STAINING CELLS FOR DIRECT EPIFLUORESCENCE COUNTING

SOLUTIONS

4', 6 diamidino-2-phenylindole (DAPI) (Sigma Chemical D9542)

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)

GLOVES AND SAFETY GLASSES REQUIRED

METHOD

1. From your preserved sample from the last lab, add 1.8 ml PBS with 0.2 ml of sample in a sterile snap cap tube. In a second snap cap tube place 2 ml of PBS. This will be your blank. Label tubes accordingly.
2. Stain your cells and PBS blank for 5 minutes with 100 µl DAPI working solution. This is in a foil wrapped microcentrifuge tubes in the refrigerator (or on your workspace).
3. While your samples incubate, 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.
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. Draw all water to through filter using vacuum.
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 the blank). 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 the older microscopes, the gridded field is 100 µm by 100 µm and the diameter of the whole field is 200 µm. On the newer scopes, the diameter of the FOV is 225 µm. If using the display capture, the screen shows a rectangle 190 × 100 µm. 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.