SOP to handle amoebas in a BSL-2 lab

Thawing amoebas.

ATCC guidelines: (https://www.atcc.org/products/30010)

Storage and Culture Initiation from ATCC stock

Frozen ampules packed in dry ice should either be thawed immediately or stored in liquid nitrogen. If liquid nitrogen storage facilities are not available, frozen ampules may be stored at or below -70°C for approximately one week. **Do not under any circumstance store frozen ampules at refrigerator freezer temperatures (generally -20°C).** Storage of frozen material at this temperature will result in the death of the culture.

1. To thaw a frozen ampule, place it in a 35°C water bath such that the lip of the ampule remains above the water line. Thawing time is approximately 2 to 3 minutes. Do not agitate the ampule. Do not leave ampule in water bath after thawed.
2. Immediately after thawing, aseptically transfer contents to a T-25 tissue culture flask or 16 x 125 mm plastic test tube containing 5 mL ATCC Medium 712.
3. Screw the cap on tightly and incubate the tube or flask at 25°C.

Culture mainteinance

1. When the culture is at or near peak density, vigorously agitate the culture.
2. Transfer approximately 0.25 ml to a fresh tube or flask containing 5 ml of fresh ATCC medium 712.
3. Screw the caps on tightly and incubate at 25°C (incubate test tubes at a 15° horizontal slant).
4. The amoebae will form an almost continuous sheet of cells on the bottom surface of the flask or test tube. Repeat steps 1-3 at 5-7 d intervals.

Side notes: The media can be washed by centrifuging the culture at 300Gx5 min at room temperature. Amoebas can be counted and prepared to freeze in 90%FBS and 10%DMSO.

Amoeba preparation for *M. abscessus* infection.

These steps are done on the same day of the infection if the goal is to infect amoebas on flask while shaking. If the goal is to infect amoebas in a plate, this protocol must be done a day before to allow the amoebas to adhere to the plastic.

1. When the culture is at or near peak density, vigorously agitate the culture. Cell scraper can be use to get all the amoebas attached to the plastic.
2. Transfer the culture to a 50 ml conical tube and centrifuge at 300g/5 minutes at RT. Discard supernatant
3. Wash the amoebas with 15-20 ml of PAS solution (PBS for amoebas) once, centrifuge at 300g/5 minutes at RT. Discard supernatant.
4. Prepare amoebas to be counted an at appropriate volume according to the size of the pellet. If the pellet is big, add more media
5. Count at 1:2 dilution with trypan blue, use automatic counter and follow the steps there.
6. Based on the number of live cells, prepare the number of cells needed. Amoeba are infected at a 1:1 ratio. For example, if you have 5x10^6 cells/ml and plan to infect 20 millions of amoebas, you will need 4 ml of the amoeba suspension.
7. Take the volume needed and centrifuge at 300g/5 minutes at RT. Discard supernatant.
8. Resuspend in HALF PYG, prepared with 50ml PAS +50ml of 1X PYG. At this step, amoebas are ready to be infected. If amoebas will be plated and infected the next day, resuspend on complete 1x PYG.

Bacterial preparation for infection

M. abscessus inoculum preparation

1. 500 uL M. abscessus (M. abs) M-cherry is cultured into 10 mL 7H9 +OADC in a small flask and allowed to grow for 48 hours. Depending on the experiment culture only one flask or 2 flasks if more bacteria is needed.
2. After 48 hours measure the OD600. The goal will be an OD between 0.5-1.0. Test OD using a 1:1 dilution. Culture is not syringe passaged, swirled and mixed with a pipette
3. 10 mL of M. abs culture is taken from the flask and spun down at 3700 RPM for 5 minutes

4. The supernatant is removed, and the cells are then washed 3x with 5 mL PBS. Smooshing cells with the pipette to resuspend the cells
5. After the final wash resuspend the cells in 5 mL of half PYG.
6. Syringe passage the cells 15x with a 5 mL syringe and 26 G needle
7. Let the culture settle for 5 minutes and take the OD using a 1:1 dilution.
    a. It is likely that the OD is higher, as the cells were concentrated from 10 mL to 5 mL, it is to account for overall cell loss through the wash steps
8. Dilute M. abs to 1-2x10^7 CFU/mL using half PYG to a final volume of 4-5 mL.
    a. Equation:
       Y=
    b. If OD is too low for this inoculum concentration, spin cells down again and resuspend in a lower volume
9. Calculate how many bacteria is needed depending on the amount of amoebas to infect. Remember, MOI is 1:1. For 1x10^6 amoebas you will need the same number of bacteria.