



Note* Make sure the lid has A1 marked and that the orientation of the lid and plate stay the same!

- 4) Place the cells on a shaker inside the incubator: 275rpm, 37°C, 8% CO₂
- 5) Once cells are in the incubator, prepare Cell Boost Feeding Media for duration of the 14-Day experiment
 - a. Calculate the amount of feeding media needed to feed all wells for duration of experiment (add some more for comfort)
 - b. Vacuum filter the appropriate amount of Cell Boost 7A and Cell Boost 7B into SEPARATE 50mL tubes. Store @ 4°C

Note* DO NOT COMBINE.

- 6) Start the daily Cell Boost feeding schedule on Day 3
 - a. 7a and 7b are separately warm for 5 minutes in 37°C water bath.
 - b. Add the feeding media at 2% of 7A: 50uL and 0.2% of 7B: 5uL per well
 - c. Continue feeding once per day, including Day 13. No feed on the final Day 14.
- 7) On Day 4, turn the temperature down to 32°C
 - a. While the incubator is lowering the temperature, feed the cells.
- 8) Glucose Measurements (Start on Day 3):
 - a. measurements should be between 3-4 g/L.
 - b. Test Glucose level on Day 3, Day 5, Day 7 and after (everyday)
 - c. If the glucose level is < 3g/L: add stock glucose (200g/L) to bring the concentration to 4g/L. And then feed with booster media to 2% (e.g. add 50uL of 7A and 5uL of 7B to 2.5ml).
 - d. If the glucose level is 3g/L – 5g/L: Do not add glucose. Feed with booster media to 2% (e.g. add 50uL of 7A and 5uL of 7B to 2.5ml).
 - e. If the glucose level is >5g/L: Do not add glucose. Do not feed with booster media.
- 9) On Day 7, Day 9 (or Day 10), Day 12 (or Day 11), and Day 14: count cells (may need to perform a dilution prior to mixing with trypan blue), collect supernatant.
 - a. Collect the entire well corresponding to the timepoint --> Mix very well by pipetting up and down (7-10x) and transfer all contents to a 15 mL falcon tube.
 - b. Count cells
 - c. Spin the entire falcon tube @ 125xg for 7 minutes
 - d. Transfer the clarified supernatant to a new falcon tube
 - e. Resuspend the cells in 1mL PBS and transfer to a 1.5mL reaction tube --> pellet cells again @ 1500xg for 5 minutes, discard supernatant and freeze cells @ -80°C
 - f. Aliquot from the transferred supernatant, 200uL to 1.5mL reaction tube for Osmolarity/Glucose measurements, 200uL to 1.5mL reaction tube for Valita (freeze this immediately with cell pellet tube @ -80°C)



- g. After collecting all contents and cells are spinning down, wash the well with 5mL PBS and aspirate. Fill with 2.5mL fresh PBS

Feeding Schedule Table

DAY	Action
DAY 3	Begin feed, add 50uL of 7A and 5uL of 7B feed media each well, 37°C
DAY 4	Add 50uL of 7A and 5uL of 7B feed media each well, Switch temperature to 32°C
DAY 5	Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 6	Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 7	Count cells, collect supernatant and aliquot 200ul for Valita, 200ul for Osmolarity and Glucose, and cell pellet: store at -80. Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 8	Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 9	Count cells, collect supernatant and aliquot 200ul for Valita, 200ul for Osmolarity and Glucose, and cell pellet: store at -80. Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 10	Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 11	Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 12	Count cells, collect supernatant and aliquot 200ul for Valita, 200ul for Osmolarity and Glucose, and cell pellet: store at -80. Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 13	Add 50uL of 7A and 5uL of 7B feed media each well, 32°C
DAY 14	Count cells, collect supernatant and aliquot 200ul for Valita, 200ul for Osmolarity and Glucose, and cell pellet: store at -80°C.

Glucose Measurements (measure this once you have supernatant aliquots ready for all samples)

- Insert strip into monitor
- Add 1.5uL into the side of the capillary
- Measurement should take 3-5 seconds per sample, and most measurements should be between 3-4 g/L

Osmolarity (OSM) (measure after feeding the cells, basically last task to do)

- Warm up the machine for 10 minutes (do not warm for longer as machine will turn off)
- Run the standards three times (three separate tubes with 30uL in each tube) NO AIR BUBBLES!

Note* Between each sample/tube, use a kim wipe to wipe off probe.

- Once the standard looks consistent over three measurements, proceed with samples.
 - Samples → 30uL of the supernatant per tube, measure twice, if numbers aren't within 5-10 units of each other, process a third sample.

