Table of Contents

1.0 Purpose

This SOP describes the materials and methods needed to label antibodies with the Thermo Scientific DyLight 650 Antibody Labeling Kit.

2.0 Scope

This SOP applies to BDP personnel performing the DyLight 650 Antibody Labeling and verification assay. The ability to detect cells that express GD2 chimeric antigen receptors by labeled 1A7 depends upon the ability of the 1A7 to access and bind to Ch14.18 paratope targeting the GD2 chimeric antigen receptor.

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 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 autoMACS Rinsing Solution (BDP PN 31183)

4.10 MACS BSA Stock Solution (BDP PN 31182 or BDP-approved equivalent)

4.11 1A7 Antibody (BDP PN 50475)

4.12 APC Labeled Antibody (Compensation) (BDP PN 31294)

4.13 15 mL Centrifuge Tube (BDP PN 20006) or 50 mL Centrifuge Tube (BDP PN 20140)

4.14 Dylight 650 Antibody Labeling Kit (BDP PN 31267)

4.15 GD2CART Positive Cells

4.16 GD2CART Negative Cells

5.0 Procedure

5.1 Antibody Labeling

5.1.1 Thaw 1A7 antibody at room temp and transfer 100µL to a microcentrifuge tube.

NOTE: Record reagents and equipment on form 22217-01

5.1.2 Add 8 µL of the Borate Buffer solution from the kit to the 100µL of 1A7 antibody.

5.1.3 Add the 108 µL antibody/borate buffer solution mixture to the DyLight 650 reagent tube.

5.1.4 Incubate the DyLight 650 antibody mixture for 1 hour ± 10 minutes in the dark. Record DyLight incubation start time on Form 22217-02

5.1.5 During the incubation, prepare the spin column by placing it in a microcentrifuge tube and add 250µL of pipette mixed purification resin from the DyLight Kit.

5.1.6 Spin the loaded spin column for 1 minute at 1000xg.

5.1.7 Transfer the spin column to a new microcentrifuge tube and add the DyLight 650 antibody mixture and spin at 1000xg for 1 minute. Record DyLight incubation end time on Form 22217-02.

5.1.8 The flow through contains the labeled 1A7 antibody which can be stored at 2-8°C for up to 4 months or 6 months at -20°C ± 2°C. Store the labeled antibody in aliquots with the labeling date and expiration date indicated on the tube.

5.2 1A7 labeled verification

5.2.1 Record reagents and equipment used on Form 22217-01.

5.2.2 Turn on the MACSQuant Analyzer flow cytometer 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.**

5.2.3 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**.

5.2.4 Adjust the FSC gain to approximately 250 on the X-axis of the FSC vs. SSC plot using a 50 µL aliquot of the GD2CAR-positive cell sample to be analyzed as described in **SOP 23131 - Operation of the MACSQuant Analyzer 10 Flow Cytometer.**

5.2.5 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. Keep in the refrigerator (2-8°C) when not in use throughout the procedure.

NOTE: This gives a solution with 1X PBS, pH 7.2, 0.5% BSA and 2 mM EDTA. Record reagent volumes on Form 22217-02.

NOTE: The Miltenyi Staining Solution should be prepared fresh for each experiment.

5.2.6 Place an aliquot of GD2CART-positive cells and GD2CART-negative cells (up to 1E7 cells) in a microcentrifuge tube and record the input sample volume and lot numbers on Form 22217-02.

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

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

5.2.9 Resuspend each cell pellet in 100 µL of cold MSS and 0.5 µl of Dylight 650 labeled 1A7 antibody.

5.2.10 Incubate the samples in the refrigerator (2-8°C) for 10 minutes ± 1 minute. Record the incubation start and end times on Form 22217-02.

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

5.2.12 Resuspend each cell pellet in an appropriate volume of Miltenyi Staining Solution (typically 200 µL to 1000µL) depending upon the number of cells stained. Record the resuspension volume on Form 22217-02 as “Post-Staining Sample Volume (µL)”.

5.2.13 Analyze each sample directly following resuspension using a MACSQuant Analyzer flow cytometer as described below (Section 5.3).

5.3 Sample Acquisition

5.3.1 Click on File and select New Workspace.

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

5.3.3 Select the correct instrument setting and click on Open.

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

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

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

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

5.3.7 Select the "1A7 analysis" Analysis template and click on Open.

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

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

5.3.10 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.

5.3.11 Single tube rack

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

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

5.3.11.3 Select a flow rate and record the flow rate used on Form 22217-02 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 cells per mL), High flow rate for low cell densities (<1E6 cells per mL), Med flow rate for samples with an intermediate cell density).

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

5.3.11.5 Select Extended in the Mode drop-down menu.

5.3.11.6 Enter an appropriate volume for analysis in the Uptake volume box (Record volume on Form 22217-02) and enter the total sample volume (Post-Staining Sample Volume from Form 22217-02) in the Sample volume box.

5.3.11.7 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel R1 = 1A7-APC

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

5.3.12 Chill 5 Rack

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

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

5.3.12.3 Click on Group.

5.3.12.4 Select a flow rate and record the flow rate used on Form 22217-02 as "Flow Rate Used".

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

5.3.12.6 Select Extended in the Mode drop-down menu.

5.3.12.7 Select the Annotations tab and modify the annotations for the flow cytometer channels as follows: Channel R1 = 1A7-APC

5.3.12.8 Select Ungroup.

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

5.3.12.10 For each tube, enter an appropriate volume for analysis in the Uptake volume box (Record volume on Form 22217-02) and enter the total sample volume (Post-Staining Sample Volume from Form 22217-02) in the Sample volume box.

5.3.12.11 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. 

6.0 Data Analysis

6.1 The data may be analyzed on the MACSQuant Analyzer 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).

6.2 Click on File and select New Workspace.

6.3 Click on the Sample tab.

6.4 Right-click and select "Add…"

6.5 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 data files have a *.mqd extension.

6.6 The flow data may be visualized using the "1A7 Analysis" analysis template in either of two ways:

6.6.1 Since the "1A7" analysis template was selected prior to data acquisition (Step 5.3.7), 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.

6.6.2 Alternatively, the "1A7" analysis template may be applied to the data file by clicking on File → Open. Select Analysis and browse through the folders to find the "1A7" analysis template and click on Open.

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

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

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

6.8 For the 1A7 histogram plot, double-click on the plot to enlarge it and adjust the interval gate as needed to include the entire population of 1A7-positive cells

6.9 Click on Edit and select “Copy plot”.

6.10 Paste the histogram for both positive and negative cells into the area indicated on Form 22217-03.

6.11 Indicate whether the sample is positive (higher than the background with GD2CAR negative cells) for 1A7 binding on Form 22217-03 and indicate expiration date based upon the date of labeling.

7.0 Documentation

Record all reagent part numbers, lot numbers and expiration dates on Form 22217-01. Record the calibration and compensation details on Form 23131-01. Record sample preparation details on Form 22217-02.Record the results of the assay on Form 22217-03.

8.0 References and Related Documents

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

Form 22217-01 1A7 Flow Cytometry and Labeling Reagents

Form 22217-02 DyLight Antibody Labeling Sample Information

Form 22217-03 1A7 Flow Cytometry Results