Application Note

Viable cell count (VCC) of bacteria in water

Discrimination of dead and living bacteria using the CyStain™ BacCount Viable kit (manual and automated)

Introduction

The quantification of the bacterial amount is important to control the microbial ecology in different water samples. Sysmex Partec instruments use an improved and standardized application to determine bacterial counts in water: The CyStain™ BacCount reagents.

The old standard – Heterotrophic plate count

Worldwide, the bacterial quality control of water is performed with the 'heterotrophic plate count' (HPC). This method goes back to Robert Koch, who more than a century ago determined the microbiological status of drinking water by counting microbial colonies on agar plates.

Today it is well known that this routine cultivation technique has severe limitations:

1. Not all microbes present in water samples grow and form colonies on solid cultivation media. These bacteria are e.g. the so-called viable but nonculturable (VBNC) bacteria.
2. The data obtained from colony counting for result interpretation is weak as it represents down to less than 0.1 % of the bacteria present in a water sample.
3. Plate counting requires considerable manpower and delivers results with some days of delay, which is caused by the necessary period of cultivation.

Fig. 1: Despite of the many disadvantages, heterotrophic plate count is still the standard methods for the quality control of drinking water.

The delay of the results (1 - 14 days) poses a risk, since the sampling of water and reporting of results are decoupled: By the time the results become available, the drinking water has already reached the consumer and, most likely, has been consumed for some time.

State-of-the-art: Flow Cytometry

More and more scientific publications recommend bacterial cell counting (Total cell count, TCC) in drinking and industrial water via flow cytometry. One advantage of this application is the potential of high throughput automation [1 - 5].

Sysmex Partec standardized and improved bacterial cell count in water samples using the accurate and convenient CyFlow™ Cube 6 V2m Flow Cytometer together with the two CyStain™ BacCount kits. The CyStain™ **BacCount Total** kit enables the detection of the total amount of bacteria in water samples (More information in the Application Note "APN_014A_CyStain BacCount Total"). On the other hand, the CyStain™ **BacCount Viable** kit discriminates between live and dead bacteria, which is important to monitor successful inactivation of harmful bacteria in water.

This procedure allows to discriminate between live and dead bacteria and to differentiate between bacterial populations with a high (HNA bacteria) and a low content of nucleic acid (LNA bacteria) within less than 15 minutes from the time of sampling. The procedure is simple, reliable, fast and samples can be analysed by the small, compact and robust CyFlow™ Cube 6 V2m Flow Cytometer that is capable of volumetric counting of particles.

Flow cytometry allows to determine the viable bacterial count within less than 15 minutes

In contrast to the HPC, this method detects not only those bacteria growing on agar plates but the entire population of bacteria, regardless of their potential to grow in an artificial or natural environment. This explains why HPC results and the total bacterial cell count determined by flow cytometry can differ by a factor of 100 to 10,000. Monitoring the VCC allows drawing conclusions on the present microbiological status of drinking water, but also of its development. The method is suitable for drinking water and surface water.

Principle of the CyStain™ BacCount Viable kit

The viable cell count is based on the labelling of bacterial DNA with two different fluorescent dyes (Fig. 2):

1. CyStain™ Green is a membrane permeable dye that unspecifically stains all bacteria in water samples (living and dead bacteria), emitting green fluorescence. Both live and dead cells will be stained.
2. CyStain™ Red is not membrane permeable and stains only bacteria with a damaged cell membrane. This is an indication of dead or dying cells.

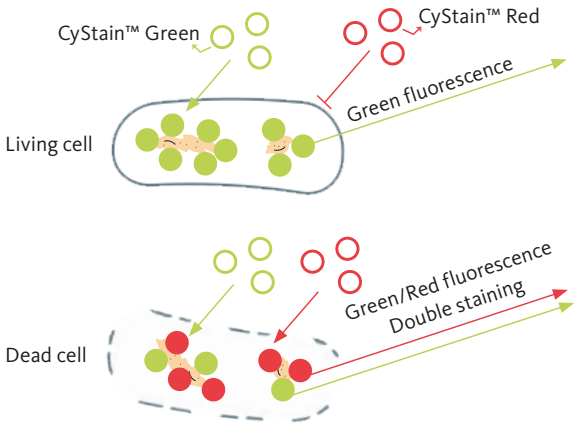


Fig. 2: The membrane-permeant CyStain™ Green stains all living and dead bacteria by emitting a characteristic green fluorescence. CyStain™ Red is only able to stain bacteria with a damaged membrane. Dead bacteria are therefore labeled with both dyes, resulting in emission of two fluorescences.

Make sample automation simple

There are 2 options using the CyStain™ BacCount Viable kit:

1. Manual measurements using the kit and the CyFlow™ Cube 6 V2m Flow Cytometer, or
2. Automated measurements using the kit, the CyFlow™ Cube 6 V2m Flow Cytometer and the CyFlow™ Robby 6 V2m Autoloading Station.

Sample automation is the key requirement for quality and process control of water samples. The CyFlow™ Robby 6 V2m Autoloading Station enables a complete automation solution, which offers simplicity, sustainable reliability and exceptional walk-away convenience. This automated system is the ideal platform to tailor all customers needs in the water industry.

Tab. 1: Advantages of flow cytometry compared to HPC

	Flow cytometry	HPC
Reproducibility	High	Low
Speed	15 min	Days
Automation	Yes, easy to scale up	No
Bacteria detected	All	Only those growing on agar
Sensitive for changes in cell count	Yes	No
Information	Total-, Living-, Dead-, VBNC-cells; Fingerprint (LNA/ HNA ratio)	Colony forming unit (CFU)

Instrument requirements

All analysis require the CyFlow™ Cube 6 V2m Flow Cytometer (Ref. No. CY-S-3061R). This flow cytometer is equipped with a blue laser (light excitation, 488 nm) and is able to analyse side scatter (SSC), green (536 ± 40 nm) and red (> 630 nm) fluorescence as detection parameters. For an automated analysis the CyFlow™ Robby 6 V2m Autoloading Station (Ref. No. CY-S-3083) is needed.

Fig. 3: The CyFlow™ Cube 6 V2m Flow Cytometer with the CyFlow™ Robby 6 V2m Autoloading Station.

Material and methods

Kit components

The CyStain™ BacCount Viable kit contains the following reagents:

- 5 Aliquots x 40 µL CyStain™ Green
- 5 Aliquots x 400 µL CyStain™ Red
- 29 mL CyStain™ Dilution Buffer

Fig. 4: CyStain™ BacCount Viable kit (Ref. No. 05-5028).

Additional required equipment

- 2 mL Reaction Tubes (Safe-Lock)
- 3.5 mL Sample Tubes (Ref. No. 04-2000)
- Heating block/water bath set to 37 °C ± 0.5 °C
- Vortex mixer

Sample preparation

NOTE: There are two options for sample preparation: Manual and automated sample analysis. In this Application Note both options are presented in parallel.

Manual analysis

1a. Prepare a working solution by mixing both stains. Dilute the 1000X stock solution of CyStain™ Green 1:100 and the 100X stock solution of CyStain™ Red 1:10 with CyStain™ Dilution Buffer. E.g. *for 10 samples mix 10 µL CyStain™ Green and 100 µL CyStain™ Red with 890 µL CyStain™ Dilution Buffer.*

2a. Mix 100 µL CyStain™ working solution with 900 µL water sample in a 2 mL reaction tube.

3a. Mix sample with a vortex mixer for 3 seconds.

4a. Incubate sample for 13 minutes at 37 °C ± 0.5 °C, protected from light in a heating block or water bath.

5a. Mix sample with a vortex mixer for 3 seconds.

6a. Pipette 850 µL of the sample into a sample tube for flow cytometry.

Automated analysis

1b. Prepare a working solution by mixing both stains. Dilute 20 µL stock solution of CyStain™ Green and 200 µL stock solution of CyStain™ Red with 1780 µL CyStain™ Dilution Buffer.

2b. Mix 20 µL CyStain™ working solution with 180 µL water sample per well in a 96 Well Plate (Ref. No. 04-2020).

3b. Mix gently by pipetting up and down the solution.

4b. Incubate sample for 13 minutes at 37 °C ± 0.5 °C, protected from light.

Analysis range

The maximum cell number for an accurate data acquisition is 2 x 10^5 cells/mL. It is recommended to dilute a water sample if the cell number is higher. Dilute the water sample with ultrapure water or 0.1 µm filtered water before staining with the CyStain™ BacCount Viable kit.

Tab. 2: Analysis range of CyStain™ BacCount Viable kit

Detection Limit	Reportable Range
200 cells/mL*	1 x 10^3 – 2 x 10^5 cells/mL

*A specific treatment of the CyFlow™ Cube 6 V2m Flow Cytometer is required if the expected viable cell count is lower than 1 x 10^4 cells/mL. Please refer to section General hints for further information.

Data acquisition

1. Switch on the CyFlow™ Cube 6 V2m flow cytometer and use if required the CyFlow™ Robby 6 V2m Autoloading Station.
2. Start the CyView™ Software after the device has booted.
3. For manual analysis load the script "CyStain BacCount Viable_..._Standalone.cvc85". For automated analysis load the script "CyStain BacCount Viable_..._Robby.cvc85".
4. For initial cleaning of the device start the Priming procedure and follow the instructions on the display until Priming is completed.
5. After completed Priming start the Quality Check (QC) procedure and follow the instructions on the display until the QC is completed.

NOTE: For further information see CyStain™ BacCount reagents – Quality Check Manual [6].

6. Two FCS Express™ reports will be automatically loaded to verify the QC. Enter QC material information for LOT N° and expiry date.
7. The analysis of stained water samples can be started after a "valid" QC procedure.

NOTE: Make sure that your device is qualified to perform measurements with the CyStain™ BacCount Viable kit. If tests fail, the device is not qualified to perform measurements. Please see section Troubleshooting.

8. For manual analysis connect the sample tube from step 6a and start measurement. For automated analysis insert 96 Well Plate from step 4b into the CyFlow™ Robby Autoloading Station and start measurement.
9. The measurement automatically stops at the end of analysis.
10. FCS Express™ automatically starts and opens analysis report.
11. All further samples are analysed as stated before.
12. Start the Shutdown procedure after the sample analysis is completed and follow the instructions on the display.

Exemplary data

Quality Check

The QC procedure controls background, laser power, optical alignment, gate positioning and counting precision. After the QC Part 1 and 2 are completed the results will be analysed automatically by FCS Express™. An exemplary report can be found in the CyStain™ BacCount reagents – Quality Check Manual (section 4.2.2) [6].

Water samples

For this application Note Evian mineral water (commercially available bottled water) samples with different cell numbers were chosen. Evian mineral water is used due to its natural content of high nucleic acid (HNA) and low nucleic acid (LNA) bacteria.

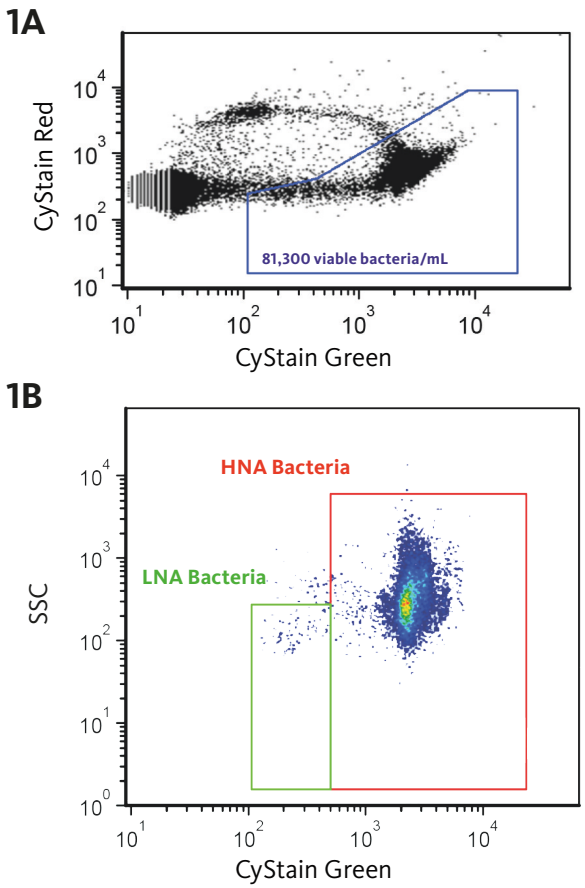
Sample 1: Evian mineral water
Sample 2: 1:1 mixture of pure and autoclaved Evian mineral water
Sample 3: Evian mineral water, autoclaved

Results

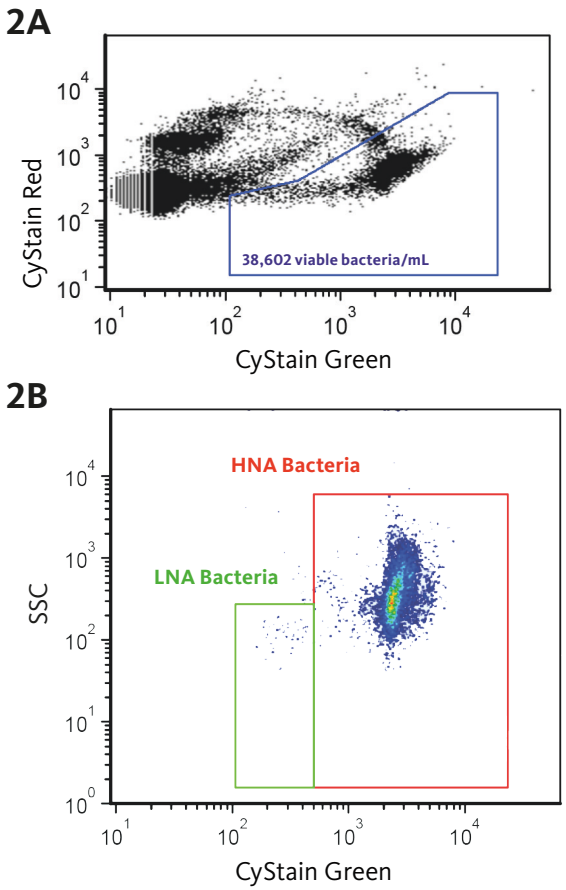
The highest cell number of viable bacteria was found as expected with Evian mineral water (81,300 viable bacteria/mL). The effectiveness of autoclavation was shown in the 1:1 mixture (38,602 viable bacteria/mL) and in the autoclaved water (only 101 viable bacteria/mL).

NOTE: The lower detection limit of the CyStain™ BacCount Viable kit is 200 cells/mL. Therefore, a detected cell count of less than 200 cells/mL represent a negative result.

Sample 1: Evian mineral water



Sample 2: 1:1 mixture of pure and autoclaved Evian mineral water



Sample 3: Evian mineral water, autoclaved

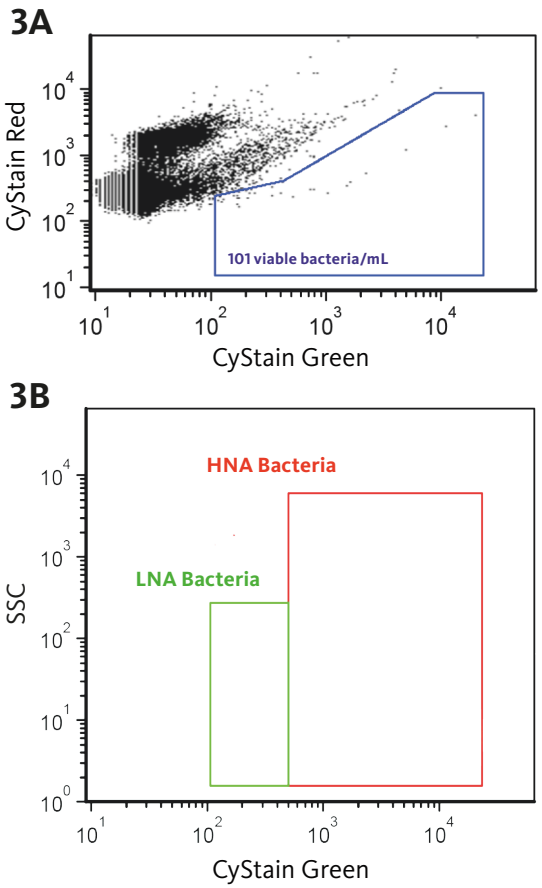


Fig. 5: Different samples of Evian mineral water stained with CyStain™ BacCount Viable kit. (1) Evian mineral water, (2) 1:1 mixture of pure and autoclaved Evian mineral water and (3) autoclaved Evian mineral water. (A): Dot plot showing CyStain™ Green vs. CyStain™ Red. All viable cells are located and automatically counted within the predefined blue gate. (B): Dot plot showing the side scatter (SSC) vs. CyStain™ Green which allows to determine the LNA and HNA numbers - the "Fingerprint" of a water sample.

Tab. 3: Amount of detected viable bacteria in the 3 water samples

Water sample	Viable bacteria
Evian mineral water	**81,300/mL**
1:1 mixture of pure and autoclaved Evian mineral water	**38,602/mL**
Evian mineral water, autoclaved	**101/mL**

General hints

Cleaning steps between different samples

Depending on the cell count of the water sample, as well as the analysis method cleaning steps between individual measurements are recommended to reduce the possibility of cross contamination and background signals.

Tab. 4: Cleaning steps between different samples

Set of water samples		Cell content		
		similar	different	unknown
cleaning step	manual analysis	not required	intermediate cleaning with Sheath Fluid	intermediate cleaning with Sheath Fluid
	automated analysis	cleaning mode none	cleaning mode normal* or intensive**	cleaning mode normal* or intensive**

*Cleaning with Sheath Fluid (Ref. No. 04-4007_R)
**Cleaning with additional Cleaning Solution (Ref. No. 04-4009_R)

In case of changing the application from viable cell count to total cell count determination on the same day no additional QC procedure or a Shutdown of the device is necessary. However, a Priming procedure is required.

Intensive cleaning procedure

The analysis of water samples with an expected viable cell count lower than 1 x 10^4 cells/mL requires a special treatment of the CyFlow™ Cube 6 V2m flow cytometer. To maintain ultra-clean conditions the following manual cleaning procedure is recommended:

NOTE: In addition to the cleaning procedure a filtration (0.22 µm filter) of Sheath Fluid is recommended.

1. Empty the Sheath Fluid bottle and rinse twice with Hypochlorite Solution (Ref. No. 04-4012_R).
2. Remove the used Inline Filter (Ref. No. 04-004-1000) of the Sheath fluid bottle.
3. Fill the Sheath Fluid bottle with 200 mL Hypochlorite Solution.
4. Fill a sample tube with 3 mL Hypochlorite Solution.
5. Start a measurement at a speed of 0.2 µl/sec until it stops automatically.
6. Wait 90 min and measure again.
7. Stop the measurement after 10 min.
8. Exchange the Hypochlorite Solution in the sheath fluid bottle with Sheath Fluid. Rinse twice with Sheath Fluid before filling up the bottle.
9. Attach a new Inline Filter to the Tubing of the Sheath Fluid bottle.
10. Fill a sample tube with 3 mL Sheath Fluid.
11. Start a measurement with Sheath Fluid at a speed of 0.2 µl/sec.
12. Stop the measurement after 30 min.

Troubleshooting

If the error you are experiencing is not described or the remedy could not solve your problem, please contact your local Sysmex representative.

Error #1: QC procedure "invalid"

Reason	Remedy
QC material poorly mixed	Shake the QC material vigorously (e.g. by vortexing) and repeat QC procedure Check the date of expiry of the QC material Perform a priming procedure and repeat QC procedure Perform an intensive cleaning procedure and repeat QC procedure
High concentration of Calibration Beads 0.5 µm (Ref. No. 05-4005)	Perform an intensive cleaning procedure, prepare a new dilution of the QC material and repeat QC procedure
LOT NO° mismatch of QC material	Make sure to use a matching FCS Express™ template and QC material LOT NO°
Deposition of CyStain™ Red inside the device	Perform an intensive cleaning procedure and repeat QC procedure

Error #2: Population(s) of viable cells outside of predefined gate

Reason	Remedy
Incubation time of the sample with CyStain™ Red is too short or too long	Repeat sample preparation and measurement
Deposition of CyStain™ Red inside the device	Perform an intensive cleaning procedure, repeat sample preparation and measurement
Air bubbles inside the device	Perform a priming procedure, repeat sample preparation and measurement Perform an intensive cleaning procedure, repeat sample preparation and measurement

Error #3: No separation between viable bacteria and background signal

Reason	Remedy
Cell number higher than 2 x 10^5 cells/mL	Dilute water sample with filtered (0.22 µm Filter) or ultrapure water, repeat sample preparation and measurement

References

1. Van Nevel S., *et al.*, 2017. *Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring*. Water Res. 113: p. 191-206.
2. Wang, Y., *et al.*, 2010. *Past, present and future applications of flow cytometry in aquatic microbiology*. Trends Biotechnol. 28, 416-424.
3. Van Nevel S., *et al.*, 2013. *Routine bacterial analysis with automated flow cytometry*. J Microbiol Methods. 94, 73-76.
4. Hammes, F., *et al.*, 2008. *Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes*. Water Research. 42, 269-277.
5. Safford, HR., *et al.*, 2018. Flow cytometry applications in water treatment, distribution, and reuse: A review. Water Research. 151, 110-133.
6. CyStain™ BacCount reagents – *Quality Check Manual*.

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