Appendix for *Treponema pallidum*

Note: For complete instructions, protocols and additional information, please refer to: Edmondson, D. G. and S. J. Norris. "*In Vitro* Cultivation of the Syphilis Spirochete *Treponema pallidum*." Curr. Protoc. 1 (2021): e44. PubMed: 33599121.

Please refer to the individual Product Information Sheets to determine if *in vitro* culture is suitable for a specific strain.

APPENDIX I: PREPARATION OF *T. PALLIDUM* CULTURE MEDIUM 2 (TpCM-2)

1. Prepare fresh TpCM-2 medium following the recipe below one day prior to culture initiation. Filter sterilize using a 0.2 µm filter.

   Note: TpCM-2 medium may be stored at −20°C for up to 6 months prior to use in a non-"frost-free" freezer (no defrosting cycle) with no loss in efficacy. Prior to use, loosen the cap of the tube containing the frozen medium and thaw overnight in a microaerophilic (1.5% O_2; 5% CO_2; 93.5% N_2) atmosphere.

Components	Volume for 50 mL	Final Concentration
CMRL1066 Medium without phenol red or L-glutamine	37 mL	0.8×
100 mM Sodium Pyruvate Solution	364 µL	0.73 mM
0.1% (w/v) Resazurin	50 µL	0.001%
1M MOPS, pH 7.5	1 mL	20 mM
7.5% (w/v) Sodium Bicarbonate Solution	1.08 mL	19.2 mM
200 mM L-Glutamine Solution	500 µL	2 mM
100× D-glucose (15% solution in water)	500 µL	to 17.6 mM
10 g/dL D-mannitol (10% in water)	80 µL	0.88 mM
5 g/dL L-histidine (5% in water)	80 µL	0.52 mM
DL-Dithiothreitol	4 mg	0.52 mM
Heat-Inactivated Fetal Bovine Serum	10 mL	20%

2. Pre-gas fresh TpCM-2 by placing the container (with a loosened lid) in a vented Brewer jar. Exchange the atmosphere in the jar five times. Connect the Brewer jar to a vacuum source and gauge with vacuum tubing and connectors (see Figure 1 in Edmondson, D. G. and S. J. Norris. "*In Vitro* Cultivation of the Syphilis Spirochete *Treponema pallidum*." Curr. Protoc. 1 (2021): e44. PubMed: 33599121.). Draw a vacuum (12 in. of Hg to 18 in. of Hg) in the jar using the vacuum source. Refill the jar with 5% CO_2 and 95% N_2. Repeat vacuum-and-refilling procedure four additional times using 1.5% O_2; 5% CO_2; 93.5% N_2 for the final refill.
3. Quickly transfer the pre-gassed TpCM-2 to a humidified incubator maintained at 34°C with a microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2) and continue pre-equilibration overnight. Leave the lid of the medium container loose to facilitate pre-equilibration. An incubator tank switch may be used, if preferred. Alternatively, after gas exchange in the Brewer jar, seal the gas inlet of the jar with a hose clamp and transfer the entire jar to a 34°C incubator.

APPENDIX II: PREPARATION OF THE HOST CELL MONOLAYER

1. Prepare the Sf1Ep medium following the recipe below. Filter sterilize using a 0.2 µm filter.

   Note: Sf1Ep medium may be stored at 4°C for up to 3 months.

Components	Volume
Minimal Essential Medium Eagle (MEM) with Earle's salts and L-glutamine	500 mL
MEM Non-Essential Amino Acids Solution	5 mL
100 mM Sodium Pyruvate Solution	5 mL
200 mM L-Glutamine Solution	5 mL
Heat-Inactivated Fetal Bovine Serum	50 mL

2. One day prior to culture initiation or passage, seed *Sylvilagus floridanus* epithelial cells (Sf1Ep; ATCC® CCL-68™) into 6-well tissue culture plates with low-evaporation lids at a seeding density of 0.5×10^5 to 1×10^5 cells/well in Sf1Ep medium.
3. Incubate the inoculated plates overnight at 37°C in an aerobic atmosphere with 5% CO_2 until the cells form a 10% confluent, adherent monolayer.

BEI Resources
www.beiresources.org
E-mail: contact@beiresources.org
Tel: 800-359-7370
Fax: 703-365-2898

© 2026 American Type Culture Collection (ATCC). All rights reserved.

APPENDIX III: INITIATION OF *IN VITRO* CULTURE OF *T. PALLIDUM*

1. Three to four hours prior to the start of an experiment or passage, obtain the 6-well plates containing the Sf1Ep monolayers. Remove Sf1Ep medium from the 6-well plates by aspiration.
2. Rinse each well with 1 mL pre-equilibrated TpCM-2 (Appendix I) to remove traces of Sf1Ep medium. Aspirate and discard the TpCM-2 rinse.
3. Add 4 mL pre-equilibrated TpCM-2 (Appendix I) to each well.
4. Place 6-well plates in a Brewer jar. Replace the air in the Brewer jar with a microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2) following the instructions in Appendix I.
5. Quickly transfer plates to an incubator maintained at 34°C with a microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2) and allow to equilibrate for at least 3 to 4 hours.
6. Thaw a frozen vial of *T. pallidum* in a 37°C water bath for 2 to 3 minutes, until thawed. Immerse the vial just enough to cover the frozen material. Do not agitate the vial. *T. pallidum* may exhibit sluggish motility following thawing.
7. Dilute the vial contents such that the inoculum contains 2×10^6 to 5×10^6 motile *T. pallidum*. The inoculum volume is typically 200 µL to 750 µL, although lower or higher volumes may be used.
8. Remove Sf1Ep cultures from the microaerophilic incubator (step 5).
9. Inoculate each well of the 6-well plate containing the Sf1Ep host cell monolayer with prepared *T. pallidum* at the appropriate inoculation volume.
10. Place the inoculated 6-well plates in the Brewer jar and replace air in the jar with the microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2) following the instructions in Appendix I.
11. Quickly transfer plates to the incubator maintained at 34°C with a microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2). Be sure that the base of the culture vessel is well supported during transport to avoid accidental spillage.
12. Incubate cultures for 7 days at 34°C with a microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2).

    Note: *T. pallidum* has a very slow doubling time of 32 hours to 40 hours. Seven days provides for 4 to 5 generations and is a convenient interval between subcultures. Culture time can be extended a few days by feeding *T. pallidum* cultures every 3 to 5 days by removing half the TpCM-2 and replacing it with fresh medium. Despite this procedure, the Sf1Ep cultures may overgrow and fail to support *T. pallidum* growth after 10 to 14 days of culture. Please refer to the individual Product Information Sheets to determine if *in vitro* culture is suitable for a specific strain.

APPENDIX IV: MAINTENANCE AND HARVEST OF *IN VITRO* CULTURE OF *T. PALLIDUM*

1. One day prior to harvest or passage, prepare fresh TpCM-2 medium (Appendix I) and Sf1Ep cultures (Appendix II).
2. On the day of passage, change the medium of the Sf1Ep cultures and pre-equilibrate following the instructions in Appendix II.
3. Immediately prior to harvest, remove *T. pallidum* cultures from the microaerophilic incubator and examine with an inverted microscope to assess confluency and overall appearance of the host cell monolayer.
4. Remove the TpCM-2 growth medium from each well by aspiration and reserve it in separate sterile 15 mL conical centrifuge tubes. Measure the volume of medium to ensure that there has not been significant evaporation.
5. Rinse each well with 0.35 mL trypsin-EDTA and transfer the rinse to the tube containing the reserved medium.
6. Add an additional 0.35 mL trypsin-EDTA to each well.
7. Incubate plates at 37°C in an aerobic atmosphere with 5% CO_2 for 5 minutes.
8. View plates with the inverted microscope to ensure that the Sf1Ep cells are rounded or floating. Rap the edge of each plate to dislodge the Sf1Ep cells from the plate and the *T. pallidum* from the cells.
9. Complete trypsinization is important to maximize *T. pallidum* recovery. Typically, ~ 50% of the *T. pallidum* are attached to the Sf1Ep monolayer in a healthy culture, and these are released by trypsinization. Hold the 6-well plate flat on the stage of the inverted microscope and rap the side of the plate with a polypropylene rack for microcentrifuge tubes to dislodge the cells from the plate. If the cells do not dislodge, add additional trypsin-EDTA and incubate at 37°C for 5 minutes more.
10. When trypsinization is complete and all the Sf1Ep cells are detached, add reserved medium (see step 5) back to the corresponding well to stop the trypsinization.

    Note: When the use of trypsin might be deleterious to an experiment, please refer to the alternative protocol provided in: Edmondson, D. G. and S. J. Norris. "*In Vitro* Cultivation of the Syphilis Spirochete *Treponema pallidum*." Curr. Protoc. 1 (2021): e44. PubMed: 33599121.

11. Rinse well with the reserved medium and return the combined trypsin and medium to the sterile 15 mL conical centrifuge tube.
    Optional: Centrifuge for 5 minutes at 125 × g to pellet Sf1Ep cells. This step reduces variability due to Sf1Ep cell "carryover."
    a) Use 125 µL to 750 µL (typically 250 µL) of the trypsinized, optionally centrifuged culture to inoculate new 6-well Sf1Ep cultures prepared in steps 1 and 2. Three replicate cultures per condition are recommended. Adjust the volume to yield an inoculum of 2×10^6 to 6×10^6 *T. pallidum*.

BEI Resources
www.beiresources.org

E-mail: contact@beiresources.org
Tel: 800-359-7370
Fax: 703-365-2898

© 2026 American Type Culture Collection (ATCC). All rights reserved.

    b) Gas the inoculated cultures in the Brewer jar following the instructions in Appendix I and return new cultures to the incubator maintained at 34°C with a microaerophilic atmosphere (1.5% O_2; 5% CO_2; 93.5% N_2).

12. Quantify harvested *T. pallidum* by darkfield microscopy. Please refer to Support Protocol 3 provided in: Edmondson, D. G. and S. J. Norris. "*In Vitro* Cultivation of the Syphilis Spirochete *Treponema pallidum*." Curr. Protoc. 1 (2021): e44. PubMed: 33599121.

APPENDIX V: CRYOPRESERVATION

1. Transfer 1 mL of the trypsinized culture from Appendix IV to a cryovial.
2. Add 250 µL of sterile 50% glycerol to yield a final glycerol concentration of 10%.
3. Mix gently by pipetting or inversion and place the vial at -80°C.

BEI Resources
www.beiresources.org

E-mail: contact@beiresources.org
Tel: 800-359-7370
Fax: 703-365-2898

© 2026 American Type Culture Collection (ATCC). All rights reserved.