Emory University
Emory Vaccine Research Center
Standard Operating Procedure

TITLE: Flow Cytometry-Based Potency Assay for Assessing Binding of 5.24.1C11 and 9.20.1C3 to *Ebolavirus* glycoproteins.

I. PURPOSE

The purpose of this SOP is to describe a procedure for measuring the binding of monoclonal antibodies 5.24.1C11 and 9.20.1C3 to Sudan ebolavirus glycoprotein (SUDV-GP) expressed on the surface of human Jurkat T cells. The assay allows for determining the maximum binding value and 50% effective concentration (EC_{50}) for each mAb production batch. These maximum binding and EC_{50} values are compared to a reference mAb stock measured in parallel.

II. DESCRIPTION OF ASSAY

mAbs 9.20.1C3 and 5.24.1C11 bind to conformation-sensitive epitopes on the glycoproteins of several members of the Ebolavirus genus. Binding of recombinant viral glycoproteins to ELISA plate surfaces may result in the degradation of these epitopes, particularly for mAb 9.20.1C3, which requires the intact glycoprotein trimer for binding. Therefore, the preferred assay for measuring antibody potency is to measure binding to glycoproteins expressed on the surface of cells and compare this binding to a reference antibody stock.

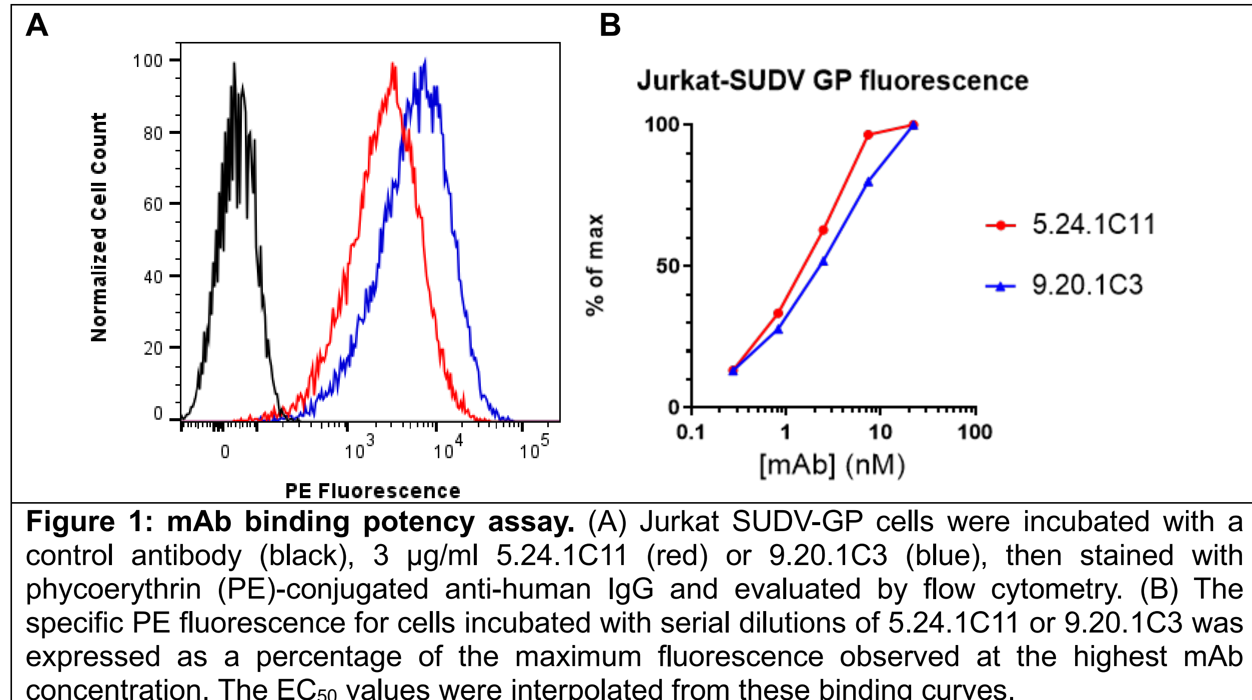


Figure 1: mAb binding potency assay. (A) Jurkat SUDV-GP cells were incubated with a control antibody (black), 3 µg/ml 5.24.1C11 (red) or 9.20.1C3 (blue), then stained with phycoerythrin (PE)-conjugated anti-human IgG and evaluated by flow cytometry. (B) The specific PE fluorescence for cells incubated with serial dilutions of 5.24.1C11 or 9.20.1C3 was expressed as a percentage of the maximum fluorescence observed at the highest mAb concentration. The EC_{50} values were interpolated from these binding curves.

In the potency assay used at Emory (Figure 1), we use a stably lentivirus-transduced Jurkat cell lines expressing the glycoprotein from the Gulu strain of Sudan ebolavirus (Jurkat SUDV-GP cells). In the assay, glycoprotein-expressing Jurkat cells are stained with serial dilutions of

5.24.1C11 or 9.20.1C3. This is followed by washing and detection of bound antibody by incubation with a phycoerythrin (PE) conjugated anti-human IgG secondary antibody. Geometric mean fluorescence (GMF) of mAb binding to live cells is then measured by flow cytometry. The effective concentration of mAb required for 50% maximal binding (EC50) is determined and compared to a reference antibody stock.

III. MATERIALS AND EQUIPMENT

Cell culture:

1) Jurkat SUDV-GP cells.
    - Note: These are human Jurkat T cells (ATCC sublone E6-1) that have been transduced by a lentiviral vector to express Sudan ebolavirus (Gulu 2000 strain) on the cell surface. See PMID: 31104840 for details on their production.
2) 37°C tissue culture incubator with 5% CO_2 atmosphere for cell culture.
3) Liquid nitrogen cell storage tank.
4) -80°C freezer.
5) Hemacytometer or cell counter.
6) 0.4% Trypan blue solution.
7) T25 and T75 tissue culture flasks with vented caps.
8) Sterile serological pipettes and controller for tissue culture work.
9) RPMI 1640 medium.
10) Fetal bovine serum (FBS).
11) Penicillin-streptomycin solution, 10000 U/ml.
12) L-glutamine solution, 200 mM.
13) Sterile dimethyl sulfoxide (DMSO).
14) Disposable 50 ml and 15 ml conical tubes.
15) 2 ml sterile cryovials for liquid nitrogen storage.
16) 0.5 M EDTA solution.
17) 10% sodium azide solution in water.
18) Dulbecco's PBS without calcium or magnesium.

Flow cytometry:

1) Flow cytometer instrument. Essentially any instrument is acceptable as the assay involves single color detection.
    - An instrument with a 96-well plate loader is recommended.
2) 5.24.1C11 and 9.20.1C3 antibody reference lots.
3) 5.24.1C11 and 9.20.1C3 production lots to be tested.
4) Goat anti-human IgG-PE (Southern Biotech catalog #2040-09), or other anti-human IgG fluorescent detection reagent compatible with the flow cytometer used.
5) 96-well round bottom plates with lids for antibody staining.
    - Use plates compatible with the cytometer plate reader, if applicable.
6) FACS tubes compatible with flow cytometer (if not using plate reader).
7) Standard micropipettes and tips.
8) Multichannel pipette and reagent reservoirs.

9) Microcentrifuge tubes.
10) Aluminum foil.
11) Tissue culture centrifuge with swinging bucket rotor and standard adapters for tubes and plates.

Buffers/media:

1) R-10 medium
   - Combine:
     i) 440 ml RPMI 1640 medium
     ii) 50 ml fetal bovine serum (10% final)
     iii) 5 ml Penicillin-streptomycin solution (100 U/ml final)
     iv) 5 ml L-glutamine solution (2 mM final)
   - Store at 2-8°C
2) Flow cytometry staining buffer:
   - Combine:
     i) 500 ml Dulbecco's PBS
     ii) 10 ml fetal bovine serum (2% final)
     iii) 2 ml EDTA solution (2 mM final)
     iv) 2.5 ml sodium azide stock (0.05% final)
   - Store at 2-8°C

IV. PROPAGATION OF JURKAT-SUDV-GP CELLS

1) Perform all cell manipulations in a biosafety cabinet using BSL-2 precautions.
2) To initiate Jurkat-SUDV-GP cell culture, thaw one cell aliquot (1 ml) at 37 degrees and then rapidly transfer into 25 ml pre-warmed (37°C) R-10 culture medium.
3) Pellet cells by centrifugation at room temperature for 5 minutes at ~450 x *g* (1500 rpm for a typical centrifuge).
4) Resuspend cells in 2 ml of pre-warmed R-10 and count an aliquot of cells using trypan blue staining to exclude dead cells.
5) Adjust cell concentration to 0.3 million viable cells per ml with warm R-10 and culture in 37°C, 5% CO_2 incubator in upright T25 or T75 tissue culture flasks with vented caps. For culture volumes up to 10 ml, use T25 flasks; use T75 flasks for volumes up to 30 ml.
6) Split cells when the cell density reaches 1 to 1.5 million viable cells per ml. Do not allow cell density to exceed 2 million per ml.
7) Split cells by diluting into fresh R-10 medium in a new flask at a density of 0.3 million cells per ml. Cells should be split 1-2 times per week depending on the growth rate.
8) For making frozen stocks:
   - Cells should be growing at ~1 million cells per ml.
   - Cells are pelleted by centrifugation and resuspended in sterile 90% FBS/10%DMSO at 5 million cells per ml.
   - 1 ml aliquots of cells (5 million cells each) are transferred into 2 ml sterile cryovials.

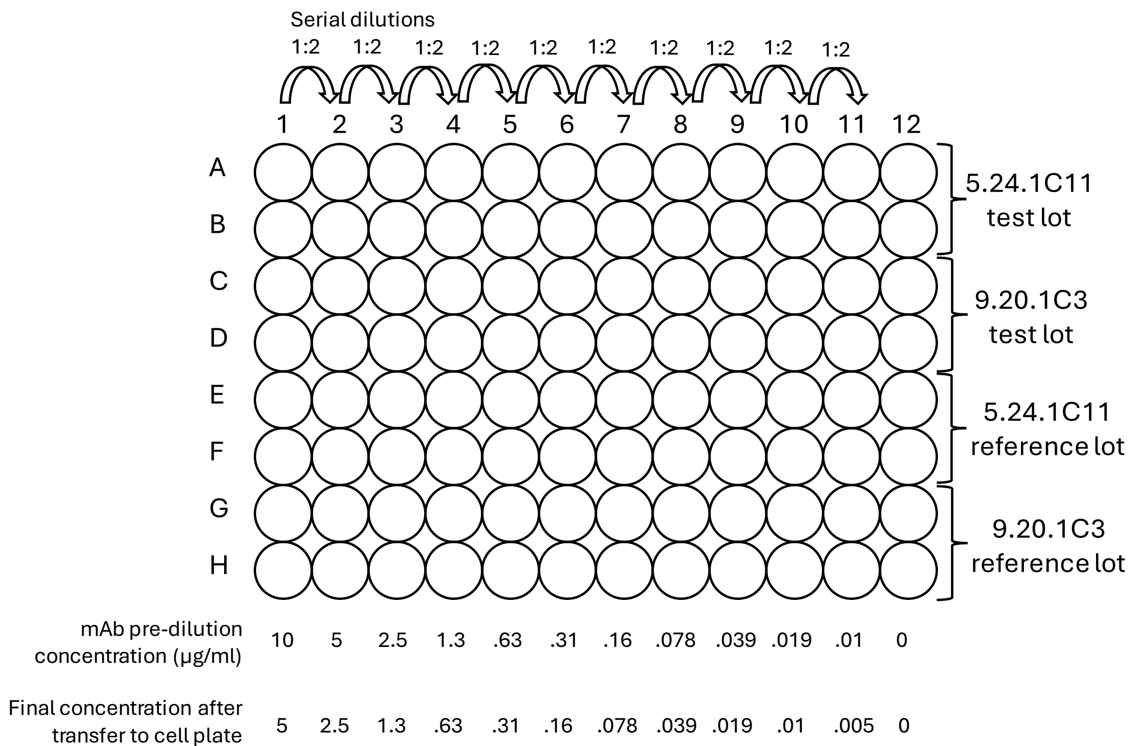
- Cryovials are closed and placed in freezing chambers, then placed in a -80°C freezer overnight.
- Cryovials are then transferred to liquid nitrogen for long-term storage.

9) SUDV-GP cells are stably transduced and single-cell cloned and generally maintain high expression levels over time. However, cells may begin to lose expression of SUDV-GP over many passages due to the faster growth of subclones with lower GP expression levels. If passaging for long periods, it is recommended to monitor expression of GP every two weeks and to restart the culture from a fresh aliquot of cells by passage 40 or if there is significant loss of GP expression.
    - A small (<5-fold) reduction in GP expression will not significantly affect the EC50 value determined in the assay.

V. CELL STAINING PROCEDURE

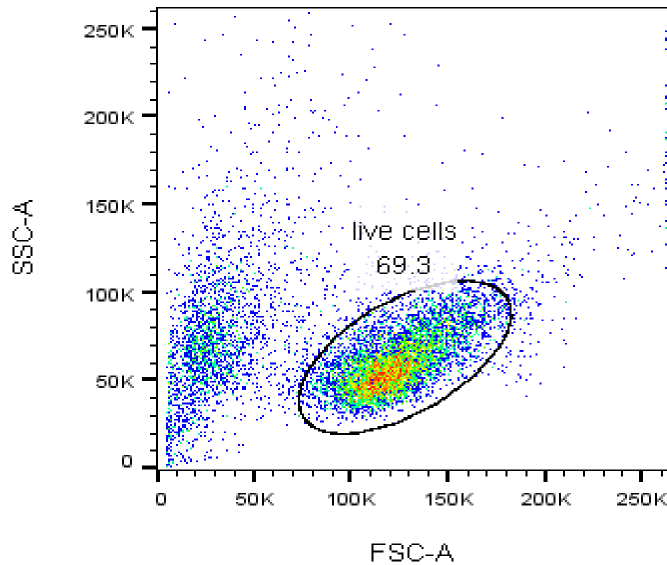
1) Split Jurkat-SUDV-GP cells 2-3 days before performing the staining assay so that they are in log-phase growth with viability of 80% or higher.
2) Collect 1.2 million cells per antibody to be tested, plus another 1.3 million cells per reference stock to test, and place cells in a conical tube(s).
    - For example, if you are testing a single production lot of 5.24.1C11 and 9.20.1C3, plus the reference stocks for each antibody, you will collect 4 x 1.3 million = 5.2 million cells into the tube.
3) Spin down the cells at 1500 rpm for 5 minutes, remove the media by decanting or aspirating, and wash cells by adding 10 ml of flow cytometry staining buffer to the cells.
4) Spin down cells at 1500 rpm for 5 minutes, remove supernatant, and resuspend cell pellet at 1 million cells per ml. Transfer to a reagent reservoir for pipetting.
5) Using a multichannel pipette, transfer 50 µl of cells to wells of a round bottom 96 well plate (50,000 cells per well). For each antibody to be tested, you will require two rows of wells containing cells.
    - For testing two mAb test lots plus the two reference stocks, place cells in all 96 wells.
6) Cover the 96 well plate the lid and place on ice.
7) Set up a second 96 well plate for pre-diluting the mAbs to be tested. Add 100 µl of flow cytometry staining buffer into each well (the number of wells used should match the cell plate; i.e. for testing 4 mAbs you will use a full plate for the pre-dilutions).
8) Add an extra 100 µl of flow cytometry staining buffer to the wells in column 1 of the pre-dilution plate, so that the final buffer volume is 200 µl in this first column.
9) For each mAb to be tested, add 2 µg/well to duplicate wells in column 1 (e.g. add 2 µg mAb #1 to A1 and B1, 2 µg mAb #2 to C1 and D1, etc.)
    - The final mAb concentration will be 10 µg/ml in this first column.
10) Perform serial two-fold dilutions in the pre-dilution plate by pipetting 100 µl from column 1 into column 2, mixing thoroughly by pipetting, then transferring 100 µl from column 2 into column 3 and mixing. Repeat this serial dilution process across the plate until column 11, but do not dilute into column 12, which will be used as a negative staining control.
    - The mAb concentration will now be 5 µg/ml in column 2, 2.5 µg/ml in column 3, etc.

- See plate layout below for an illustration of the dilution process.



11) Transfer 50 µl from each well of the pre-dilution plate into the corresponding well of the plate containing Jurkat-SUDV-GP cells.
    - The final volume in each well should now be 100 µl and the final concentration of mAb in the cell plate is now 50% of the original concentration in the pre-dilution plate (i.e. 5 µg/ml in column 1, 2.5 µg/ml in column 2, etc.)
12) Wrap the cells + mAb plate with aluminum foil and place on ice for 45 minutes.
13) During this 45-minute stain with the GP-specific antibodies, prepare a working solution of the anti-human IgG PE antibody by diluting it 1:500 in flow cytometry staining buffer. Prepare 110 µl of this working solution per well to be stained. Keep on ice, protected from light.
14) After the 45-minute incubation step is complete, remove the cells+ mAbs plate from the foil and spin in the tissue culture centrifuge in a plate holder for 2 minutes at 2000 rpm (~800 x *g*).
15) Remove the buffer from each well by flicking plate into biohazard trash or container with bleach. While the plate is still in the inverted position after flicking it, blot the plate on a paper towel to remove excess medium from the lips of the wells before turning the plate to the right side up position.
16) Wash the plate by adding 200 µl per well of flow cytometry staining buffer, covering with the lid, spinning the plate down again for 2 minutes at 2000 rpm, and flicking to remove the supernatant as above.
17) Repeat this wash step one more time.
18) Resuspend the cells in each well in 100 µl of anti IgG-PE working solution. Cover the plate with the lid, wrap in foil, and incubate for 45 minutes on ice.

19) After the 45 minute incubation is complete, spin down cells, flick off supernatant, and wash with 200 µl flow cytometry staining buffer.
20) Repeat this wash step.
21) Resuspend cells in 110 ul per well flow cytometry staining buffer.
22) Perform flow cytometric analysis of anti human IgG-PE fluorescent signal for each mAb concentration by measuring geometric mean fluorescence (GMF) on live cells in the PE channel.
    - Live cells can be identified by forward and side scatter as shown below:



23) Determine the GMF at each concentration of mAb and subtract off the average GMF of cells incubated without GP-specific antibody (cells from column 12 of the plate) to determine the specific fluorescence.
24) Determine the average specific fluorescence for each pair of replicate wells at each mAb concentration.
25) Determine the maximum binding signal for each mAb and compare to the reference antibody stock. There should be less than 20% variation in this maximum signal between the test lot and the reference material if both are run in the same assay.
26) Determine the concentration of mAb required for 50% maximum binding (EC_{50}) by interpolation from the binding curve data and compare to the reference antibody stock. There should be less than 25% variation in this EC_{50} value between the test lot and the reference material if both are run in the same assay.