STAINING CELLS FOR DIRECT EPIFLUORESCENCE COUNTING

SOLUTIONS

4', 6 diamidino-2-phenylindole (DAPI)

Stock solution: 1000 µg/ml (dissolve 5 mg in 5 ml 0.2 µm filtered DI water).

Working Solution: 200 µg/ml (add 1 ml stock solution to 4 ml DI).

Filter the working solution through a 0.2 µm acrodisc

(**A filtered working solution has been prepared for you**)

Phosphate buffered saline (PBS)

METHOD

1. Place a glass fiber (Whatman GF/F) backing filter, which simply serves as a gasket, on the Millipore frit, and dampen with a little DI water (GF/F filter should be reused). Place on top of the backing filter a 0.22 µm pore size black polycarbonate (PC) filter on the backing filter. Clamp on the 15 ml filter tower.
2. Mix 1.8 ml PBS and 0.2 ml of preserved sample in a sterile snap cap tube. In another snap cap tube place 2 ml of PBS. This will be your blank. Label accordingly.
3. Stain your cells and PBS blank for 5 minutes with 100 µl DAPI working solution. This is located in foil wrapped microcentrifuge tubes in the refrigerator (or on your workspace).
4. After a 5 minute incubation, pour or pipette the sample into the Millipore filter tower. Pull the water through the filter using the hand pump, but DO NOT ALLOW THE FILTER TO GO DRY PRIOR TO ADDING THE PBS RINSE BELOW. Always use low pressure (<12 cm Hg) filtration to avoid breaking cells.
5. Rinse tube and sides of the filter tower with approximately 1-3 ml PBS (volume used is not critical) using squirt bottle or pipette. Allow all water to pass through filter.
6. Put a small drop of microscope immersion oil on a microscope slide.
7. Remove the PC filter from the frit and place it on top of the oil. KEEP FILTER FACE UP, as that is where the bacteria are. Dot another drop of oil on a glass cover slip and place it on top of the filter, oil side down. Add a third drop of oil to the top of the glass cover slip.
8. Label the slide with your initials, and the column number (or blank if it is one). Remove gloves before using the microscopes.
9. Count at least 5 fields in the ocular grid using the epifluorescence microscope using the 100x oil-immersion objective. If there are only a few bacteria per field then you would normally prepare another filter, but use a larger sample volume (1-2 ml, without PBS), and add DAPI proportionately (50µL/mL). Likewise, you would reduce sample volume if there are too many bacteria to count. However, there may not be time to prepare another filter.
10. While waiting, measure the inside diameter of the filter tower needed for calculations.
11. Calculate the average number of cells per field (note if using an older microscope without the Zen software, the area of the gridded field is 100 µm by 100 µm; otherwise, use area calculated by software), the working area of the filter, and the number of cells per milliliter. The volume of your sample should not include the PBS buffer that was added in step 2. Also make sure to account for dilution of your sample by the formaldehyde preservative.