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Application Note

EZ-Fluo™ System

For rapid detection of spoilage organisms in wine

Many beverage manufacturing processes are susceptible to spoilage organisms like yeast or bacteria contamination. Contamination can alter the odor, flavor or turbidity of a beverage, resulting in customer dissatisfaction and, in some cases, in product recall. For these microorganisms, traditional monitoring methods require up to 10 days to obtain microbiological results allowing the release of the product. A rapid microbiology system that can detect potential contamination 3 times faster than traditional monitoring methods would result in a significant cost saving and preserved company reputation. The EZ-Fluo™ System uses fluorescence-based technology and is a convenient and a sensitive platform for the quantitative detection of contaminants in filterable samples. This rapid microbiological method is based on a universal enzymatic fluorescent staining of viable and culturable microorganisms. The fluorescent staining procedure is non-destructive, allowing microorganism identification following a positive result.

The EZ-Fluo™ system offers a fast and reliable alternative for the rapid detection of spoilage microorganisms in wines. This evaluation study, performed by the accreditated lab Centro de Investigación y Asistencia Técnica a la Industria (CIATI AC) in Argentina, shows that the system enables a faster response and corrective action when used during the wine manufacturing process. It improves process control, product yield and the faster release of final product to market.

Materials:

EZ-Fluo™ system Reader (EZFKIT001WW) Membrane Filtration systems (EZFTIMIC01)

Equipment:

EZ-Fluo™ system Reagent Kits (EZFREAG57)

Media:

MRS agar + tomato juice
Brettanomyces agar
Carr agar
YEPD agar

Matrices tested:

Red Wine

Microorganisms:

Lactic Acid Bacteria / Oenococcus oeni strain
Brettanomyces spp. Strain
Acetic acid bacteria (*AAB*)
Yeast Counts / Saccharomyces cerevisiae strain

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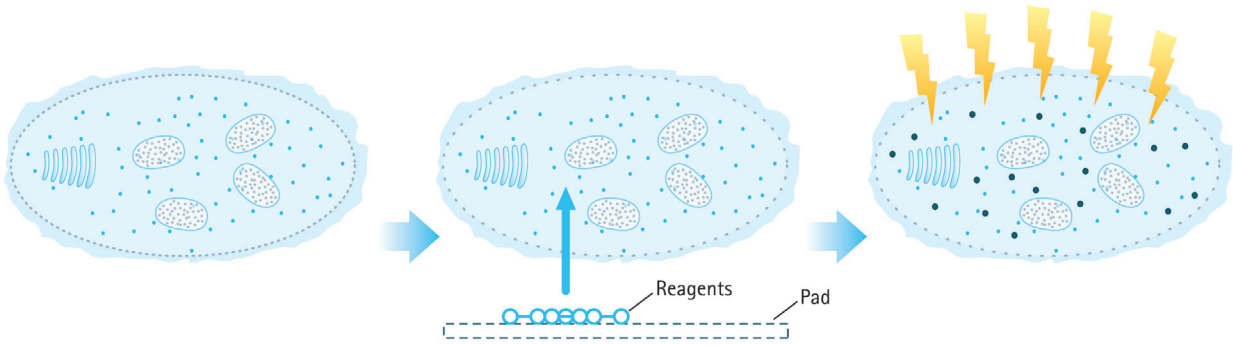
Principle of detection

The principle of the fluorescence detection is based on an enzymatic reaction. The fluorogenic substrate used is a non–fluorescent viability marker which is cleaved by non-specific ubiquitous intracellular enzymes resulting in a fluorescent product. Natural amplification of fluorescence by accumulation inside cells is an indicator of microbial metabolism. The dye is diluted in a staining buffer allowing cell membrane permeability and thus dye introduction into cells.



Note: Fluorescence detection is a non-destructive method that enables the microorganisms to continue to grow after they have been stained in order to identify them using standard ID technology.

Protocol for rapid detection

The procedure used was a standard protocol to detect spoilage microorganisms in samples of interest with the fluorescence detection:

- A filtration unit is installed onto the filtration system
- The appropriate volume of sample is poured into the filtration unit
- After filtration, the membrane is disconnected from the device and aseptically transferred onto a media cassette
- The incubation is performed according to the specifications
- After the incubation, the membrane is stained with the fluorogenic reagent for 30 min at 32.5°C (± 2.5)
- The fluorescent micro-colonies are counted using the fluorescence reader
- After detection, the stained membrane can be re-incubated on fresh media for traditional plate count and identification if required

Definition of a rapid incubation time

An appropriate incubation time is defined as the minimal time which allows a percentage of recovery above 70% compared to the traditional method. The calculation is based on both formulas:

- The fluorescence recovery is the fluorescent dot count compared to the traditional method count. Fluorescence recovery (%) = (average of fluorescence counts / average of traditional method count) x 100
- The viability recovery is the colony count on stained membranes after re-incubation compared to the traditional method count. Viability recovery (%) = (average of Colony-Forming Units counts after re-incubation / average of traditional method counts) x 100

An optimal incubation time should allow a sufficient fluorescent signal intensity and fluorescence and viability recoveries above 70%.

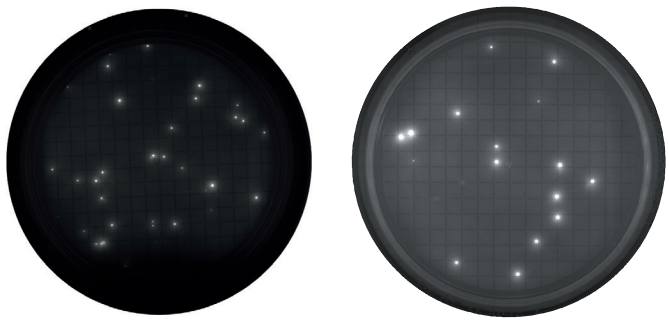


Figure 2:

The picture on the right illustrates a sufficient fluorescent signal intensity translating to an appropriate incubation time. The picture on the left shows that an accurate count is not possible if the intensity of fluorescence is too low due to an insufficient incubation time.

CIATI AC Evaluation Results

A series of tests on red wine were carried out to determine the performance of the rapid count system compared to traditional microbiological analysis, as established by the OIV and wine industry. Results in the tables are average counts of the performed tests.

1) *Brettanomyces* spp. counts

a) Inoculated samples

Three (n = 3) 500 mL samples of red wine were inoculated with different concentrations (N_0) of a *Brettanomyces* spp. strain previously isolated and characterized in CIATI AC microbiology laboratory.

Counts of the test yeast were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on *Brettanomyces* agar at 25°C (± 2) for 10 days under aerobic conditions) and the EZ-Fluo™ rapid system. The results were read at several times during incubation.

Results

Sample details	Days of incubation (25 °C)	Traditional microbiological test (CFU/10 mL)	EZ-Fluo™ System (CFU/10 mL)
Sample 1, estimated N_0 = 3 CFU/10 mL	3	<1	<1
	5	<1	3
	7	2	2
	10	3	4
Sample 2, estimated N_0 = 30 CFU/10 mL	3	<1	6
	5	<1	28
	7	13	33
	10	29	30
Sample 3, estimated N_0 = 30 x 102 CFU/10 mL	3	<1	52
	5	<1	295
	7	252	283
	10	304	307

b) Naturally contaminated samples

Five (n = 5) samples of red wine naturally contaminated with *Brettanomyces* spp were analyzed.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on *Brettanomyces* agar at 25°C (±2) for 10 days) and the EZ-Fluo™ rapid system (with incubation at 25°C (±2) for 5 days).

Results

Samples	Traditional microbiological test (CFU/10 mL) (10 days)	EZ-Fluo™ System (CFU/10 mL) (5 days)	Visible colonies at counting time with EZ-Fluo™ System (5 days)
1	49	45	0
2	570	601	0
3	1	2	0
4	2	1	0
5	55	37	0

2) Lactic acid bacteria (LAB) counts

a) Inoculated samples

Three (n = 3) 500 mL samples of red wine were inoculated with different concentrations (N0) of an *Oenococcus oeni* strain previously isolated and characterized at the CIATI AC.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on MRS agar + tomato juice at 30°C (± 2) for 10 days under aerobic conditions) and the EZ-Fluo™ rapid system. The results were read at several times during incubation.

Results

Sample details	Days of incubation (25 °C)	Traditional microbiological test (CFU/10 mL)	EZ-Fluo™ System (CFU/10 mL)
Sample 1, estimated N_0 = 3 CFU/10 mL	3	<1	2
	5	1	3
	7	2	2
	10	4	5
Sample 2, estimated N_0 = 30 CFU/10 mL	3	<1	14
	5	3	21
	7	23	30
	10	36	31
Sample 3, estimated N_0 = 30 x 102 CFU/10 mL	3	<1	42
	5	17	310
	7	254	303
	10	311	320

b) Naturally contaminated samples

Five (n = 5) samples of red wine naturally contaminated with lactic acid bacteria were analyzed.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on MRS agar + tomato juice at 30°C (± 2) for 10 days) and the EZ-Fluo™ rapid system (with incubation at 25°C (± 2) for 5 days).

Results

Samples	Traditional microbiological test (CFU/10 mL) (10 days)	EZ-Fluo™ System (CFU/10 mL) (5 days)	Visible colonies at counting time with EZ-Fluo™ System (5 days)
1	144	152	9
2	3	2	0
3	623	489	21
4	105	97	6
5	18	22	0

3) Acetic acid bacteria (AAB) counts

a) Inoculated samples:

Three (n = 3) 500 mL samples of red wine were inoculated with different concentrations (N0) of an *Acetobacter* spp. strain previously isolated and characterized at the CIATI AC.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on Carr agar at 25°C (± 2) for 4 days under aerobic conditions) and the EZ-Fluo™ rapid system. The results were read at several times during incubation.

Results

Sample details	Days of incubation (25 °C)	Traditional microbiological test (CFU/10 mL)	EZ-Fluo™ System (CFU/10 mL)
Sample 1, estimated N_0 = 3 CFU/10 mL	1	<1	<1
	2	<1	3
	3	2	4
	4	5	2
Sample 2, estimated N_0 = 30 CFU/10 mL	1	<1	<1
	2	<1	17
	3	11	25
	4	27	22
Sample 3, estimated N_0 = 30 x 102 CFU/10 mL	1	<1	<1
	2	<1	252
	3	199	277
	4	246	280

b) Naturally contaminated samples

Five (n = 5) samples of red wine naturally contaminated with acetic acid bacteria were analyzed.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on Carr agar at 25°C (± 2) for 4 days) and the EZ-Fluo™ rapid system (with incubation at 25°C (± 2) for 2 days).

Results

Samples	Traditional microbiological test (CFU/10 mL) (4 days)	EZ-Fluo™ System (CFU/10 mL) (2 days)	Visible colonies at counting time with EZ-Fluo™ System (5 days)
1	6	9	0
2	70	101	9
3	26	18	3
4	12	16	1
5	137	145	11

4) Yeast counts

a) Inoculated samples

Three (n = 3) 500 mL samples of red wine were inoculated with different concentrations (N0) of a *Saccharomyces cerevisiae* strain previously isolated and characterized at the CIATI AC.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on YEPD agar at 25°C (± 2) for 4 days under aerobic conditions) and the EZ-Fluo™ rapid system. The results were read at several times during incubation.

Results

Sample details	Days of incubation (25 °C)	Traditional microbiological test (CFU/10 mL)	EZ-Fluo™ System (CFU/10 mL)
Sample 1, estimated N_0 = 3 CFU/10 mL	1	<1	<1
	2	<1	2
	3	2	3
	4	3	2
Sample 2, estimated N_0 = 30 CFU/10 mL	1	<1	<1
	2	<1	32
	3	18	35
	4	33	29
Sample 3, estimated N_0 = 30 x 102 CFU/10 mL	1	<1	<1
	2	<1	289
	3	125	307
	4	296	301

b) Naturally contaminated samples

Five (n = 5) samples of red wine naturally contaminated with yeasts were analyzed.

Counts were performed using both the traditional method (filtration through a 0.45 µm membrane, and incubation on YEPD agar at 25°C (± 2) for 4 days) and the EZ-Fluo™ rapid system (with incubation at 25°C (± 2) for 2 days).

Results

Samples	Traditional microbiological test (CFU/10 mL) (4 days)	EZ-Fluo™ System (CFU/10 mL) (2 days)	Visible colonies at counting time with EZ-Fluo™ System (5 days)
1	28	24	7
2	1.97 x 103	2.33 x 103	46
3	115	175	15
4	42	36	9
5	5	3	0

Main outcomes of the CIATI AC evaluation study

- According to the results obtained, the EZ-Fluo™ rapid system performed very well in obtaining counts of wine spoilage microorganisms, since it reduced the incubation time by at least 50% in every case analyzed, in comparison to that required for traditional methods (OIV).
- The EZ-Fluo™ rapid system enabled the detection and quantification of *Brettanomyces* spp. and lactic acid bacteria (LAB) in as little as 5 days of incubation, and of acetic acid bacteria (AAB) and yeasts in as little as 2 days of incubation.
- The EZ-Fluo™ system is a useful tool for the rapid (and non-destructive) detection and quantification of wine spoilage microorganisms.

Conclusion

Using our fluorescence-based technology as a microbiology quality control tool reduces the time needed to detect yeast and bacterial contaminations in wine dramatically. This study demonstrated that this technology could easily replace the compendial microbiological method with a 2 to 4 times faster time to result, and a full compatibility with the standard culture media traditionally used for the detection of spoilage organisms in beverages.

Moreover, as the method is non-destructive, each fluorescent micro-colony detected will continue to grow to yield visible colonies allowing the identification of the contaminants using available identification methods.

With the EZ-Fluo™ system, beverage manufacturers can improve their quality control by detecting contaminations earlier, and implementing corrective actions faster. This early answer creates savings of raw materials and manufacturing capacities. It can also help in the root cause analysis of a process failure, giving a better knowledge and confidence in the process and an increased quality control. Most importantly the product release can be accelerated and storage time decreased, resulting in a financial saving for the manufacturer.

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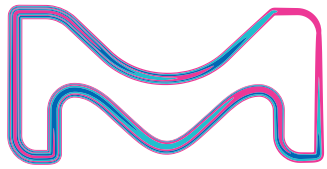

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