**Protocol title:***Quantification of colony forming units (CFU) using 6x6 drop plating*

**Aim of the protoco****l:** *to accurately count the concentration of viable single-celled microbes (e.g., bacteria or yeast) in a sample. This protocol uses a 10-fold dilution series that is plated for 6 different concentrations. It plates a larger volume for each dilution step (30uL) because this is more accurate (but you can adjust it to plate a single 5uL drop for each dilution step to yield a total of 6 different samples at 6 dilutions each). This protocol plates the same dilution series at three different incubation temperatures. This protocol should primarily be used for samples where you have no prior estimate of the CFU’s. If you have some idea of the concentration of your sample, you will get more accurate estimates by reducing the dilution series (e.g., 5-fold).  
Note that you should do* at least 3 *replicates for each sample to estimate variance of the mean.*

**Materials**

* Biosafety cabinet
* Bacterial cultures/samples that need to be quantified:  
  Single-use inoculum shots, kept at -80 and thawed only before use.
* Sterile LB
* A sterile 96 deep-well (2mL) dilution plate
* P1000 single channel & sterile pipettes
* P100 single & multichannel & sterile pipettes  
  *Note: If using a multi-channel, make sure it is properly calibrated, has tight fitting tips, and watch the tips carefully as you pipette to see that the volumes are all even.*
* Agar plates with appropriate media (e.g., circular 94mm plates):  
  You will need 9 for each sample + extra for mistakes + extra for controls.  
  *Note: Use well dried plates (i.e.,* ***not*** *freshly poured plates!). Dry plates for ~1 hour under laminar flow or leave them overnight at RT to dry sufficiently.*
* Incubator(s)
* -20 cold block (for keeping inoculum shots frozen until use)

**Step by step procedure**

1. If using freshly poured plates, make sure the plates are dried out sufficiently so that the inoculum will absorb readily into the agar.  
   If using previously prepared plates, be sure to warm the plates up to room temperature.
2. Label the plates with the sample and incubation temperature before you begin making the dilution series.
3. Prepare the dilution series by aliquoting 900uL of LB to rows A-F of the deep-well plate.  
   *Note1: different samples and/or different replicates for the same sample will go in the different columns.  
   Note2: it is ideal to prepare different samples sequentially as it will take you a little while to plate each one.*
4. Using the P100, add the 100uL inoculum shot to row A. Discard the pipette tip.
5. Pipette up and down 5 times to mix. Aliquot 100uL into row B. Discard the pipette tip.
6. Repeat step 4 for rows C through F.  
   **One of the most common sources of inaccuracy in this protocol is the dilution series. Therefore, it is critical that you discard the tip when going from a higher to lower dilution. And that you mix sufficiently before aliquoting to the next dilution.**
7. Aliquot the 6x6 grid of drops onto the plate:
   1. Check that the label on the agar plate matches your sample.
   2. Set to P100 multichannel to 30uL and securely load 6 tips.
   3. Insert the tips into wells A-F (or, if using, C-H) of the same column. Pipet up and down several times to mix the 6-point dilution series.
   4. Pick up 30uL of liquid. Drop microdroplets in ~6 rows onto the agar plate. Make sure that **all the liquid** is aliquoted onto the plate, even if >6 rows needed.
   5. Allow the plate to sit uncovered for 5-10 minutes until all liquid is absorbed into the agar.  
      *Note: Drops may sometimes merge; that’s okay, try your best to prevent this. When this happens, discard the whole plate and drop again.  
      If you find that the drops are often merging or that you need to pipette >>6 rows very close to the edge of the plate, consider reducing the total volume. But DO NOT reduce the total volume to <20uL!! (Alternatively, you may consider increasing the total volume for more accurate counts.)*
8. Repeat step 7 eight more times (for a total of 9 identical plates per sample).
9. Then, repeat steps 3-8 for each sample.
10. Plate 180uL (6 x 30uL, or adjust as needed) of LB on 3 agar plate as negative controls. These will be incubated at a different temperature each.
11. Seal plates with parafilm and incubate 3 replicates at 28C, 3 replicates at 35C, and 3 replicates at 40C with agar face-down. Check on the cultures as appropriate (e.g., overnight for fast growers, in the afternoon for slow growers) for up to 7 days until the colonies are large enough to be counted by eye.
12. Count the colonies:
    1. For each sample, select the concentration where there are >18 but <180 colonies *in total* for all the drops in that column (i.e., in the total volume of 30uL plated).
    2. Count the total number of colonies observed at this concentration.
    3. Calculate the CFU of the initial culture by using the total volume (30uL) and the dilution of the counted concentration,  
       *For example, 36 CFUs counted at gives*

**References**

*The spread plate method is considered the CFU “gold standard” (i.e., aliquoting 100uL of diluted cells onto an agar plate, spreading the drop evenly across the agar using an inoculum-spreader, repeating this for multiple dilutions, incubating overnight, then counting the plates with 20-200 or 30-300 colonies) but it requires a lot of plates (and spread plating may be too aggressive for some organisms). 6x6 drop or tilt-dilution plating are alternative methods that use fewer plates and a multichannel pipette for more efficient CFU quantification. For both methods a total inoculum of ~50-60uL of diluted cells should be plated (a lower volume will lead to less accurate CFU estimates!). The main problem with tilt-dilution plating is merging of tracks; but instead of tilting it’s also possible to gently drag the multichannel along the agar surface as the liquid is ejected. I chose not to use this method because I personally find it difficult to make the tracks and then later to count colonies corresponding to the same track… Instead, I think it’s easier to do the drop method. Finally, another alternative is the single plate-serial dilution spotting (SP-SDS). This method does not use a multichannel and it uses a smaller total inoculum (20uL total aliquoted in 10-12 microdroplets); this means that it is more laborious and perhaps more inaccurate (but see the reference for this method below). The reference details other advantages of the SP-SDS method as compared to the drop or tilt methods but I didn’t understand why this would be the case.*

Wise 2006 “Preparing spread plates protocols” American Society for Microbiology <https://asm.org/ASM/media/Protocol-Images/Preparing-Spread-Plates-Protocols.pdf?ext=.pdf>   
Chen et al. 2003 <https://doi.org/10.1016/S0167-7012(03)00194-5>

Thomas *et al.* 2015 <https://doi.org/10.1016/j.btre.2015.08.003>

**Modifications**

*If you’re interested in comparing cell viability for different environments, then you will want to compare CFU’s for the same dilution series but using different media or different temperatures (for example). The protocol above yields a total volume of 900uL per concentration but only uses 60uL per plate; this means that you can compare up to 14 different environmental conditions without any adjustments to the volumes. If you need to compare more environments, then you will have to adjust the volumes. Remember that the deep-well 96-well plates hold maximum 2.2mL but you should leave a few hundred uL “head” room to prevent contamination between wells.*

*If using soil directly, make sure that you spin down and allow the debris to settle for the 10^0 dilution. Then dilute by carefully taking only the liquid phase from the well plate. Plate only 10^-2 onwards.*

*Another modification is to use a different dilution series. Dilutions smaller than 10-fold (e.g., 5-fold) will make your CFU estimate more accurate. If you already have some information about the expected concentration (e.g., from OD values), then you can try to dilute with smaller fold and plate the concentrations such that you expect to see 50 colonies in the 3rd or 4th plated dilution.*