

**Protocol for Preparation of Cells for Detection of *Mycoplasma* Species
(Montefiori Lab)
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I. INTRODUCTION

Serum and plasma samples are tested for the presence of neutralizing antibody responses by using a specific assay, as described in “Protocol for Measuring Neutralizing Antibodies Against HIV-1, SIV, and SHIV Using a Luciferase Reporter Gene Assay in TZM-bl Cells,” that utilizes molecularly cloned Env-pseudotyped viruses generated in 293T/17 cells, as described in “Protocol for the Preparation and Titration of HIV-1 Env-pseudotyped Viruses.”

To comply with GCLP regulations, cell culture lines 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 of the neutralizing antibody assay and the production of Env-pseudotyped viruses. The testing is performed by a third-party, contract testing laboratory using a highly sensitive Multiplex PCR assay [1].

II. DEFINITIONS

GCLP: Good Clinical Laboratory Practice

PCR: Polymerase Chain Reaction

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

DMEM: Dulbecco’s Modified Eagle Medium

FBS: Fetal Bovine Serum

PBS: Phosphate Buffered Saline

HEPES: N-2-Hydroxyethylpiperazine-N’-2-Ethanesulfonic Acid

III. REAGENTS AND MATERIALS

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

Antibiotic-free Growth Medium*

DMEM, with L-glutamine, sodium pyruvate, glucose and pyridoxine. Sterile, store refrigerated at 4°C.

Gibco BRL Life Technologies

Fetal bovine serum. Heat-inactivated at 56°C for 30 minutes, sterile. Store at -20°C. Once thawed, store at 4°C.

Hyclone

HEPES Buffer, 1 M. Sterile, store at 2°C – 8°C

Gibco

***Antibiotic-free GM consists of DMEM containing 10% heat-inactivated FBS.** To make a 500 ml of antibiotic-free GM, combine 437.5 ml DMEM, 50 ml FBS, and 12.5 ml HEPES in a sterile bottle, mix, store at 4°C for up to 2 months. Warm medium to 20-37°C prior to use.

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

Invitrogen

PBS, sterile

Invitrogen

Disposable pipettes, sterile, individually wrapped

Falcon/VWR

1 ml pipettes

5 ml pipettes

10 ml pipettes

25 ml pipettes

50 ml pipettes

Culture flasks with vented caps, sterile

Costar/VWR

T-75 flask

Conical tubes, sterile

Costar/VWR

15 ml capacity

50 ml capacity

“Mycoplasma Testing Record” (Appendix A)

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

NuAire

Incubator

Forma Scientific

Pipettor

Drummond

Light Microscope

Olympus

Centrifuge, low speed capable of up to 500 x g

Jouan

15 ml tube holder

50 ml tube holder

Specimens:

TZM-bl and 293T/17 cell lines

IV. PROTOCOL

Initial Qualification of Cell Lines

1. The Laboratory must maintain an archived inventory of frozen cells, designated as “Master Archive Stock” and “Working Archive Stock,” for both the TZM-bl and 293T/17 cell lines, that have been tested for the presence of *Mycoplasma* in order to determine baseline purity. All cell lines in this archive should have tested negative for the presence of *Mycoplasma* species and be recorded in the appropriate laboratory log book.
2. During the initial qualification run, a vial of cells from the Working Archive Stock is thawed and cultured in vitro. Cells are tested for *Mycoplasma* contamination at Weeks 0, 2, 4, 8, 12, 18, and 24. 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, the routine testing schedule can be reduced to a period of time not to exceed every 3 months.
3. In the event that a cell culture tests positive for *Mycoplasma* during the qualification process, the culture must be discarded immediately, a new *Mycoplasma*-free cell line must be established, and another period of qualification testing performed as indicated above. This qualification run is also necessary if the laboratory begins culturing a new cell line.

Protocol for Preparation of Cells for *Mycoplasma* Testing

1. At the predetermined time for routine *Mycoplasma* testing, the technician trypsinizes a confluent flask of TZM-bl cells and/or 293T/17 cells in accordance to “Protocol for Measuring Neutralizing Antibodies Against HIV-1, SIV, and SHIV Using a Luciferase Reporter Gene Assay in TZM-bl Cells,” and “Protocol for the Preparation and Titration of HIV-1 Env-pseudotyped Viruses.”

NOTE 1: Cell cultures must be discarded after either 60 passages or 5 months in culture, whichever comes first.

2. Cells to be tested for *Mycoplasma* must be maintained in antibiotic-free GM for a minimum of two passages over at least a 7 day period.

*NOTE 2: The presence of antibiotics could reduce the *Mycoplasma* copy numbers, if present, to below detection limits [1].*

3. To prepare the cells for submission to the testing facility, each set of cells grown in antibiotic-free GM is trypsinized once more, spun down, and resuspended at 1×10^7 /mL. The cell suspensions are then transferred into a pre-labeled tube and stored at -80°C .

4. The tubes to be tested are shipped on dry ice to the third-party contract testing facility.

Pass/Fail Criteria for *Mycoplasma* Testing

Pass

The cell samples test negative for the presence of *Mycoplasma* species, i.e., less than 20 copies of *Mycoplasma* species by Multiplex PCR assay.

Fail

The cell samples test positive for the presence of *Mycoplasma* species, i.e., 20 or greater copies of *Mycoplasma* species by Multiplex PCR assay.

Procedure for Recording and Reviewing Results

1. All appropriate information pertaining to the cells submitted for testing, as well as the “Pass” or “Fail” results, must be recorded on the *Mycoplasma* Testing Record (Appendix A).

NOTE 3: The testing facility will assign an accession number to each vial of cells submitted for testing. The official test results report will list the sample ID of the cells exactly as it is written on the tube along with the assigned accession number. Both the accession number and the sample ID should be indicated on the Mycoplasma Testing Record (Appendix A).

2. The *Mycoplasma* Testing Record (Appendix A) will be reviewed, initialed and dated by a Lab manager or appropriate personnel designated by the Principal Investigator.
3. The official test results from the testing facility and the *Mycoplasma* Testing Record (Appendix A) must be filed in the Laboratory. Previous test results must be stored in the Laboratory’s Archives.

V. REFERENCES

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

