NovoCyte® Flow Cytometer

Application Note No. 12

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Detection of Bacteria in Environmental Waters using the NovoCyte Flow Cytometer

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 either 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, aren't 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 utilizing microbial detection cover everything from drinking water /waste water system monitoring, industrial biotechnology, food and drug quality control, 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.

ACEA NovoCyte® flow cytometer can detect very small particles with high sensitivity. Combining multi-parameter analytic capability and convenient fluidic maintenance, the NovoCyte flow cytometer can be easily applied to a variety of microbial studies.

Determination of total bacteria count in water samples from multiple sources

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

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

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

1. NovoCyte flow cytometer instrument setup - determining light scattering of bacteria and background noise
2. Clean instrument as best as possible – use clean, filtered DI water to dilute NovoFlow sheath fluid , run cleaning and rinsing cycles
3. Carry out routine QC test to evaluate instrument performance
4. Test blank sample (0.1um filtered deionized water) with the lowest threshold setting (FSC-H at 10) to determine background noise on FSC vs SSC and on any applicable fluorescence detection channels
5. Identify proper thresholds for either the scatter or fluorescence detection channels. Run blank sample to verify correct settings by observing a substantial decrease in the events collected
6. With the applied thresholds from above, run small particle samples

*Note: some small particles are difficult to detect only using FSC or SSC, so if this is the case, it is best to use a fluorescent label to identify the small particles of interest

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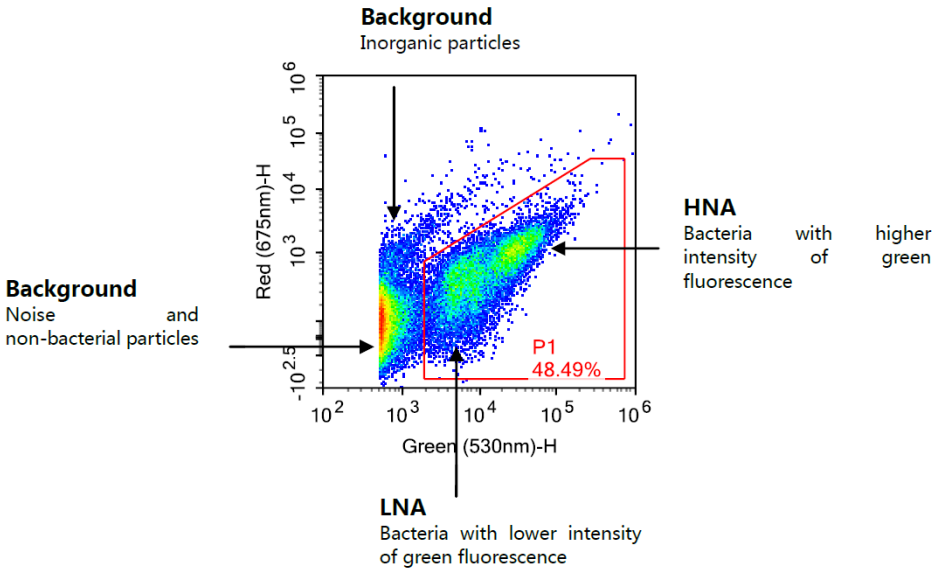


Figure 1. Detection of bacteria in natural waters. Fresh natural water (or stock at 4 degree) was filtered through a 300 mesh sieve, diluted with deionized water (filtered through 0.1µm membrane) to a desired concentration. A 100x SYBR® Green I dye (Invitrogen, S7563, diluted 100-fold in DMSO) was added to the sample, and the mix was incubated at 37 degree for 13min. NovoCyte settings: 30ul 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.

Water	TCC (total cell count) (cells/µl)
0.1um filtered deionized water	<1
Bottle water	<1
Tap water	195
Mountain stream	206
Spring	784
Lake 1	3,655
Lake 2	7,893
Wetland	9,200

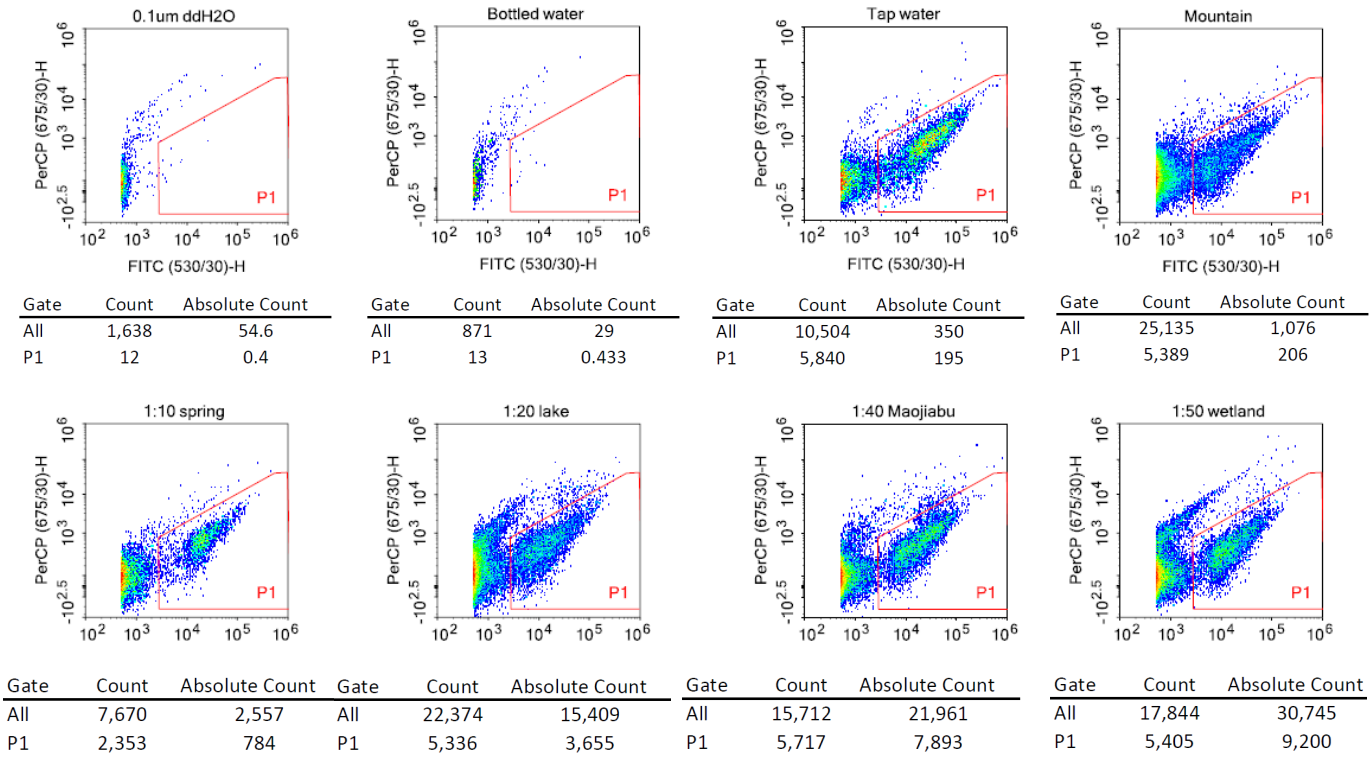
Table 1. Total cell count of bacteria in various water samples obtained automatically on the NovoCyte flow cytometer.

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) **(Fig. 1)**. It is broadly accepted that HNA is active bacteria, whereas LNA is inactive, dead or dormant population. Whether LNA and HNA are different types of bacteria or physiologically in different states is still unclear. In addition, the fluorescence spectra of bacteria are adopted by some researchers as bacterial fingerprints, which can be valuable for detection of population changes that are not reflected in the cell concentration. (Hammes et al, 2012, De Roy et al, 2012.)

In this application note, by using ACEA NovoCyte® flow cytometer, we precisely quantified the total counts of bacteria in multiple environmental waters, 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).

The deionized water we sampled was filtered through 0.1 µm membrane and stained with SYBR® Green I. Absolute cell counts were obtained using the NovoCyte flow cytometer and equal to less than 1 cell/µl (P1 gate) in the unfiltered bottled water, equivalent to the filtered deionized water **(Figure 2)**. Total cell counts for all samples are listed in **Table 1.** Specified in guidelines of drinking water analysis from Swiss Federal Office of Public Health, the acceptable limit of bacteria in drinking water is 2.0 x 10^2 cells/µl. We found that natural waters contain the highest bacterial counts of the samples we tested and bottled water the lowest. Bacterial counts in still water were 10-100X higher than that of spring and mountain stream sources.

In addition, as a measure of precision, lake water was serial diluted and quantified for total bacteria count using the NovoCyte flow cytometer. In our studies, 8 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)**.



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

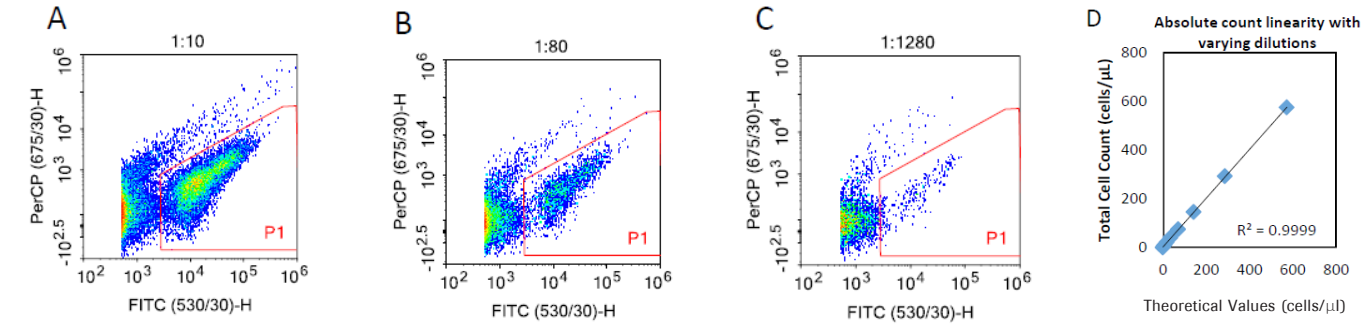
Gate	Count	Absolute Count
All	7,670	2,557
P1	2,353	784

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 by 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.



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 by 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

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 half an hour, and EDTA (5 mM) was added prior to staining with SYBR® Green I and PI . EDTA was added to disrupt the outer membrane of Gram-negative bacteria to increase staining efficiency (Berney et al, 2007, Nebe-von-Caron et al, 2000). A dose-dependent decrease of HNA, LNA and increase of damaged bacteria was observed with increasing chlorine concentrations, while the total count remains constant (Figure 4). Moreover, we noticed that the HNA bacteria are more sensitive to chlorine treatment than that of the LNA bacteria.

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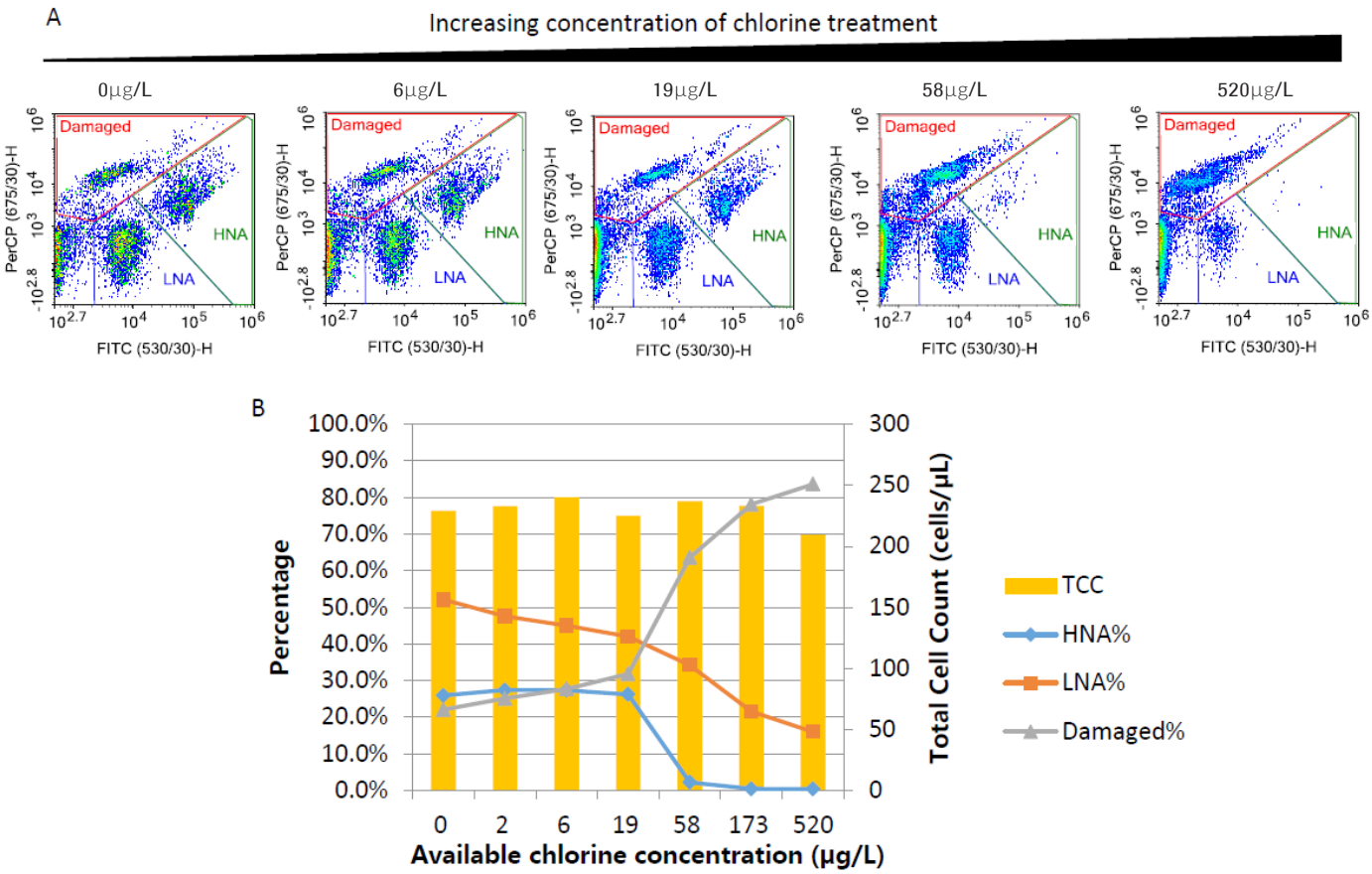

Figure 4 . Total count and viability of bacteria of spring water after chlorine treatment. A. SYBR® Green I and PI staining on water samples with treatment by increasing concentrations of chlorine; B. Total cell count of HNA, LNA and the damaged upon chlorine treatment from A.

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

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