Application Note

# Microbial QC in wine

## CyStain™ OenoCount – Fast, Sensitive, Robust
## The choice for microbial QC in wine production

## Introduction

Wine is a foodstuff and for the entire production process from harvest to bottling the Hazard Analysis and Critical Control Point system, or HACCP has become an important safety standard for the wine industry. One of the advantages of establishing a HACCP plan is that it allows the winemaker to integrate chemical, physical, sensory, and microbiological analyses, all along the wine production process.

Such analyses allow a precise control of each single step in vinification.

Harvest, alcoholic- and malolactic-fermentation are targets as well as maturation, filtration and last but not least bottling. If you succeeded in creating a great wine, it would be a shame to fail at this last important step before serving it to customers.

Beside microbial contaminants, there are crystalline (commonly calcium or potassium bitartrate), and amorphous/colloidal particulates (protein, tannin, polysaccharides, or metal complexes) in the wine. These could be removed either by centrifugation or Coarse depth filters.

But to ensure microbial sterility after bottling a sterile (0,45µm) filtration is needed. Those days roughly 80% of all wine, by volume, is sterile filtered compared to just 20% ten years ago (Bowyer, 2018). To reduce the risk of insufficient filtration and to guarantee a smooth bottling, it´s common to determine the Filtration index or FI. The FI indicates the time until a specific filter medium is blocked during filtration.

To do so, the flow rate of wine through a filter (0.45µm) at a constant pressure (2 bar) and temperature (preferably between 15°C and 20°C) over a time period of 5-10 minutes is measured and the volume filtered every 30 seconds is determined.

Equation 1: Calculation of Filtration Index (FI)

$$FI = \frac{Volume\ from\ time\ 30 - 90\ seconds}{Volume\ from\ time\ 120 - 180\ seconds}$$

An FI ratio from 1-1,5 counts as good while 1,5-2 is moderate and every result above 2 as poor or difficult to filter (needs prefiltration).

Such a filtration is often done twice, before and during the bottling process at the bottling line. Sounds like a sure bet? In most cases it is but who would take the residual risk of a contamination because of imperfectly cleaned bottles, a ruptured or blocked filter membrane, which can lead to contaminations to? Nobody will!

Therefore, spot checks of bottled wine are commonly taken during bottling to check for microbial contaminations. The most common method is plating. While yeast is cultivated on WL agar, for bacteria MRS/A/C plates (de Mann, Rogosa and Sharpe (MRS) nutrient agar supplemented with apple juice and cycloheximide solution) are common (The Australian Wine Research Institute, 2023).
The plates are incubated at 27°C ± 1°C and examined for growth after 3, 8 and 10 days.

Fig. 1: Despite of the many disadvantages, growth-based methods are still the standard methods for the detection of microbes in wine.

The main disadvantages of plating are:
The very long time to result, the fact that not all yeast and bacteria will grow comparably well on the same agar and that bacteria or yeast which are in the so called VBNC status (Viable but non-Culturable) will not grow and therefore not detected at all.
An ideal counting method would be able to detect individual cells in the wine, differentiate between viable and dead, independently of the physiological status (VBNC).
This describes exactly the characteristics of the CyStain™ OenoCount kit in combination with the CyStain™ Elution Buffer, the choice for microbial QC in wine production.

## Required instrument

For the analysis the CyFlow™ Cube 6 V2m flow cytometer (Ref. No. CY-S-3061R) is recommended, offering all scripting and templates for automated data analysis of the sample. A flow cytometer with equivalent configuration can be used, too.

Fig. 2: The CyFlow™ Cube 6 V2m flow cytometer and needed reagents

Optionally, the CyFlow™ Robby 6 Autoloader can be used for automated sampling from 96-well plates.

## Material

### Kit components

The CyStain™ Elution Buffer (Ref. No.: BN701127) contains the following reagents:

- 4 x 220 mL CyStain Elution Buffer

Fig. 3: CyStain™ Elution Buffer (Ref. No.: BN701127)

The CyStain™ OenoCount (Ref. No.: AN192657) contains the following reagents:

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

Fig. 4: CyStain™ OenoCount (Ref. No. AN192657).

### Additional required equipment

- Vacuum filtration unit with vacuum pump, e.g., WELCH MPC 090E
- 0.4 µm membrane filter, e.g., Sartorius polycarbonate track-etched membrane filter type 23006
- Forceps
- 5 mL test tube, e.g., Sarstedt screw cap tube
- Vortex mixer
- 2 mL reaction tubes (Safe-Lock)
- Heating block / water bath set to 37 °C ± 0.5 °C
- Sample tubes compatible with the flow cytometer, e. g. sample tubes 3.5 mL (Ref. No. 04-2000, available from Sysmex Partec)
- Optional: centrifuge, e.g., Eppendorf 5430 centrifuge

## Sample preparation

*NOTE: Detailed information for the sample preparation is available in the CyStain™ Elution Buffer Package Insert* (Sysmex Partec GmbH, 2023) *and CyStain™ OenoCount Package Insert* (Sysmex Partec GmbH, 2023).

The sample preparation includes an enrichment of the sample via vacuum filtration. The CyStain™ Elution Buffer ensures an optimal elution from filter bound microorganisms. After an optional washing and second enrichment step via centrifugation, the sample is ready to be transferred into the staining process using the CyStain™ OenoCount kit. The standardized and automated analysis with the Cube 6 V2m ensures objective clear-cut results.

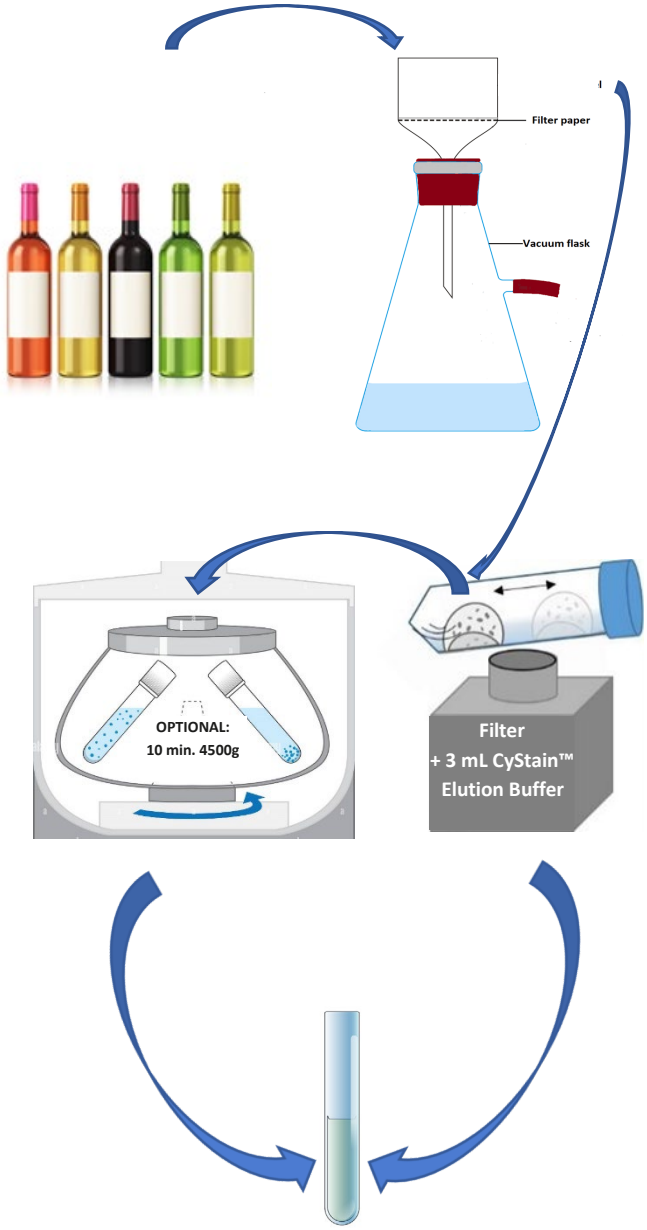


Fig. 5: Workflow

## Staining principle

The CyStain™ OenoCount is based on the labelling of yeast and bacterial DNA with two different fluorescent dyes (Fig. 6)

1. CyStain™ OenoCount Green is a membrane permeable dye that stains all yeast and bacteria cells, emitting green fluorescence. Both viable and dead cells will be stained.

2. CyStain™ OenoCount Red is not membrane permeable and stains only yeast and bacteria with a damaged cell membrane. This is an indication of dead or dying cells.

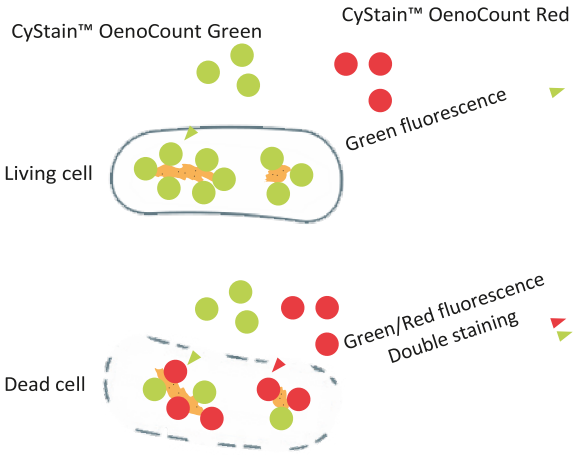


Fig. 6:Staining principle

After preparation of the working solutions for staining, the staining procedure of the eluted wine sample is finished within 13 minutes only and the sample is ready to be analysed.
A total time to result including sample preparation, sample staining, sample acquisition and sample analysis of less than 30 minutes is expected for initial volumes of approximately 250 mL.

## Data Acquisition & Analysis

A quality check procedure (QCP) is carried out before the sample is acquired. Passing the QCP ensures that the instrument is in good working order (Fig. 7). Therefore, the specific script for the CyStain™ OenoCount kit must be loaded on your CyFlow™ Cube6 V2m and after the initial priming, a QC procedure can be selected. Sample loading can be performed manually or using the CyFlow™ Robby 6 Autoloader. The software guides you through the steps and a final report in FCS Express shows the results of the QC procedure.
*NOTE: Further information is found in the CyStain™ OenoCount – Quality Check IFU* (Sysmex Partec GmbH, 2023).

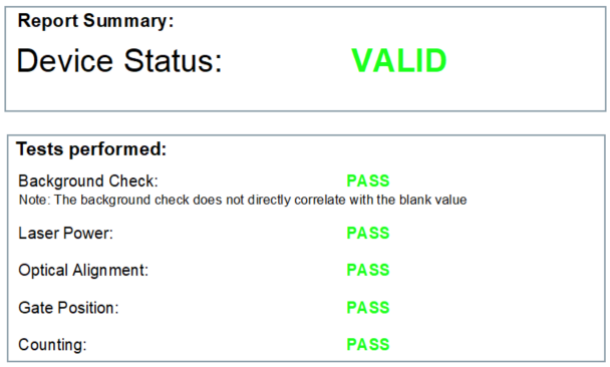


Fig. 7: Snapshot from FCS Express QC template: Valid results for the measurement of Count Check Beads green show functionality of the device.

Once the quality check procedure is completed, select the [Work] batch. As with the Quality Control procedure, sample loading can be performed manually or by the CyFlow™ Robby 6 Autoloader.
Press the 'Start' button and sample aspiration is initiated and processed automatically. Real-time data is displayed on the screen within the CyView™ software. Once the pre-set volume has been collected, the measurement stops, and an FCS Express template is automatically opened. The data is analysed, and the results are displayed (see Fig. 8).

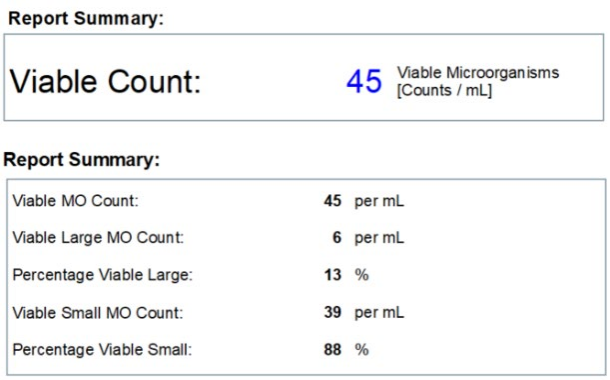


Fig.8: Snapshot from FCS Express Report: Result is calculated by dilution via staining only. No further dilution or enrichment is factorized.

As the initial volume of wine brought into the enrichment assay is not fixed, the template offers the option of entering a dilution or enrichment factor in addition to the automatic factoring by the staining protocol (see Fig. 9). When the factor is added, the values change accordingly. In this example 250 mL of red wine was vacuum filtered and the filter eluted in 3 mL of CyStain™ Elution Buffer. No further sample washing or enrichment by centrifugation was performed. Therefore, the enrichment factor was calculated as follows (see Equation 2):

Equation 2: Calculation of enrichment factor

$$EF = \frac{V_{initial}}{V_{EB}} = \frac{250\ mL}{3\ mL} = 83.33$$

whereas EF is the enrichment factor, $V_{initial}$ is the volume of wine brought into the vacuum filtration and $V_{EB}$ is the volume of CyStain™ Elution Buffer.

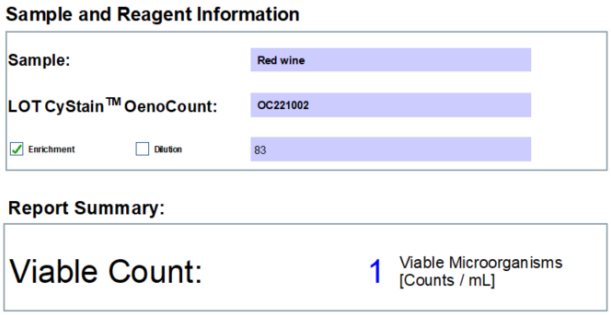


Fig. 9: Snapshot from FCS Express Report: Result is re-calculated after entering of enrichment factor of 83. 250 mL wine vacuum filtrated, and filter was eluted in 3 mL CyStain™ Elution Buffer

A calculation of the initial concentration of microorganisms per millilitre wine based on the enrichment factor is showing a result of 1.
By entering a dilution/enrichment factor used within the preparation of the sample, a calculation of microorganisms in your initial wine sample is easily possible.

## Results

The Gusmer Fermentation Center, in collaboration with Sysmex Partec GmbH and Sysmex America, Inc., tested the CyStain™ OenoCount kit under development as a replacement or complement to traditional bottled wine sterility testing. The CyStain™ OenoCount kit was used to quantify live bacteria and yeast that may be present in very low concentrations in sterile filtered wine bottles.
In a first approach, twenty-five bottles of recently sterile-filtered and bottled wine were acquired from an industry partner and tested for potential microbial contamination. All showed microorganisms at concentrations of less than 10 cells/ml. The industry partner simultaneously used traditional microbial plating methods on all bottles and found no microbial growth. This suggests that the limit of detection (LOD) for the CyStain™ OenoCount kit is 10 cells/mL, establishing a baseline for what can be expected in 'clean' wine (see Fig. 10 and Fig. 11).

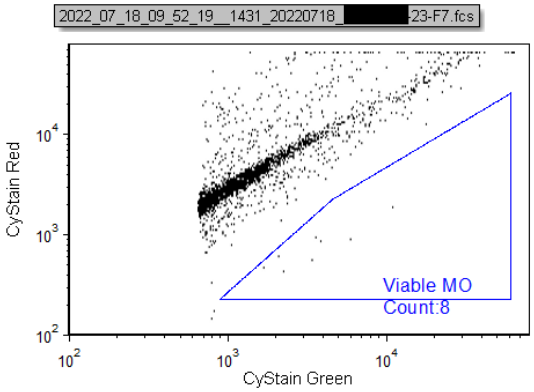


Fig. 10: FL1 vs. FL3 dot plot highlighting all viable microorganisms within a red wine sample

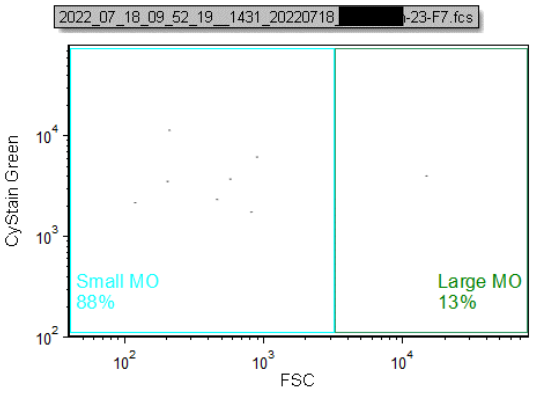


Fig. 11: FSC vs. FL1 dot plot separating between small (mainly bacteria) and large (mainly yeast) organisms

In a second approach, further testing was carried out on bottles of wine most likely to have been contaminated during the bottling process, approximately 2 months prior testing by flow cytometry. Two samples were positive using the CyStain™ OenoCount kit. With an enrichment factor of 83, the initial concentration was calculated to be 567 viable cells/ml and 287 cells/ml respectively (see Fig. 12 and Fig. 13).

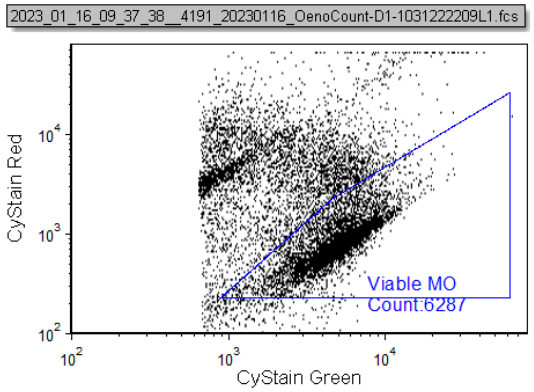


Fig. 12: Red wine (1031222209L1) after bottling: Natural contamination of microorganisms can be seen in a FL1 vs. Fl3 dot plot.

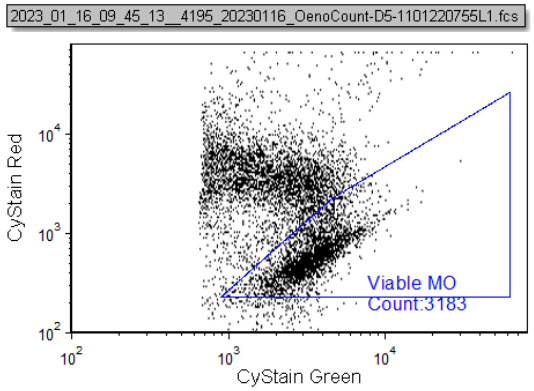


Fig. 13: Red wine (1101220755L1) after bottling: Natural contamination of microorganisms can be seen in a FL1 vs. Fl3 dot plot.

PCR (Scorpion Wine Spoilage Panel, a proprietary multiplex PCR test) testing was carried out directly at the customer's site at time of bottling and the PCR test showed contamination with acetic acid bacteria and *Oenococcus oeni* as shown in Fig. 14

**1031222209L1**

"Scorpion" Yeast & Bacterial Panel

| | |
|---|---|
| Acetic acid bacteria | 70 cells/mL |
| L. brevis/hilgardii/fermentum | < 10 cells/mL |
| Lactobacillus plantarum/casei/mali | 20 cells/mL |
| Lactobacillus kunkeei | < 10 cells/mL |
| Oenococcus oeni | 880 cells/mL |
| Pediococcus species | < 10 cells/mL |
| Brettanomyces bruxellensis | < 10 cells/mL |
| Saccharomyces cerevisiae | < 10 cells/mL |
| Zygosaccharomyces specis | < 10 cells/mL |

**1101220755L1**

"Scorpion" Yeast & Bacterial Panel

| | |
|---|---|
| Acetic acid bacteria | 300 cells/mL |
| L. brevis/hilgardii/fermentum | < 10 cells/mL |
| Lactobacillus plantarum/casei/mali | < 10 cells/mL |
| Lactobacillus kunkeei | < 10 cells/mL |
| Oenococcus oeni | 250 cells/mL |
| Pediococcus species | < 10 cells/mL |
| Brettanomyces bruxellensis | < 10 cells/mL |
| Saccharomyces cerevisiae | < 10 cells/mL |
| Zygosaccharomyces specis | < 10 cells/mL |

Fig. 14: Results of "Scorpion" PCR test at time of bottling put in order by industry partner

Plating results at time of bottling did show overwhelming growth (>300 Cfu/250mL) of bacteria, presumptive to be acetic acid bacteria. It is estimated that there was a little growth of *Oenococcus oeni* as well on the plates, but it was covered by acetic acid bacteria, as *Oenococcus oeni* is notoriously hard to culture with an unspecific agar.

Simultaneously to the flow cytometric analysis, Gusmer Fermentation Center put in order a second PCR testing. Essentially, the Scorpion Wine Spoilage Panel showed levels of *Oenococcus oeni* in ranges consistent with the CyStain™ OenoCount measurements (see Fig. 15).

| Sample# | Sample description | Acetic Acid Bacteria | Pediococcus ssp. | Oenococcus Oeni | Brettanomyces bruxellensis Brettanomyces anomala |
|---|---|---|---|---|---|
| 2301431 | 1031222209L1 2023-01-16 | 33,800 cells/ml | <10 cells/ml | 650 cells/ml | <10 cells/ml |
| 2301432 | 1101220755L1 2023-1-16 | 58,200 cells/ml | <10 cells/ml | 210 cells/ml | <10 cells/ml |

Fig. 15: Results of Scorpion PCR test at time of flow cytometric analysis put in order by Gusmer Fermentation Center

The PCR detected a high number of acetic acid bacteria that were not visible in the CyStain™ OenoCount measurements. These contaminants are expected to be non-viable as after time of storage of the wine bottles the population grew up and with consumption of all present oxygen died off leaving the *Oenococcus oeni* in vital status.
Plating on Wallerstein Laboratory Agar at time of flow cytometric analysis also showed growth for *Oenococcus oeni* only, but none for acetic acid bacteria.

Experiments carried out by the Sysmex-Partec GmbH laboratory have shown that some bottled red wine samples, such as Shiraz Cabernet Sauvignon and Dornfelder semi-dry, contain significant viable microorganisms. Fig. 16 - Fig. 19 show the results obtained after enrichment and analysis of 250 mL wine.

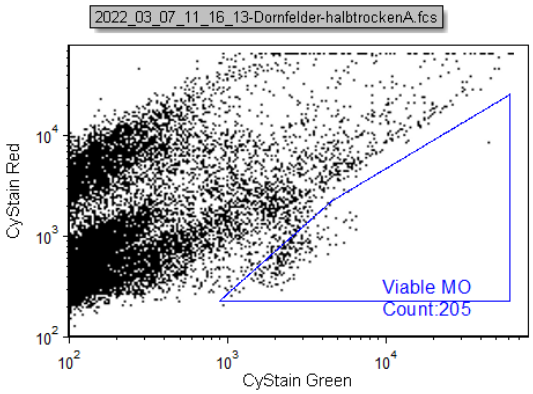


Fig. 16: FL1 vs. FL3 dot plot highlighting all viable microorganisms within a red wine sample

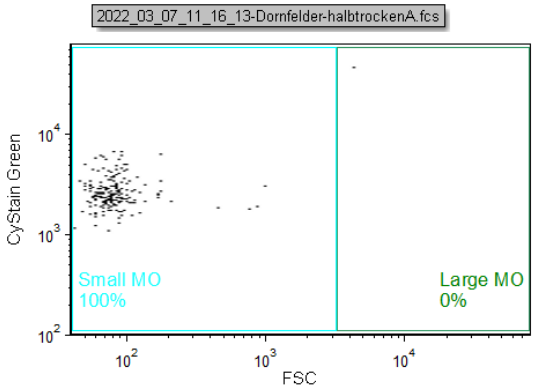


Fig. 17: FSC vs. FL1 dot plot separating between small (mainly bacteria) and large (mainly yeast) organisms

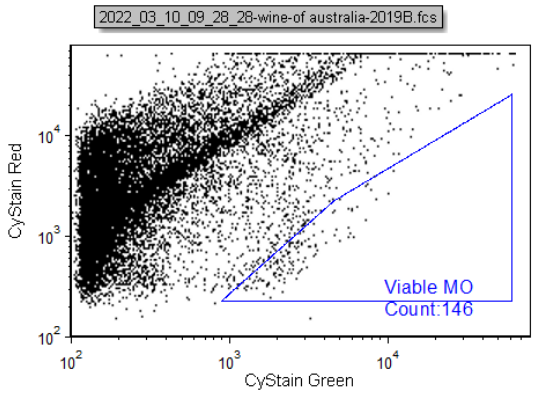


Fig. 18: FL1 vs. FL3 dot plot highlighting all viable microorganisms within a red wine sample

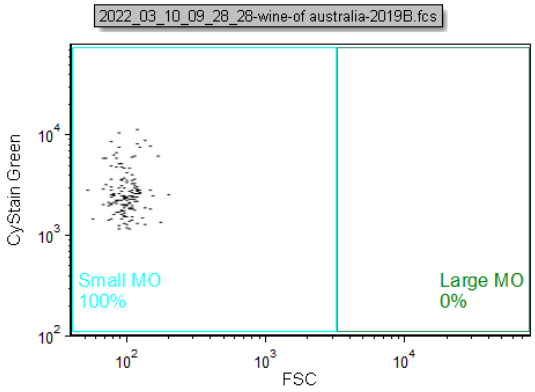


Fig. 19: FSC vs. FL1 dot plot separating between small (mainly bacteria) and large (mainly yeast) organisms

A total of eight wine samples were analysed during the study but only two samples with viable microorganisms could be cultured in liquid media (results not shown).
A study by V. Millet and A. Lonvaud-Funel (Millet & Lonvaud-Funel, 2000) showed that the analysis of wine bacteria after filtration by using the counting method only enumerates the part of population that is cultivable at a given time. Counting and flow cytometry are often reported to give different results due to the ability of

some microorganisms to survive in a viable but non-culturable state (Roszak & Colwell, 1987).

Further wine samples were spiked by the Gusmer Fermentation Center. These wines spiked with *Oenococcus oeni* prior to analysis with the CyStain™ OenoCount kit gave highly accurate results (see Fig. 20 and Fig. 21).

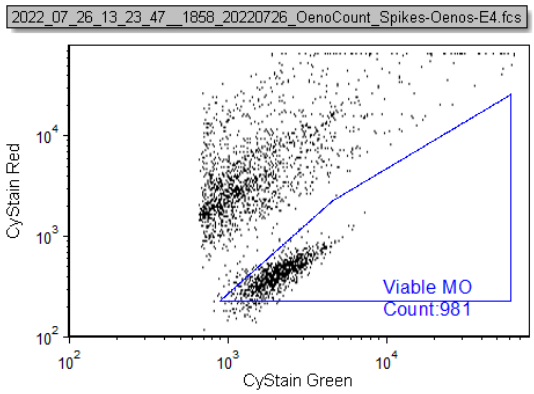


Fig. 20: FL1 vs. FL3 dot plot showing a cluster of Oenococcus oeni in a red wine sample

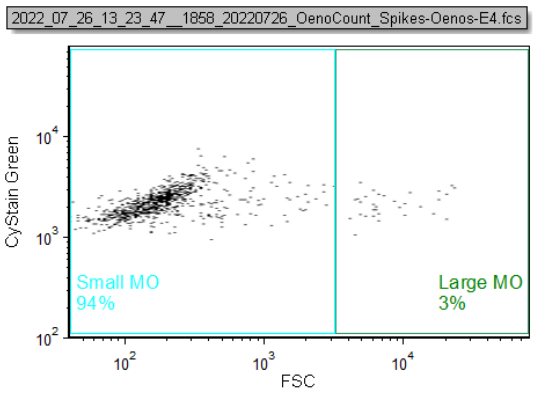


Fig. 21: FSC vs. FL1 dot plot showing that mainly small microorganisms are present in the sample

In addition, other species of viable wine microorganisms such as *Oenococcus oeni*, *Pediococcus damnosus*, *Acetobacter aceti* and *Brettanomyces bruxellensis* were spiked at Sysmex-Partec GmbH. All microorganisms used were grown to the end of the exponential growth phase and stored at 2-8°C in a refrigerator. The wine sample "Dornfelder semi dry" frequently used for the study was spiked with a total of 100 cell/mL, enriched and analysed (See Fig. 22 and Fig. 23).

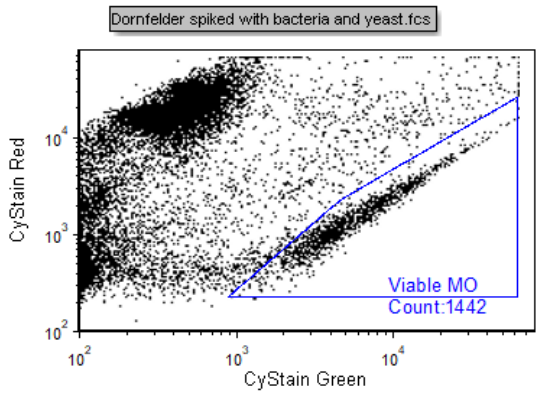


Fig. 22: FL1 vs. FL3 dot plot showing all viable microorganisms

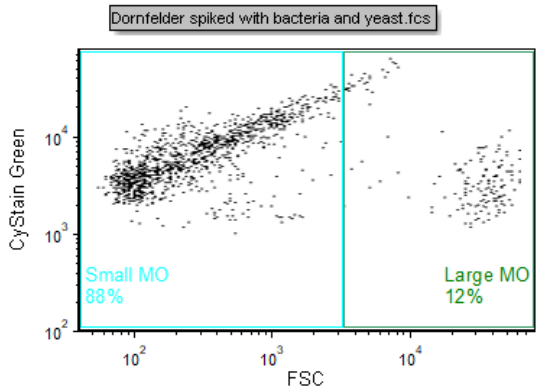


Fig. 23: FSC vs. FL1 dot plot showing small (Oenococcus oeni, Pediococcus damnosus and acetobacter aceti) and large (Brettanomyces bruxellensis) microorganisms recovered by filtration of 250 mL wine sample.

## Conclusion

In the first hand, only small amounts of microorganisms were detectable. Since the industrial partner did not see any microbial growth, we believe that the positive signals at these low concentrations are either spill-over signals from background noise (Fig. 10 ), possibly the colour matrix and other small particles present in the wine, or VBNC cells at very low concentrations. Focusing only on the contaminated bottles, 2 bottles contaminated with microorganisms could be identified in the second hand. *Oenococcus oeni* was detected using the CyStain™ OenoCount kit and the number of viable cells was correlating with a PCR test. Additionally, plating was showing a significant growth of *Oenococcus oeni*, while there was no growth of acetic acid bacteria.

Spiking samples with *Oenococcus oeni* at a concentration of 100 cells/mL resulted in a positive count that was 96.4% accurate (Fig. 20). Further spiking experiments with a variety of different species such as *Pediococcus damnosus*, *Acetobacter aceti* and *Brettanomyces bruxellensis* demonstrated the functionality of the detection of different classes and species.

It can be argued that the CyStain™ OenoCount kit can successfully quantify wine microorganisms at concentrations greater than 10 cells/ml. This concentration is likely to be exceeded if the bottling process is not working as expected.

It is important to note the inherent differences between traditional microbial plating and flow cytometry. While plating can differentiate between species and types of bacteria depending on the agar used, flow cytometry is much faster and more accurate at counting. A colony forming unit on a plate may represent multiple cells and cannot be accurately quantified. At the same time, flow cytometry reads and counts each event individually, distinguishing microorganism types based on size, internal complexity, and fluorescent signal. Traditional plating determines microorganism type based on colony structure and appearance, often requiring further analysis under the microscope and additional plating. Because of these differences, results between the two technologies will never line up exactly.

## References


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# Troubleshooting

### 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 |
| LOT NO° mismatch of QC material | Make sure to use a matching FCS Express™ template and QC material LOT NO° |

### Error #2: Poor vacuum generation

| Reason | Remedy |
|---|---|
| Filter membrane is inserted correctly within the vacuum filtration device | Insert the filter membrane correctly, place it in the middle of the steel frit |
| Lack of connection between vacuum filtration unit and vacuum pump | Proof proper connection of vacuum tubing in between vacuum pump and filtration unit |
| The outline filter is blocked / got in contact with wine sample | Replace the outline filter |

### Error #3: Low filtration speed (lower than expected)

| Reason | Remedy |
|---|---|
| Filter membrane is blocked | Replace the filter membrane and filtrate wine sample again |
| | Filter a maximum volume of 250 mL wine sample |

### Error #4: Fast filtration speed (higher than expected)

| Reason | Remedy |
|---|---|
| Filter membrane is damaged | Replace the filter membrane and filtrate wine sample again |
| filter is not placed in the middle of the steel frit | Insert the filter correctly, place it in the middle of the steel frit |

### Error #5: Cell number is too high

| Reason | Remedy |
|---|---|
| The filtration unit is not clean | Clean the complete filtration unit by filtration of 500 ml Ethanol [70%] in a first step and 500 ml ultra-pure water in a second step without any filter membranes. Then repeat wine sample filtration with a new filter membrane |
| The filter membrane is not sterile | Use a new filter |
| CyStain™ Elution Buffer is contaminated | Sterilize the CyStain™ Elution Buffer by filtration through a 0,2 µm filter |
| CyStain™ OenoCount kit component(s) is/are contaminated | Sterilize the CyStain™ OenoCount component(s) by filtration through a 0,2 µm filter |
| The flow cytometer is contaminated / background signals are too high / cross contamination | Clean the device according to the troubleshooting cleaning protocol of the CyFlow™ Cube6 V2m |

### Error #6: Viable cells outside predefined gate

| Reason | Remedy |
|---|---|
| Staining is incomplete | Increase the incubation time with CyStain™ OenoCount Green to a maximum of 20 minutes |
| | Check incubation temperature is set to 37°C |
| Incubation with CyStain Red is too long | Analyse the sample immediately after addition of Cystain™ OenoCount Red solution |
| Residues of CyStain Red inside the device | Perform a priming procedure, repeat sample preparation and measurement |
| Air bubbles inside the device | Perform a priming procedure, repeat sample preparation and measurement |

### Error #7: No separation between viable cells and background

| Reason | Remedy |
|---|---|
| Cell number too high | Dilute sample with filtered (0.22 µm filter) Dilution Buffer, repeat sample preparation and measurement |
| | Problem in bottling procedure: Defect in filtration prior bottling |



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