Protocol for the Preparation of Cells for Detection of *Mycoplasma* Species

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I. Introduction

Serum and plasma samples are tested for the presence of neutralizing antibody responses by using assays as described in various Protocols (Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells as well as other cell lines that utilize molecularly cloned Env-pseudotyped viruses and IMC viruses generated in 293T/17 or 293S GnTI- cells).

Cell line cultures must be screened for *Mycoplasma* contamination as *Mycoplasma* can cause alterations in cell growth rates, morphology, and cell viability as well as can spread to other cell cultures [1]. Maintaining the integrity of these key cell lines is critical for ensuring the validity and quality of the neutralizing antibody assay and the production of viruses.

II. Definitions

PCR: Polymerase Chain Reaction

Antibiotic-free GM: Growth Medium without the presence of antibiotics

IMC: Infectious Molecular Clone

GM: Growth Medium

FBS: Fetal Bovine Serum

DPBS: Dulbecco's Phosphate Buffered Saline

EDTA: Ethylenediaminetetraacetic acid

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

Antibiotic-free Growth Media for TZM-bl Cells (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)

Antibiotic-free Growth Media for TZM-bl Cells (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA)
Sterile
Vendor: Invitrogen

DPBS
Sterile
Vendor: Invitrogen

Disposable pipettes, sterile, individually wrapped
1 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes
Vendor: Falcon/VWR

Culture flasks with vented caps, sterile
T-75 flask
Vendor: Corning/Neta Scientific

Conical tubes, sterile
15 ml capacity
50 ml capacity
Vendor: Corning/Neta Scientific

"*Mycoplasma* Testing Record" (Attachment #1)

MycoAlert Mycoplasma Detection Kit
Vendor: Lonza

Cryogenic vials, 2.0 ml sterile screw cap, frosted label
Vendor: Starstedt Brand Products

IV. Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Biological Safety Cabinet
Manufacturer: Baker Co.

Incubator
Manufacturer: Panasonic

Pipettor
Manufacturer: Drummond

Light Microscope
Manufacturer: Olympus
Manufacturer: Advanced Microscopy Group (AMG)

Centrifuge
Manufacturer: Jouan
(low speed capable of up to 500 x g)
15 ml tube holder
50 ml tube holder

4°C Refrigerator
Manufacturer: LabRepco

Water Bath
Manufacturer: VWR

Hemacytometer
Manufacturer: INCYTO

> ***NOTE 1:*** An automated cell counting device (e.g., Countess, Manufacturer: Invitrogen) may be used in lieu of a light microscope / hemacytometer for cell counting and viability calculation.

Low Temperature Freezer
Manufacturer: Generic Brand

V. Specimens

TZM-bl, 293S GnTI- and 293T/17 cell lines listed in various Protocols.

VI. Protocol

1. Initial Qualification of Cell Lines

1.1 The laboratory must maintain an archived inventory of frozen cells, designated as "Master Archive Stock" and "Working Archive Stock," for the TZM-bl, 293T/17, 293S GnTI- and any other applicable cell lines. During the initial qualification of a cell line, the baseline purity of the cell line must be determined by testing for *Mycoplasma* at certain time points. For a cell line to be qualified to be used in the Laboratory, the cell line must be negative for *Mycoplasma* at every time point tested.

1.2 To determine the baseline purity, cells from the Master Archive Stock and Working Archive Stock should be cultured in vitro in antibiotic-free GM and tested for *Mycoplasma* contamination at Weeks 2, 4, 8, 12, and 18. Cell lines that are only kept in culture for 3 months should be tested at Weeks 2, 4, 8, and 12.

1.3 During each round of testing, the cells must be found negative for the presence of *Mycoplasma* species. If no positive results are obtained by the end of week 24 (week 12 for cells kept 3 months only), the routine testing schedule can be reduced to a period of time not to exceed every 3 months.

1.4 In the event that a cell culture tests positive for *Mycoplasma* during the process of establishing baseline purity, the culture must be discarded immediately. A new cell vial must be thawed and cultured in vitro in antibiotic-free GM (as described above) to establish the baseline purity. Repeating the baseline purity process is also necessary if the laboratory begins culturing a new cell vial from an outside source for the creation of a new Master Archive Stock.

> ***NOTE 2:*** TZM-bl, 293T/17, 293S GnTI- or other applicable cell cultures must be discarded after either 60 passages or 5 months in culture, whichever comes first.

2. Preparation of Cells (TZM-bl, 293T/17, 293S GnTI-, or other applicable cell lines) for *Mycoplasma* Testing

NOTE 3: Cell lines that are being used in the laboratory must undergo *Mycoplasma* testing during a minimum of two different passages.

NOTE 4: *Mycoplasma* testing can be performed using a variety of commercially available kits. Refer to the manufacturer's instructions for the use of each individual kit. *Mycoplasma* testing can be performed by a third-party laboratory. Refer to the third-party laboratory's instructions for the proper preparation of cells for testing.

2.1 Cells should be maintained according to the various Protocols for Trypsin-EDTA Treatment for Disruption of Cell Monolayers.

2.1.1 If required by the *Mycoplasma* testing kit/company, cells should be carried for at least 10 days or 3 passages in antibiotic-free GM.

2.2 After culturing cells for at least 10 days in antibiotic-free GM, cells are harvested. For applicable adherent cells, retain the growth media in the final culture flask for use in resuspension of cells after treatment with trypsin. Growth media is also tested for the presence of *Mycoplasma*.

2.3 Wash cells with PBS. Trypsinize according to the aforementioned protocol. Use approximately 10 mL of saved old growth media to quench trypsin, resuspend the cell layer. Perform cell count. Aspirate cell mixture and dispense into a sterile conical tube.

2.4 Centrifuge conical vial for approximately 3-5 minutes at no more than 1000 rpm. Cells will form a pellet. Carefully discard old media back in conical tube, and resuspend pellet at testing concentration (i.e. TZM-bl cells are sent for testing at a concentration of 1×10^7 cells/ml.

2.5 Samples should be kept in -80°C freezer until shipment date.

2.6 A positive and negative control should be run in parallel with the testing of the cells if performing in-house testing. Positive and negative controls are commercially available. If sending the cells out for testing, ensure that the laboratory performing the test includes the appropriate controls.

2.7 In the event that a cell culture tests positive for *Mycoplasma*, the culture must be discarded immediately and a new *Mycoplasma*-free cell line must be established.

NOTE 5: Cell lines that test positive for *Mycoplasma* contamination must not be used for any assay.

3. Procedure for Recording and Reviewing Results

3.1 All appropriate information pertaining to the cells that are being tested, as well as the "Pass" or "Fail" results, must be recorded on the *Mycoplasma* Testing Record

(Attachment # 1). The *Mycoplasma* Testing must be reviewed, initialed, and dated by a Lab Manager or appropriate personnel designated by the Principal Investigator.

3.2 The *Mycoplasma* Testing Record (Attachment # 1) must be retained in the laboratory.

VII. References

1. Kilani, A. "Mycoplasma Testing – An Overview." Clongen Laboratories, LLC. http://www.clongen.com/mycoplasma_testing_services2.htm.

2. MycoAlert User Manual

VIII. Attachments

1. "*Mycoplasma* Testing Record"

Technician	Cell Type	Date of Cells	Passage #	Test Date	Results	Pass / Fail[1]	Reviewer Initials	Date