

Protocol for the Preparation of Cells for Detection of *Mycoplasma* Species
(October 2021)

I. Introduction

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 Env-pseudotyped and IMC viruses.

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).

II. Definitions

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

DPBS: Dulbecco's Phosphate Buffered Saline

EDTA: Ethylenediaminetetraacetic acid

FBS: Fetal Bovine Serum

GM: Growth Medium

IMC: Infectious Molecular Clone

PCR: Polymerase Chain Reaction

RCF: Relative Centrifugal Force

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.

TZM-bl Cells

Manufacturer: NIH AIDS Reagent Program

Catalog Number: 8129

293T/17 Cells

Manufacturer: American Type Culture Collection

Catalog Number: ATCC CRL-11268

293S/GnTI-

Manufacturer: American Type Culture Collection

Catalog Number: ATCC CRL-3022

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)

Dulbecco's Phosphate Buffered Saline (DPBS)

Manufacturer: Thermo Fisher Scientific

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

Manufacturer: Thermo Fisher Scientific

Disposable pipettes, sterile, individually wrapped

Vendor: Corning

1 ml pipettes

2ml pipettes

5 ml pipettes

10 ml pipettes

25 ml pipettes

50 ml pipettes

Culture flasks with vented caps, sterile

Vendor: Corning/Neta Scientific

T-25 flask

T-75 flask

Conical tubes, sterile

Vendor: Corning

15 ml capacity

50 ml capacity

MycoAlert Mycoplasma Detection Kit

Vendor: Lonza

Cryogenic vials, 2.0 ml sterile screw cap, frosted label

Vendor: Starstedt Brand Products

IV. Instrumentation

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

Biological Safety Cabinet

Manufacturer: Baker Co., sterilGARD e3

Incubator (37°C, 5% CO₂ standard requirements)

Manufacturer: Panasonic

Pipettor

Manufacturer: Drummond

Light Microscope

Manufacturer: Olympus

Centrifuge

Manufacturer: Jouan C412 or Sorvall RT6000B

(low speed capable of up to 500 x g)

15 ml tube holder

50 ml tube holder

4°C Refrigerator

Manufacturer: LabRepCo, Inc

Water Bath

Manufacturer: VWR

Hemocytometer

Manufacturer: INCYTO

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

Low Temperature Freezer (-70°C or lower)

Manufacturer: Thermo Scientific

V. Specimens

TZM-bl, 293S GnTI- and 293T/17 cell lines

VI. Protocol

1. Cell Line Mycoplasma Baseline Purity Test

- 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 should be cultured in vitro and tested for *Mycoplasma* contamination at Weeks 2, 4, 8, 12, 18 and 24, following procedures outlined in Preparation of Cells (TZM-bl, 293T/17, 293S GnTI-, or other applicable cell lines) for Mycoplasma Testing. Cell lines that are only kept in culture for less than 24 weeks should be tested at designated time points, listed above, up to the maximum length the cells are kept in culture (see NOTE 4).

NOTE 2: Baseline purity testing also can be performed using a cell line’s Working Archive Stock, in lieu of the Master Archive Stock, provided the Working Archive Stock was derived from the Master Archive Stock, and there is clear documentation of associated cell passage and creation of the archive stocks.

NOTE 3: Baseline purity for each cell line must be re-established if a new Master Archive Stock is developed or the laboratory begins culturing cells from a different lot/source.

NOTE 4: TZM-bl and 293T/17 cell cultures must be discarded after either 60 passages or 5 months in culture, whichever comes first. The 293S/GnTI- cell line must be discarded after 45 passages.

- 1.3 During each round of testing, the cells must be found negative for the presence of *Mycoplasma* species to proceed to the next round of testing.
- 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. It is recommended to decontaminate the cell culture flask directly by adding bleach to the flask before discarding.
- 1.5 If mycoplasma are detected during the baseline purity testing period, a new mycoplasma-free cell line would need to be established. A new vial of cells must be thawed and cultured in vitro to establish the baseline purity followed by another period of mycoplasma testing according to the scheduled outlined in 1.2.

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

NOTE 5: Cell lines that are being used for current assays in the laboratory must undergo quarterly *Mycoplasma* testing (at passage 26 (± 2) and at the end of their cycle.

NOTE 6: *Mycoplasma* testing can be performed using a variety of commercially available kits, e.g., MycoAlert Mycoplasma Detection Kit. Refer to the manufacturer's instructions for the use of each individual kit. *Mycoplasma* testing can be performed by a third-party laboratory, e.g., Clongen. 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 Protocol for Thawing, Expanding, Maintaining, and Cryopreserving Adherent Cell Lines.
 - 2.1.1 If required by the *Mycoplasma* testing kit / third party laboratory, 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. Remove and retain the growth media from the final culture flask in separate tube for use in re-suspension of cells after treatment with trypsin. Growth media is also tested for the presence of *Mycoplasma*.
- 2.3 Wash cells with 10ml DPBS.
- 2.4 Trypsinize adherent cells according to the aforementioned protocol.
- 2.5 Perform cell count.
- 2.6 Aspirate cell mixture and dispense all into sterile, 15ml conical tube.

- 2.7 Centrifuge cells in a conical tube for approximately 3-5 minutes at no more than 380 xg. Cells will form a pellet. Carefully aspirate the supernatant without disturbing the cell pellet and transfer it to the tube of media reserved from step 2.4.
- 2.8 Re-suspend pellet in reserved media at testing concentration and aliquot 1ml. TZM-bl cells are sent for testing at a concentration of 1×10^7 cells/ml.
- 2.9 Samples should be kept in Ultra Low Temperature Freezer until shipment date, no longer than two weeks if the cells are in the first quarterly test.
- 2.10 A positive and negative control should be assessed 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.
- 2.11 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 7: 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, should be recorded. Records should be reviewed, initialed, and dated by a Lab Manager or appropriate personnel designated by the Principal Investigator.

VII. References

1. Kilani, A. “Mycoplasma Testing – An Overview.” Clongen Laboratories, LLC. http://www.clongen.com/mycoplasma_testing_services2.htm.
2. MycoAlert User Manual
3. Ozaki, D.A., Gao, H., Todd, C.A., Greene, K.M., Montefiori, D.C. and Sarzotti-Kelsoe, M. (2012) International transfer of a GCLP-compliant HIV-1 neutralizing antibody assay for human trials. PLoS One, 7(1): e30963.
4. Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells
5. Protocol for Preparation and Titration of HIV-1 Env-pseudotyped Viruses
6. Protocol for Preparation and Titration of IMC Viruses
7. Protocol for Thawing, Expanding, Maintaining and Cryopreserving Adherent Cell Lines
8. Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells