1850. CULTIVATION MEDIUM FOR TREPONEMA PALLIDUM

Complex culture conditions: Coculture with Sf1Ep cottontail rabbit epithelial cells in a modified CMRL 1066 medium with 20% fetal bovine serum. Incubation at 34C in an atmosphere of 1.5% O2, 5% CO2, 93.5% N2. Please see Literature 41127 (doi: 10.1002/cpz1.44) for details.

CULTURE OF Sf1Ep CELLS

Sf1Ep medium

500 ml Eagle's MEM (Sigma M4655)
5 ml MEM Non-Essential Amino Acids (Gibco 11140-050)
5 ml L-glutamine (Sigma G7513)
5 ml sodium pyruvate (Sigma S8636)
50 ml fetal bovine serum, heat inactivated (any high quality FBS)

Filter sterilize and store at 4° C. It is not necessary to use the prescreened *T. pallidum* permissive FBS for routine culture of Sf1Ep cells.

Cell Culture Procedure

Sf1Ep cells obtained directly from ATCC® are derived from primary cells and have a finite lifespan of about 27-30 passages. Thus, it is important to make a large frozen seed stock of Sf1Ep cells. These cells have an epithelial-like morphology and take on a polygonal or cobblestone appearance when they reach confluence. These low passage Sf1Ep grow slowly and are subcultured 1:5 every one-two weeks.

We have an apparently immortal cell line of Sf1Ep cells that arose spontaneously from the ATCC® cells. These cells grow much faster than the original cells, grow to higher densities, and take on a spindle-shaped cell morphology when they reach high densities. Initially, these support *T. pallidum* growth as well as the original Sf1Ep cells and are easier to produce in large quantities. However, after reaching passage 65, *T. pallidum* growth becomes reduced and they should be discarded and a lower passage culture of cells should be thawed. We can supply this cell line to anyone wishing to initiate in vitro cultivation. These are subcultured at 1:15 every week.

Perform all manipulations in a biosafety cabinet using aseptic technique.

Materials

75 cm^2 tissue culture flasks (e.g. Corning 43061U)
6-well cell culture plates for *T. pallidum* cultivation (low evaporation plates such as Falcon 353046)
Frozen stock of Sf1Ep (NBL-11) cells from ATCC® (CCL-68™) or colleague
Sf1Ep medium
Freezing medium (Sf1Ep medium + 10% [v/v] DMSO)
70% ethanol
Tissue culture incubator maintained at 37° C, 5% CO_2
Sterile PBS
Trypsin-EDTA (e.g. Sigma T4049)
15 and 50 ml conical sterile disposable centrifuge tubes (e.g. Falcon 353046 and Nunc 339653)
Inverted microscope
Hemocytometer
DMSO (sterile cell culture grade e.g. Sigma D2650)
2 ml cryogenic vials (e.g. Corning 430659)
Cell freezing container (e.g. ThermoFisher Scientific 5001-0001)
5-, 10-, and 25-ml sterile disposable pipets and pipetting aid
Cryogenic Dewer tank or similar liquid nitrogen storage device

Thawing Sf1Ep stocks

1. Pre-warm the Sf1Ep medium in the 37° C, 5% CO_2 incubator.

2. Quickly thaw a frozen stock of Sf1Ep cells at 37° C.

3. Wipe outside of vial with 70% ethanol.

4. Slowly add 1 ml of Sf1Ep medium to the cryogenic vial and gently mix with the thawed cell stock.

   The newly thawed cells are fragile and should be handled gently.

5. Move cell stock to a 15 ml sterile conical centrifuge tube, add an additional 5 ml of Sf1Ep medium and gently mix with a sterile 5 ml pipet.

6. Centrifuge the Sf1Ep cells in a low speed centrifuge for 7 minutes at 125 x g.

7. Remove and discard supernatant from the cell pellet.

8. Gently resuspend the cell pellet in 5 ml of Sf1Ep medium and transfer the entire amount to a T75 flask containing 10 ml of Sf1Ep medium.

   Steps 6-8 are optional but improve recovery of the newly thawed cells. If omitting steps 6-8, place the 7 ml of cells from step 5 directly into the T75 flask containing 8 ml of Sf1Ep medium. The following day, remove one-half of the medium and replace with fresh warmed Sf1Ep medium to dilute the residual DMSO used for freezing.

9. Incubate in a humidified incubator maintained at 37° C and 5% CO_2. If using non-vented flasks, make sure the lid is loosened.

Maintenance of Sf1Ep stocks and preparation of Sf1Ep cells for T. pallidum culture

Growth of S1Ep cell cultures is monitored by observation with an inverted microscope. When the cells become ~90% confluent, they are ready to be used for *T. pallidum* culture or to be sub-cultured. Cell life can be extended by feeding the cell culture once a week by replacement of one-half the medium with fresh medium. When the cultures grow to very high density the cells become quiescent and take longer to recover after sub-culture.

1. Aspirate the cell culture medium and discard.

2. Add 5 ml of sterile PBS to the flask, close the flask and rinse the cell monolayer to remove the Sf1Ep medium from the monolayer.

   Serum in the medium will inhibit trypsinization.

3. Aspirate and discard the PBS.

4. Add 2.5 ml of trypsin-EDTA to the flask.

5. Seal the cap, spread the trypsin-EDTA over the cell monolayer and place the flask in the 37° C incubator for 5 minutes.

6. Observe the cell monolayer under the inverted microscope to confirm complete removal of cells from the tissue culture flask. Rap the flask sharply to dislodge the Sf1Ep cells from the growth surface.

7. Add 5 ml of pre-warmed Sf1Ep medium to the trypsin in the flask and rinse the surface that once hosted the monolayer.

8. Remove the trypsin-medium mix to a conical centrifuge tube. Gently mix with a pipet to disperse the cells.

9a. To maintain working stocks of Sf1Ep cells, transfer an aliquot of trypsin-EDTA mixture to a new T75 tissue culture flask. Split the cells about 1:15 for transformed Sf1Ep cells (500 µl per 15 ml Sf1Ep medium) or 1:5 for low-passage, untransformed Sf1Ep cells (1.5 ml per 15 ml Sf1Ep medium).

9b. For *T. pallidum* culture, quantitate the cells with a hemocytometer. Calculate the required number of Sf1Ep cells and dilute the cells in Sf1Ep medium such that each 2 ml aliquot will contain 0.5-1.0 x 10^5 cells. Distribute the diluted cells to 6-well dishes.

Gently pipet up and down before removing a sample to load the hemocytometer or distributing the diluted sample into the 6-well dishes. The cells settle to the bottom of the tube very quickly.

9. Incubate 7 days in a 37° C, 5% CO_2 incubator for routine Sf1Ep passage. For *T. pallidum* culture, use within 1-2 days.

Freezing Sf1Ep cells

Sf1Ep cells can be conveniently frozen at passage or when seeding for *T. pallidum* culture.

1. Trypsinize Sf1Ep cultures as described in steps 1-8 of the above protocol.

2. Pellet the trypsinized Sf1Ep cells in a low speed centrifuge for 7 minutes at 125 x g.

3. Remove and discard the supernatant being careful not to disturb the small whitish pellet.

4. Resuspend gently in Sf1Ep freezing medium. Use 1 ml freezing medium for each 1-4 x 10^6 cells.

5. Aliquot the resuspended cells into cryovials.

6. Place cells in a cell freezing container and freeze overnight at -80.

Mammalian cell viability is improved by slow freezing, typically at about -1°C/min. Specialized freezing racks and programmable coolers are available. A less expensive low-tech alternative is to use a styrofoam test-tube rack. The styrofoam rack should be one inch deep to slow cooling of the cryovials when they are inserted into the rack.

7. Move to liquid nitrogen tank after freezing.

Frozen stocks of mammalian cells should be stored in liquid nitrogen or liquid nitrogen vapor in a cryogenic Dewer tank or similar. The cells retain viability for at least several weeks at -80°C but should be maintained at < -130° C for longer storage.

INFECTION OF Sf1Ep CELLS WITH TREPONEMA PALLIDUM

For the infection protocol please see D.G. Edmondson and S.J. Norris, University of Texas Health Science Center at Houston, Current Protocols "In Vitro Cultivation of the Syphilis Spirochete *Treponema pallidum*" (doi: 10.1002/cpz1.44 (Literature 41127))