# LIQUID NITROGEN FREEZING PROTOCOL (MODIFIED)

1. **Preparation of cells to be frozen**
   Grow cells on TAP plate, 3-5 days.

2. **Day before freezing: prepare Nalgene box**
   Pour 250mL isopropanol in box and store at 4°C.

3. **Day of freezing: prepare cell solutions**
   - Resuspend cells to $6x10^6$cells/mL in 10 mL TAP.
   - Aliquot 750μL of TAP + 10% methanol (filter sterilized) into labeled 2mL cryotubes.
   - Make one aliquot in Eppendorf tube if doing pre-freeze titer.
   - Make one cryotube for post-freeze titer.

4. **Mix of cell culture and TAP + 10% methanol under hood, <u>lights off</u>**
   - Check that no isopropanol is on the rack inside the Nalgene box.
   - Transfer 750μL of cell culture into cryotube.
   - Keeping tube hidden in hand, invert gently.
   - Place tubes in Nalgene box and close lid, keeping in dim light for remainder of procedure.
   - Set aside mixture in E. tube and plate dilutions after placing cryotubes in -80°C incubator.

5. **First freezing at -80°C for 1h 30m**

6. **Second freezing at -170°C in liquid nitrogen**
   - Prepare freezer box: place in container with ~1 inch liquid nitrogen so tubes stay cold.
   - Take tubes out of -80°C and immediately place in pre-chilled freezer box.
   - Place box into liquid nitrogen freezer. Alternatively, use forceps to transfer tubes to a stock box in freezer.

# THAWING PROTOCOL

1. **Take cryotube out of freezer and keep on dry ice**

2. **Thaw in 35°C water bath**
   - Set a water bath at 35°C.
   - Place tubes in a rack and put in bath for 5 min. <u>Shield from light.</u>
   - Take tubes out of water bath and wipe. Place in box to shield from light.

3. **Elimination of TAP-methanol mix**
    - Under hood, lights off, transfer cells into 2 Eppendorf tubes (split sample in half) with a sterile filter tip.
    - Centrifuge 2 min. at slow speed (1,500 rpm-just enough to pellet).
    - Carefully remove most of the supernatant (be careful; pellet is loose). May leave some supernatant as pellet is loose.
    - Resuspend each pellet in 1mL TAP (may use TAP+Tween as cells tend to stick to tubes) and combine into a sterile 13x100 mm tube. Let sit in dim light overnight (on bench is OK).

4. **Plating cells**
    - Post-freeze titer: If recovery will be measured, then cells need to be counted, serially diluted and plated. The cfu can be compared to the total number of cells or to control samples from the same culture that were not frozen.
    - Spread 300μL of each aliquot of cells onto a TAP plate.
    - Optional: Spin down remaining cells in a Eppendorf tube at low speed and plate.
    - Resuspend remaining cells in 300μL TAP and plate on a third TAP plate, or try to recover cells in liquid by diluting into 10 mL TAP.