Quantification of Bacteria by Flow Cytometry

Bacteria are small and the intensity of scattered light/SSC signal is close to the detection limit of standard benchtop flow cytometers. Therefore, flow cytometric enumeration of bacteria requires the use of nucleic acid-specific stains, such as SYBRGreenI often combined with a non-membrane permeable staining like Propidium Iodide (PI) for live/dead discrimination. One of the benefits of flow cytometry is that the viability of individual cells can be examined even when those cells are ‘‘non-culturable’’. However, dye penetration and efficiency of staining can greatly differ depending on the type of bacteria and staining protocols. Optimal detection of bacteria therefore requires optimization of various factors such as concentration of the fluorescent dye, the solution used to dilute the sample or the need of a pretreatment, e.g. EDTA can be used to permeabilize the outer membrane of the gram-negative cell wall or the addition of a carbon source in starved cells, etc. (1). To reduce background in undefined samples taken from the environment caused by particles like algae, several centrifugation and filtration steps might be needed prior to any treatment.

Note: Because bacteria are not much bigger than the wavelength of the 488 nm laser light, the SSC signal cannot be used as an indicator of size and granularity, but can be useful as a discriminator of different bacterial populations.

Dyes

Cell Permeable Stains

SYBRGreenI has a strong affinity for dsDNA, medium affinity for single stranded DNA and low affinity for RNA.

The SYTO dyes are lower-affinity nucleic acid stains that passively diffuse through the membranes of most cells. These UV- or visible light–excitable dyes can be used to stain RNA and DNA in both live and dead gram-positive and gram-negative bacteria.

For discrimination between gram-positive and gram-negative bacteria a combination of hexidium iodide and a SYTO dye of a different emission wavelength can be used. Hexidium

iodide is selectively cell permeant for gram-positive bacteria and displaces the SYTO dye.

Cell Impermeable Stains

Propidium Iodide (PI) is a DNA-intercalating dye and a widely used dead cell stain, as it is impermeable to living cells.

But there are also other dyes, e.g. of the monomeric TO-PRO™ family, often improved as dimers like the TOTO™ family, excited by and emitting in various wavelengths and therefore giving more opportunities for detection depending on the instrument used. All these dyes are dead cell stains, except YO-PRO™-1 which can already stain apoptotic cells.

Also the SYTOX dyes are are especially useful for staining both gram-positive and gram-negative bacteria as dead cell stains and available in several colors.

The following instructions in principle refer to all dyes but might be more important for some of them and should also be checked in the manufacturer´s protocol:

- ☑ Limit number of freeze-thaw cycles (two or three) to prevent loss of staining efficiency.
- ☑ Briefly spin stock solution to reduce noise (~20.000 x g)
- ☑ To limit noise, dilute stock for further dilutions not in DMSO.

Samples

Dilution

Salts present in the samples can interfere with the efficiency of the staining. Therefore, cultured bacteria should be centrifuged and washed twice in buffer. Samples from sea water which cannot be centrifuged should be diluted to a final dilution factor greater than 10-fold. Undiluted samples from fresh water need to be tested for staining efficiency.

- ☑ Dilution of the sample in TE Buffer (10 mM Tris-HCl and 1 mM EDTA, pH8) is recommended instead of PBS, Milli-Q water to avoid electronic coincidence. TE buffer should be sterile filtered (0.2 µm) to maintain low background fluorescence.

Staining

All staining procedures are usually performed at room temperature and samples should be kept in the dark during incubation. As the dyes are little to non-fluorescent in aqueous medium they can be used without washing steps.

- ☑ SYBRGreen I commercial stock should be diluted 1:10000 and incubated for 15 min (3).

- ☑ For staining of bacteria with cell permeable SYTO dyes it is recommended to use a concentration of 50 nM – 20 µM for 1-30 minutes. For combination with a dead cell stain single stains of each dye should be added.
- ☑ For dead cell stains also a positive control should be prepared by incubating a sample in 70 % ethanol for 60 min, washed twice in buffer and stained together with the samples.
- ☑ PI should be used at a concentration of 3 µM (1:500 of the 1 mg/mL = 1.5 mM stock) and incubated for 15 min (3).
- ☑ The nucleic acid stains of the TO-PRO™ and the TOTO™ family should be used at a concentration of 0.1 µM for 10-20 minutes (3).
- ☑ SYTOX dyes should be diluted 1:1000 and incubated for 15 min before measuring (3).

Instrument Setup and Measurement

Blanks and References

- ☑ Optimal control blanks consist of buffer and a bacteria free (0.2 µm filtered) sample with the dye(s) added at the same dilution factor as the natural sample.
- ☑ Very low coincidence and background fluorescence levels should be detected before proceeding with sample analysis. Blanks ideally show a total amount of 400-1100 events in 1 min of acquisition at a flow rate of 40 µl / min.
- ☑ During the analysis, always add one blank to every batch of samples to monitor whether the noise level stays low.

Instrument Setup and Measurement

- ☑ First determine the maximum voltage for the relevant fluorescence PMT at which no electronic or laser noise is detected. Optimal PMTV can be assessed using freshly prepared Milli-Q water as sample and increasing the voltage for the fluorescence PMT until noise is detected; the voltage that can be used is just below this. In case bacteria populations go off-scale the voltage should be decreased until all stained populations are completely visible in the plot.
- ☑ To obtain optimal sample flow, sample should be diluted before analysis to minimize electronic coincidence of the bacteria (two or more bacteria pass the analysis window of the laser simultaneously, reducing accuracy of the analysis). Coincidence is minimized at event rate <1000 events s-1.
- ☑ In case of a high event rate sample (i.e. >1000 events s-1), rinse shortly with TE-buffer or Milli-Q before the next samples.

Acquisition

- ☑ Fluorescence and scatter signals are collected on a logarithmic scale (4-decade dynamic range) for best results.
- ☑ Threshold is set on the used fluorescence, at minimum level. Data are acquired on a dot plot displaying the respective fluorescence versus side scatter signal.
- ☑ Outer sleeve of the sample injection port should be cleaned between samples to prevent cross-contamination.
- ☑ Allow the flow rate to stabilize for 10-15 sec. before recording the sample. Acquisition time is typically 1 min.

References

1. Life, Death, and In-Between: Meanings and Methods in Microbiology; Hazel M. Davey; Applied and Environmental Microbiology, Aug. 2011, p. 5571–5576; doi:10.1128
2. An Optimized SYBR Green I/PI Assay for Rapid Viability Assessment and Antibiotic Susceptibility Testing for Borrelia burgdorferi; Jie Feng, Ting Wang, Shuo Zhang, Wanliang Shi, Ying Zhang; PLoS ONE 9(11): e111809. doi:10.1371/journal.pone.0111809
3. Additional information on the dyes and staining protocols can be found at https://www.thermofisher.com.