Application Note

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Detection of Bacteria in Environmental Waters using the NovoCyte Flow Cytometer

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Introduction

Rapid detection of bacteria in aquatic environments has been a challenging task in microbiology studies, especially for natural water containing complex microbial populations. The difficulties are further compounded by examining the activity, size, and physical properties of individual populations. Traditional methods of bacterial identification are based on observations of the morphology of single cells or colony characteristics when grown on agar. However, the microbes grown on agar medium with visible colonies are less than 1% of the total, and most bacteria, though obviously present and active, are not efficiently cultured.

Flow cytometers with a high sensitivity of detection provide tools for detecting and analyzing microbes independent of their cultivability. The size, number, nucleic acid content, activity, and classification of bacteria can be derived from scattered light and fluorescence signals using flow cytometry. Applications using microbial detection cover everything from drinking water/waste water system monitoring to soil and water microbial ecology. This method allows precise and rapid determinations of microbial bulk parameters and delivers detailed information on the general microbial state.

The Agilent NovoCyte flow cytometer can detect very small particles with high sensitivity. Combining multiparameter analytic capability and convenient fluidic maintenance, the NovoCyte flow cytometer can easily be applied to various microbial studies.

Materials and methods

Determination of total bacteria count in water samples from multiple sources

Flow cytometry allows the discrimination of very small microbial cells from background signals after staining with a fluorescent dye that binds to nucleic acids (for example, SYBR Green I, SYTOX Green I). In addition, precise determination of the absolute bacterial count is made possible by the automatic cell counting incorporated into the NovoCyte system. This is based on the use of a volumetric syringe pump for sample acquisition.

To efficiently detect bacteria by flow cytometry, fluorescence triggering was used to identify the bacteria and separate them from inorganic particles in water samples on the NovoCyte flow cytometer. To achieve this, nucleic acid dye was added to the samples so that only fluorescent particles representing DNA-containing organic material were recorded. Using an unstained water sample, a fluorescence threshold value was set to distinguish background noise from events triggered by fluorescent particles. For this application with SYBR Green dye, a 488 nm laser for excitation and detector with a 530/30 filter was used to set the threshold and measure fluorescence.

Tips for detection of sub-micron particles with the NovoCyte system

Set up the NovoCyte flow cytometer instrument for determining light scattering of bacteria and background noise:

1. Clean the instrument as best as possible. Use clean, filtered DI water to dilute NovoFlow sheath fluid, and run cleaning and rinsing cycles.
2. Carry out routine QC tests to evaluate instrument performance.
3. Test a blank sample (0.1 µm filtered DI water) with the lowest threshold setting (SCC at 10) to determine background noise on FSC versus SSC and on any applicable fluorescence detection channels.
4. Identify proper thresholds for either the scatter or fluorescence detection channels. Run a blank sample to verify correct settings by observing a substantial decrease in the events collected.
5. With the applied thresholds from the previous steps, run small particle samples.

Note: Some small particles are difficult to detect using only FSC or SSC. If so, it is best to use a fluorescent label to identify the small particles of interest.

Using flow cytometry, the bacteria in natural water can be differentiated into two groups: bacteria of low nucleic acid content (LNA) and of high nucleic acid content (HNA) (Figure 1). It is broadly accepted that HNA is active bacteria, whereas LNA is inactive, dead, or a dormant population. Whether LNA and HNA are different types of bacteria or physiologically in different states is still unclear. The fluorescence spectra of bacteria are also adopted by some researchers as bacterial fingerprints, which can be valuable for detection of population changes that are not reflected in the cell concentration.[3,4]

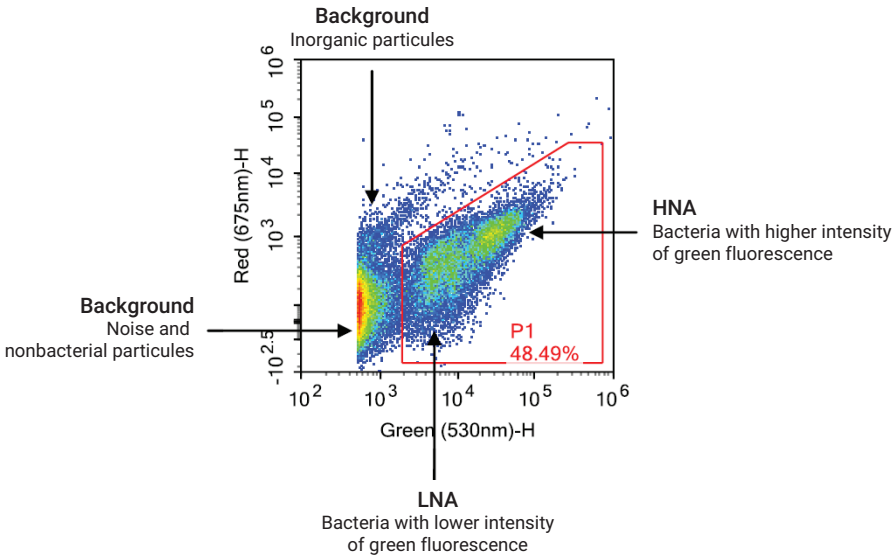


Figure 1. Detection of bacteria in natural waters. Fresh natural water (or stock at 4 °C) was filtered through a 300 mesh sieve, diluted with deionized water (filtered through a 0.1 µm membrane) to a desired concentration. A 100x SYBR Green I dye (Invitrogen, S7563, diluted 100-fold in DMSO) was added to the sample, and the mix was incubated at 37 °C for 13 minutes. Agilent NovoCyte settings: 30 µL collection volume, medium flow rate, threshold of FITC-H at 500. Bacteria were differentiated from background by FITC versus PerCP plots, and HNA was separated from LNA by the intensity of green fluorescence. Absolute counts were obtained automatically in each sample.

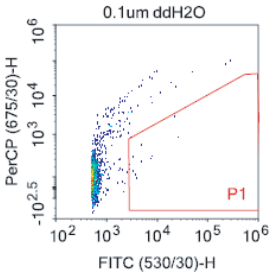
Results and discussion

In this application note, using a NovoCyte flow cytometer, the total counts of bacteria in multiple environmental waters were precisely quantified, including deionized water (ddH_2O), bottled water, tap water, water from a mountain stream, spring (1:10 spring), lake (1:20 lake, 1:40 Maojiabu), and wetland (1:50 wetland) water.

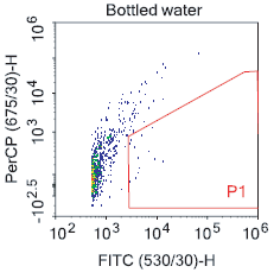
The deionized water sample was filtered through a 0.1 µm membrane and stained with SYBR Green I. Absolute cell counts were obtained using the NovoCyte flow cytometer. The counts in the P1 gate that identifies bacteria showed less than 1 cell/µL for unfiltered bottled water, which correlates to the values for filtered DI water (Figure 2). Total cell counts for all samples are listed in Table 1. Specified in guidelines of drinking water analysis from the Swiss Federal Office of Public Health, the acceptable limit of bacteria in drinking water is 2.0×10^2 cells/µL. Results showed that natural waters contained the highest bacterial counts of the samples tested, and bottled water the lowest. Bacterial counts in still water were 10 to 100x higher than that of spring and mountain stream sources.

Table 1. Total cell count of bacteria in various water samples obtained automatically on the Agilent NovoCyte flow cytometer.

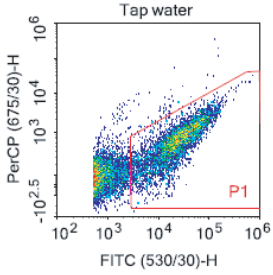
Water	TCC (Total Cell Count) (Cells/µL)
0.1 µm filtered deionized water	<1
Bottled water	<1
Tap water	195
Mountain stream	206
Spring	784
Lake 1	3,655
Lake 2	7,893
Wetland	9,200



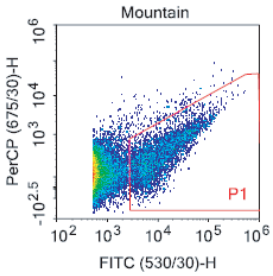
Gate	Count	Absolute Count
All	1,638	54.6
P1	12	0.4



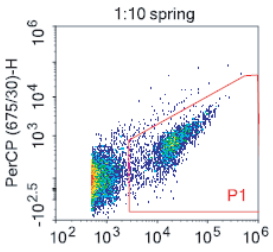
Gate	Count	Absolute Count
All	871	29
P1	13	0.433



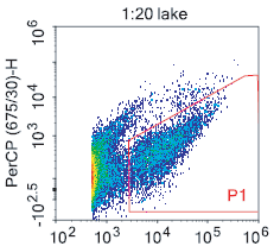
Gate	Count	Absolute Count
All	10,504	350
P1	5,840	195



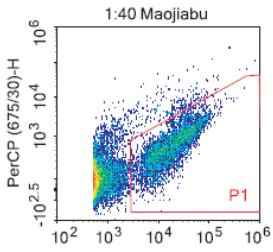
Gate	Count	Absolute Count
All	25,135	1,076
P1	5,389	206



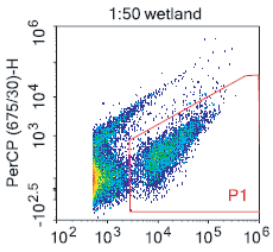
Gate	Count	Absolute Count
All	7,670	2,557
P1	2,353	784



Gate	Count	Absolute Count
All	22,374	15,409
P1	5,336	3,655



Gate	Count	Absolute Count
All	15,712	21,961
P1	5,717	7,893



Gate	Count	Absolute Count
All	17,844	30,745
P1	5,405	9,200

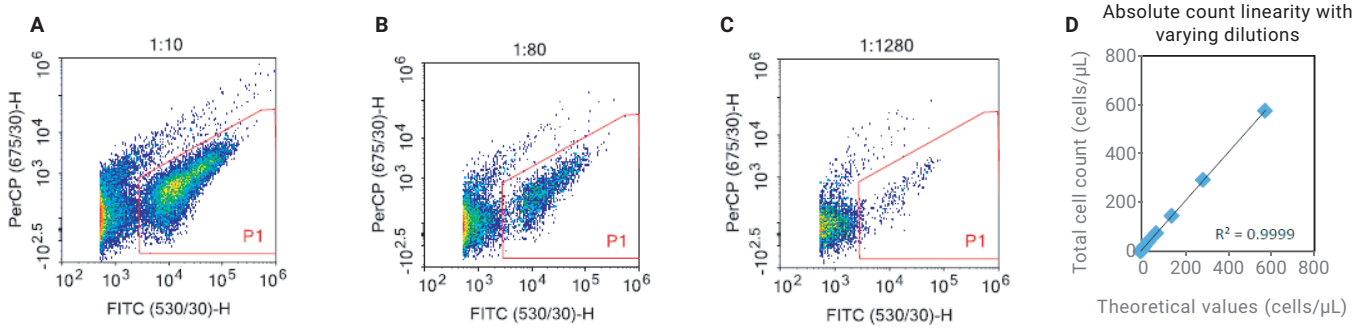
Figure 2. Bacteria counts from various water sources. Total bacterial counts were obtained using SYBR Green nucleic acid dye in either undiluted or diluted water samples. Counts are displayed underneath plots (Abs. Count) and displayed in units of cells/µL.

As a measure of precision, lake water was also serially diluted and quantified for total bacteria count using the NovoCyte flow cytometer. In these studies, eight serial dilutions were acquired on the NovoCyte. A good linearity (R^2 = 1) indicates that the volumetric absolute counting allows accurate quantification of small particles, such as these bacteria (Figure 3).

Detection of bacteria viability

Natural water is disinfected to be suitable for human consumption. To inactivate the harmful bacteria, chemical processes are performed such as flocculation and chlorination. To demonstrate the effect of chlorination on bacteria, spring water was treated with increasing concentrations of chlorine for 30 minutes, and EDTA (5 mM) was added before staining with SYBR Green I and PI. EDTA was added to disrupt the outer membrane of gram-negative bacteria to increase staining efficiency.[6,7] A dose-dependent decrease of HNA and LNA, and an increase in damaged bacteria was observed with increasing chlorine concentrations, while the total count remained constant (Figure 4). Results showed that the HNA bacteria are more sensitive to chlorine treatment than LNA bacteria.

The detection of bacteria in various water samples is essential to maintaining sanitary and healthy drinking conditions. The NovoCyte flow cytometer can easily and efficiently detect and quantify bacteria in water from several sources. With detection sensitivity coupled to the automatic cell counts measured for each sample, the NovoCyte is an efficient instrument for this application.



Gate	Count	Absolute Count
All	28,880	1,045
P1	16,143	573

Gate	Count	Absolute Count
All	10,357	356
P1	2,203	74.9

Gate	Count	Absolute Count
All	4,041	135
P1	180	6.03

Figure 3. Bacteria count accuracy across multiple dilutions. Total bacterial counts were obtained using SYBR Green nucleic acid dye in diluted water samples (A,B,C). Total cell count was plotted versus theoretical cell count using several dilutions of lake water (D). A best fit line and linearity were calculated with a R^2 value of 0.9999.

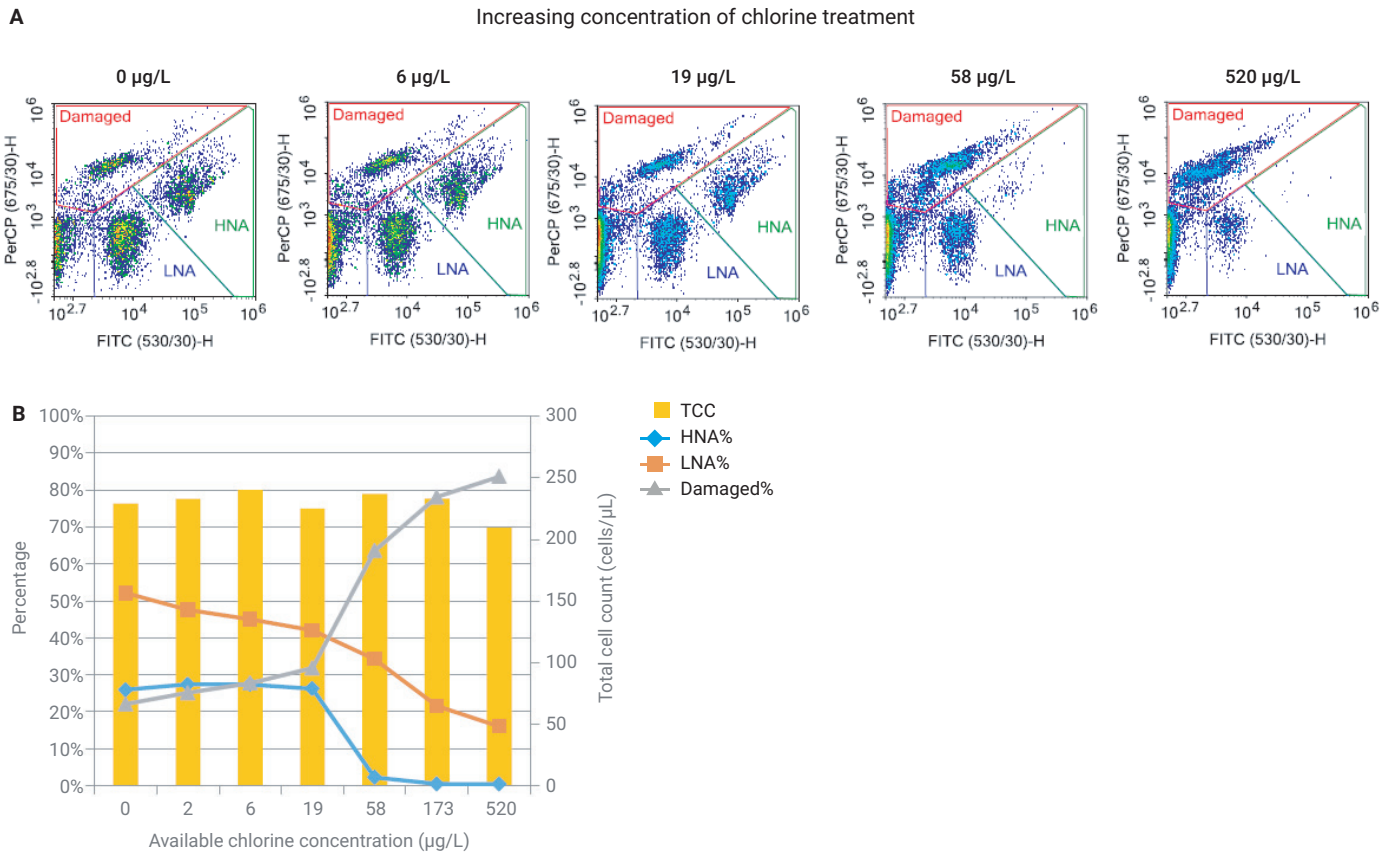


Figure 4 . Total count and viability of bacteria of spring water after chlorine treatment. (A) SYBR Green I and PI staining on water samples with treatment by increasing concentrations of chlorine; (B) Total cell count of HNA, LNA and the damage after chlorine treatment from A.

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