

9.3 Click on the Sample tab.

9.4 Right-click and select “Add…”

9.5 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.

9.6 The flow data may be visualized using the "EGFR" analysis template in either of two ways:

9.6.1 Since the "EGFR" analysis template was selected prior to data acquisition (Section 8.9), 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.

9.6.2 Alternatively, the "EGFR" analysis template may be applied to the data file by clicking on File → Open. Select Analysis and browse through the folders to find the "EGFR" analysis template and click on Open.

9.7 Double-click on each plot to enlarge it and check to ensure that the targeted population of cells is included in the gate.

9.7.1 Use the gate edit points to adjust the size and shape of the gate.

9.7.2 Click and drag a gate to move it to a different area of the plot.

9.8 For the EGFR histogram plot, double-click on the plot to enlarge it and adjust the interval gate as needed to include the entire population of EGFR-positive and EGFR-negative cells as depicted for an EGFR-positive sample in Attachment 1.

9.9 An EGFR-negative sample would exhibit only a single peak, as depicted in Attachment 2.

9.10 Record the percent of CD3-positive cells that are EGFR-positive, as indicated by %-#, on **Form 22980-04**. If the sample is EGFR-negative, record N/A for the %-#.

9.11 Click on Edit and select "Copy plot".

9.12 Paste the histogram into the area indicated on **Form 22980-04**.

10. DOCUMENTATION AND RECORDS

10.1 Record all reagent part numbers, lot numbers, and expiration dates on **Form 22980-01.**

10.2 Record the calibration and compensation details on **Form 23131-01**.

10.3 Record the completion of each step on the procedural checklist on **Form 22980-02.**

10.4 Record sample preparation details on **Form 22980-03**.

10.5 Record the results of the assay on **Form 22980-04**.

11. REFERENCES AND RELATED DOCUMENTS

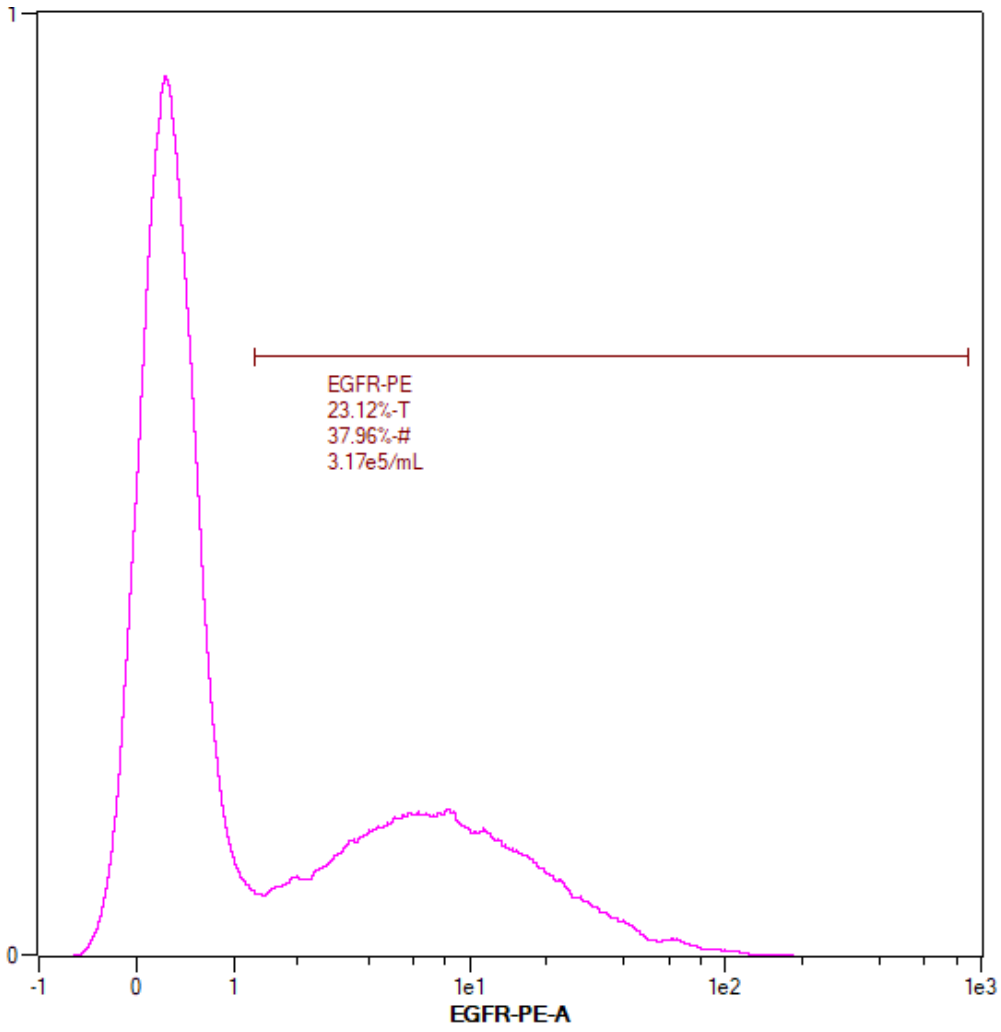
Document Number	Title
23131	Operation of the MACSQuant Analyzer 10 Flow Cytometer
22980-01	EGFR Flow Cytometry Assay Reagents
22980-02	EGFR Flow Cytometry Assay Procedural Checklist
22980-03	EGFR Flow Cytometry Assay Sample Information
22980-04	EGFR Flow Cytometry Results

12. ATTACHMENTS

Attachment 1 Representative EGFR-Positive Histogram Plot

Attachment 2 Representative EGFR-Negative Histogram Plot

Attachment 1 **Representative EGFR-Positive Histogram Plot**



Attachment 2 Representative EGFR-Negative Histogram Plot