Protocol for Neutralizing Antibody Assay Reagent Bridging Studies (October 2021)

I. INTRODUCTION

The Duke Neutralizing Antibody Assay Laboratory (NAb Lab) is responsible for assessing neutralizing antibody responses in clinical trial samples to determine efficacy of vaccines and immunotherapies. Bridging studies must be performed when new lot numbers of key reagents or preparations of cells or viruses are available. This will ensure the integrity of the reagents and the validity of the assay. All current and new reagents for bridging studies will be evaluated using the neutralizing antibody assay in TZM-bl cells.

II. DEFINITIONS

FBS: Fetal Bovine Serum

GM: Growth Medium

IMC: Infectious Molecular Clone

NIH: National Institutes of Health

PI: Principal Investigator

RLU: Relative Light Units

TCID: Tissue Culture Infectious Dose

III. SPECIMENS

Control reagents, Fetal Bovine Serum (FBS), Env-pseudotyped viruses, Env.IMC.LucR viruses, cells (TZM-bl, 293T/17, 293S GnTI-) listed in various protocols.

IV. REAGENTS AND MATERIALS

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality may be used when necessary.

Control Reagents

sCD4
Supplier: Catalent (Madison, Wisconsin)

4E10
Supplier: Polymun Scientific (Austria)

CH01
Supplier: Catalent (Madison, Wisconsin)

VRC-CH31 (referred to as CH31)
Supplier: Catalent (Madison, Wisconsin)

CH01-31
Supplier: The Duke NAb Lab prepared the final 1:1 mixture of CH01:CH31

10-1074
Supplier: Celldex Therapeutics (Hampton, New Jersey)

VRC01
Supplier: Leidos Biomedical Research, Inc (Frederick, Maryland)

Fetal Bovine Serum
Nucleus Biologics

TZM-bl Cells
NIH AIDS Reagent Program

293T/17 Cells
American Type Culture Collection

293S GnTI- Cells
American Type Culture Collection

Env-pseudotyped viruses
Duke Neutralizing Antibody Assay Laboratory
Fraunhofer Institute for Biomedizinische Technik (IBMT)

Env.IMC.LucR Viruses
Duke Neutralizing Antibody Assay Laboratory
Fraunhofer Institute for Biomedizinische Technik (IBMT)

Growth Medium
Invitrogen, Sigma-Aldrich, Nucleus Biologics

DEAE-Dextran, hydrochloride, avg. Mol. Wt. 500,000
Sigma

Trypsin-EDTA (0.25% trypsin, 1mM EDTA)
Sigma

Dulbecco's Phosphate Buffered Saline (DPBS), Sterile
Invitrogen

Trypan Blue (0.4%)
Invitrogen

Britelite Plus Reporter Gene Assay System
PerkinElmer Life and Analytical Sciences

Viviren Live Cell Substrate
Promega

Microliter pipettor tips

Eppendorf, RAININ, Biohit

Disposable Pipettes, sterile, individually wrapped (1 ml, 5 ml, 10 ml, 25 ml, 50 ml)
Costar / VWR

Flat-bottom culture plates, 96-well, low evaporation, sterile
Corning

Flat-bottom black solid plates, 96-well, Costar brand
Perkin Elmer

Reagent reservoirs, 25 ml, 50 ml, 100 ml
Denville Scientific

Culture flasks with vented caps, sterile (T-75)
Denville Scientific

V. EQUIPMENT

Luminometer
Perkin Elmer Life Science

Biological Safety Cabinet
The Baker Company, Inc.

Incubator
Panasonic

Pipettor
Sartorius, RAININ, Eppendorf, Drummond

Light Microscope
Olympus, ThermoFisher Scientific

Centrifuge and Microcentrifuge
Jouan, Eppendorf

Hemacytometer
INCYTO

Water Bath
Precision Scientific

Laboratory Refrigerator / -20°C Freezer
Sci-Cool, LABRepCo

Low Temperature Freezer
Revco/Harris, ThermoFisher Scientific

Liquid Nitrogen Freezer Tank
MVE, Inc.

VI. PROTOCOL

Bridging Procedure for Assay Control Reagents

NOTE 1: Antibodies and proteins used as control reagents (e.g., sCD4, IgG1b12, 2F5, 4E10, 2G12 and CH01-31) for HIV-1 neutralization assays bridged only in TZM-bl cells. As per the Principal Investigator (PI), it is sufficient to perform the bridging of control reagents in one cell line.

1. A bridging study should be performed each time a new lot of a control reagent is received from the manufacturer.

2. Perform a neutralizing antibody assay test of the current lot of assay control reagent set in parallel with the new lot of the assay control reagent using the neutralizing antibody assay for HIV-1 in TZM-bl cells.

3. At least three viruses should be used to bridge Control Reagents. Perform the assay with HIV-1 SF162.LS/293T/17, HIV-1 QH0692.42/293T/17 and any other viruses assigned by the PI.

    a. For routine set up, use a 0.5 mg/ml stock solution of the assay control. Start at a 1:20 dilution and do 5-fold dilutions (final starting concentration = 25 µg/ml).

NOTE 2: The starting concentration of controls will vary as indicated by the neutralizing antibody sensitivity of the virus.

4. Pass/Fail Criteria

    a. Plates and data points must be inspected and approved based on the pass/fail criteria set for neutralization assays. In addition the following criteria must also be met.

    b. Pass: For all viruses tested, IC50 values of old and new control reagents must be within 3-fold of each other

    c. Fail: For any virus tested, IC50 values of old and new control reagents are >3-fold different

        i. Failed bridging results should be repeated as necessary
        ii. If multiple failures occur, the issue should be investigated. Until the issue is resolved, the control agent should not be used for experiments.

Virus Preparation

1. A bridging study should be performed each time a new batch of an existing virus is prepared and after the Tissue Culture Infectious Dose (TCID) is completed. A bridging assay should also be performed when viruses are harvested on multiple days. Env.IMC.LucR viruses and pseudoviruses will be bridged in appropriate cell lines sensitive to each particular virus.

NOTE 3: If available, the new batch of virus should be run against a previous batch of the virus that has already passed the bridging criteria (see below).

NOTE 4: A phenotyping assay should be performed each time a novel virus (i.e., no other batches available for comparison) is made. It will be up to the discretion of the PI (or the designee) to determine if unique viruses are to be phenotyped.

2. Perform a neutralizing antibody assay test of the current lot or first harvest of virus set in parallel with the new lot or second harvest of the virus at the dilution indicated by the virus database.

3. Assay the viruses against any five out of the following control reagents: sCD4, 4E10, CH01, CH31, 10-1074, and VRC01. The PI may also assign alternate control reagents to use in the bridging/phenotyping assay. The starting concentration of controls may vary depending on the sensitivity of the virus to neutralization by the antibody.

4. For a standard set up, use a 0.5 mg/mL stock solution of the antibody controls. Start setting at a 1:20 dilution and do 5-fold dilutions (final starting concentration 25 µg/mL).

5. Pass/Fail Criteria

    a. Plates and data points must be inspected and approved on the Pass/Fail Criteria set for the neutralization assay. In addition the following criteria must also be met.

    b. Pass: Test results for ≥80% of assayed reagents agree within 3-fold between the two pseudoviruses.

    c. Fail: Test results for >20% of assayed reagents are >3-fold between the two sets of data. The mean RLU of the virus control wells are less than 10x the background for the plate. The test will be repeated as necessary.

NOTE 5: Sometimes new batches of viruses are made in other labs and shipped to Duke NAb Lab. If the other lab bridges the virus and the virus passes, then it will be considered bridged and ready to be used in neutralization assays.

293T/17 and 293S/GnTI- Cell Integrity

1. Each time a new batch of 293T/17 cells is thawed, and before discarding the old cells, a virus should be grown in parallel using the old cells and the new cells and the viruses should be bridged as described in "Virus Preparation" above. The yield of virus grown in the new batch of cells should not be lower than 3-fold compared to the virus grown in the old batch of cells.

TZM-bl Cell Integrity

1. A bridging study should be performed each time a new aliquot of cells is passed into a culture from liquid nitrogen storage.

2. Perform the neutralization assay with the current culture of TZM-bl cells set in parallel with the newly established culture of TZM-bl cells.

3. Assay the cells with HIV- SF162.LS/293T/17 and HIV-1 QH0692.42/293T/17 (or alternate viruses assigned by the PI) when testing TZM-bl cells.

4. Assay the viruses against any five of the following control reagents: sCD4, 4E10, CH01, CH31, 10-1074, and VRC01or use alternate control reagents assigned by the PI.

    a. The starting concentration of controls will vary as indicated by the neutralizing antibody sensitivity of the virus.

b. Consult with the PI in order to proceed with the appropriate antibody concentration with that will yield a full concentration curve at 50% neutralization.

5. Pass/Fail Criteria

a. Plates and data point must pass Pass/Fail Criteria set for the neutralization assay. In addition, the following criteria must also be met.

b. Pass: Test results for at least four/five assayed reagents agree within 3-fold between the two sets of data. The mean RLU of the virus control wells must be at least 10x the background for the plate.

c. Fail: Test results for at least two/five assayed reagents are > 3-fold between the two sets of data. The mean RLU of the virus control wells are less than 10x the background for the plate. The test will be repeated as necessary.

NOTE 6: If cells fail repeated bridging assays, a new batch will be retrieved from liquid nitrogen storage.

Fetal Bovine Serum

1. Perform a bridging study each time a new lot number of FBS is received from the manufacturer.

2. Perform the neutralization assay with the current lot of FBS set in parallel with the new lot of FBS using the neutralizing antibody assay for HIV-1 in TZM-bl cells.

NOTE 7: Growth medium (GM) should be prepared using the new lot number of FBS and a flask of cells kept in culture in the new growth medium for at least two passages prior to performing the test. Perform the neutralizing antibody assay in parallel using cells kept in growth medium prepared with the old lot number of FBS and using cells cultured in growth medium with the new lot of FBS. Use GM with the matching FBS for the neutralization assays.

3. Assay the cells kept in media made with the old and new FBS with HIV-1 SF162.LS/293T/17 and HIV-1 QH0692.42/293T/17 using any five out of the following list of control reagents: sCD4, 4E10, CH01, CH31, 10-1074, and VRC01. Alternate control reagents to use in the bridging, phenotyping assay can be used. The starting concentration of controls may vary depending on the sensitivity of the virus to neutralization by the antibody.

4. For standard setup, use a 0.5 mg/mL stock solution of the antibody controls. Start setting at a 1:20 dilution and do 5-fold dilutions (final starting concentration 25 µg/mL).

5. Pass/Fail Criteria

a. Each assayed plate must pass Pass/Fail Criteria established for the neutralization assay. In addition, the following criteria must also be met.

b. Pass: Test results for at least four of the five assayed control reagents (or two/two reagents in Control Reagent Bridging testing) agree within 3-fold between the two sets of data. The mean RLU of the virus control wells must be at least 10x the mean RLU value of the cell control wells of the plate.

c. Fail: Test results for at least two/five assayed reagents (or one/two reagents in Control Reagent Bridging testing) are > 3-fold between the two sets of data. The mean RLU values of the virus

control wells are less than 10X than the mean RLU values of the cell control wells of the plate. The test will be repeated as necessary. If a failed reagent cannot pass the bridging test, the reagents should not be used.

Procedure for Recording and Reviewing Results

1. The technician should record the bridging results on the appropriate Bridging Testing sheet.

2. The technician performing the bridging assay should sign the Bridging Testing sheet(s).

3. The technician should submit the Bridging Testing sheet(s), along with the raw data, to the PI (or designee) for review and signature. The reviewer should indicate on the sheet(s) whether the reagent/virus/cell line used in the bridging test has passed or failed the established criteria.

4. The Bridging Testing sheet(s), along with the appropriate raw data and any additional communication material, should be filed within the Bridging Studies notebook. Additionally, Virus bridging results (and assay number) should be entered in the Virus Database (if applicable).

VII. REFERENCES

1. TZM-bl assay protocols