**Protocol title:***Calibration of spectrophotometer to colony-forming-units (CFU)*

**Aim of the protoco****l:** *The aim of this protocol is to be able to convert from OD readings to CFU. As detailed in the references below, the OD depends on the size of the cells, the refractive index of the media, and the optics of the spectrophotometer. This means that it is necessary to perform a calibration for each machine, each media, each strain, and each growth condition. Since cells tend to be larger during the exponential phase and then shrink in size during the stationary phase, it may even be necessary to do calibration for cells in different growth phases…*

*Calibration can be done either for the cuvette spectrophotometer or for the microplate spectrophotometer (or both). Here we follow the recommendations laid out by:* [*https://doi.org/10.1371/journal.pone.0276040*](https://doi.org/10.1371/journal.pone.0276040)*. The protocol is for stationary phase cultures but it is possible to use exponential phase culture if the starting culture volume is increased.*

**Materials** *[List of equipment used, buffers, suspensions etc. Include information of the make and company.]*

* Bacterial liquid culture in medium of interest and at growth stage of interest  
  *For calibration of cuvette reader, you will need ~10.0mL of culture.  
  For calibration of both microplate readers, you will need ~8.0mL of culture.*
* Sterile medium of interest (~30mL)  
  *For minimal media, it is possible to omit the carbon source.*
* 50mL (“Falcon”) centrifuge tube
* Centrifuge
* Sterile 96-well, deep-well reservoir  
  *For calibration of cuvette reader, you can use a single 12-well reservoir instead.*
* P200 12x multi-channel and appropriate sterile tips
* P1000 pipette and appropriate sterile tips
* Sterile microplates and optically clear sealing membrane
* Cuvettes
* Agar plates
* Ethanol, plate spreader, and Bunsen burner

**Step by step procedure**

1. Turn on the microplate readers and load the program called “CFU\_calibration.prt”.
2. Put 18mL of the stationary phase bacterial culture in the 50mL centrifuge tube and spin down: 4000 rpm for 15min.
3. While you’re waiting, you can prepare to make the dilution series:
   1. For calibrating the 2 microplate readers: Fill 23 wells (i.e., 2 rows) of a 96-well reservoir with 900µL of sterile, room temperature media. Leave the first well empty!
   2. For calibrating the cuvette reader: Fill 11 wells (i.e., 1 row) of a 96-well reservoir with 1350µL of sterile media. Leave the first well empty!
4. Once centrifuge is finished, discard the supernatant, and resuspend the pellet in 4.5mL of fresh media. *This yields a 4x concentrated bacterial culture.*

Calibrating the 2 microplate readers:

1. Use the concentrated culture to make a 21-point, 2-fold dilution series:
   1. Load 900µL of the concentrated culture to the 1st well (this should be empty).
   2. Then load 900µL of the concentrated culture to the 2nd well.
   3. Discard the tip.
   4. With a fresh tip, pipette up and down in the 2nd well to mix several times.
   5. Aliquot 900µL from the 2nd well into the 3rd well.
   6. Repeat steps c)-e) 18 more times until you reach well B9 (i.e., you should have filled 21 wells in total with culture).
   7. Leave wells B10-B12 empty. These will be negative controls used for baselining.
2. Using the multichannel, fill 2 microwell plates with 200uL from the reservoir.  
   Cover the plates with Breathe-Easy optically clear seals.
3. Put the microwell plates in each of the plate readers and start the program.  
   The program will first incubate for 20min at 25°C, reading the absorbance at 600nm every 4min. Then it will increase the temperature to 40°C for 20min, again reading the absorbance at 600nm every 4min.
4. Finally, plate 100µL from each of the following wells: B3 (dilution=2-14), B6 (dilution=2-17), and B9 (dilution=2-20).

Calibrating the cuvette reader:

1. Use the concentrated culture to make a 12-point, 4-fold dilution series:
   1. Load 450µL of the concentrated culture to the 1st well (this should be empty).
   2. Then load 450µL of the concentrated culture to the 2nd well.
   3. Discard the tip.
   4. With a fresh tip, pipette up and down in the 2nd well to mix several times.
   5. Aliquot 450µL from the 2nd well into the 3rd well.
   6. Repeat steps c)-e) 9 more times until you reach the end of the row (i.e., you should have filled 12 wells in total with culture).
2. Turn on the cuvette reader and baseline it with 1.0mL of sterile culture.
3. Starting from the most concentrated well, load 1.0mL into a fresh cuvette and get the OD at 600nm. Measure the OD 3 times for each dilution.
4. When the OD measurement from the cuvette reader reaches 0.000 for all 3 replicates, there’s no need to acquire the OD readings of the more diluted samples.  
   Instead, plate 100µL from all remaining dilutions (it’s okay to skip the first and second dilutions after the threshold of detection was reached).  
   *Be sure to plate at least 5-6 dilutions down from the last detectable OD reading!* Depending on the concentration of the concentrated culture, you may need to make further dilutions by following the ratio of 1350µL sterile media to 450µL diluted culture.

**References** *[Include references of related methods and references to research articles using the protocol developed.]*[*https://doi.org/10.1371/journal.pone.0276040*](https://doi.org/10.1371/journal.pone.0276040)

This article is a highly recommended reading. However, it points out more problems than solutions so beware that is may be demotivating.  
Stevenson, K., McVey, A., Clark, I. *et al.* General calibration of microbial growth in microplate readers. *Sci Rep* **6**, 38828 (2016). <https://doi.org/10.1038/srep38828>

Another method of calibration is using beads: <https://doi.org/10.1038/s42003-020-01127-5>

**Modifications** *[Include date, which material/step was modified and by who.]*

It is possible to calibrate to exponential phase cells, but you will need to start with a long more bacterial culture. For example, you can try pelleting 50mL of exponential phase culture and resuspending that into 4.5mL.