Assessment of a Rapid Microbial Detection System

1. Background

The objective of this project was to evaluate the Milliflex Quantum system for the detection of beer spoilage organisms in filterable matrices. Beer is generally considered to be microbiologically stable, only a limited number of species are capable of growing in packaged beer. Beer contains insufficient nutrients and oxygen to sustain many microbes. Low pH, the presence of hop agents and alcohol is also inhibitory to most bacteria. However, some spoilage species (generally known as the lactic acid bacteria) are tolerant of these conditions and their proliferation in beer can lead to acidity, turbidity and the formation of off-flavours. Traditional plate counting methods are used widely in the brewing industry to detect beer spoilage organisms and although they are cheap and reliable they are limited because most beer spoilage organisms grow slowly. For example, detection of lactobacilli can take between 5 to 7 days.

With increasing production demands most companies would like to reduce the time until result to gain better control of the manufacturing process. Millipore has developed an easy-to-use, non-destructive, fluorescent staining-based system for faster microbial detection in filterable matrices. This system uses industry standard membrane filtration techniques and a fluorescent-based technology to detect viable and culturable microorganisms. The sample is filtered through pre-sterilised, disposable filter units. The filter is then placed onto a pre-filled agar cassette and incubated. After a period of incubation (typically one third to one half of the standard incubation time for any given organism) the membrane is transferred to a pad pre-wetted with fluorescent agent and incubated for 30 minutes. The fluorescent colonies are then counted through a reader. After reading the membrane and detecting any colonies, the membrane can be transferred to a fresh agar plate and incubated for the remainder of the traditional incubation period and the organisms can be identified using existing ID methodology.

Millipore claims that the increase in price per test associated with implementing most rapid technologies is often off-set against the savings achieved by releasing stock earlier to market and by the reduction in amount of stock held in quarantine. These kinds of savings are not necessarily “visible” to the Laboratory Manager and in this case it is essential to involve people from the finance department within an organisation in order to make a calculation of the Return on Investment (ROI) associated with implementing a rapid technology.

2. Summary of results

In the validation phase beer spoilage organisms (*Lactobacillus brevis*, *Lactobacillus lindnerii* and *Pediococcus damnosus*) were spiked into lager beer at approximately 1, 10 and 100 CFU per 100ml beer, filtered and incubated an-aerobically on NBB or Raka Ray agar plates at 25°C. After a set period of time varying between 40h to 72h, plates were removed from the incubator, stained and the number of fluorescent cells counted.

Although some *Lactobacillus* colonies were clearly visible after 64h, the slower growing pediococci were not visible. For *L. lindnerii* and *P. damnosus* it was not possible to detect >70% of viable cells after 64h and a longer incubation time was needed (both with Quantum and traditional).

In the second phase of the study the incubation time was extended to 72h for the Milliflex Quantum and 7 days for the traditional analysis. Tests were repeated using a mixture of test microorganisms and a number of different beer styles. The lactic acid bacteria grew more slowly or not at all when spiked into beers with high hop acids (actual bitterness units were not tested) and

took longer to detect irrespective of the detection method used. Mixed cultures of all three microbes were spiked into lager and here fluorescent recovery was around 100% after 72h incubation and staining.

This evaluation clearly demonstrates the compatibility between the Quantum and the beers. It allows the detection of slow growing beer spoilers in approximately half the time taken for traditional plate counting analysis. And the cassettes can be easily re-incubated in case of contamination to perform an identification of the microbe.

3. Materials and Methods

Isolates of *Lactobacillus brevis*, *Lactobacillus lindnerii* and *Pediococcus damnosus* were chosen for the study because they are representative of the types of micro-organisms that might be found in packaged beer. They were obtained from the Campden BRI liquid nitrogen culture collection. These micro-organisms were grown in MRS broth statically at 25°C for 5 days. A total cell count was made using a Helber bacterial counting chamber. The cultures were diluted serially in quarter-strength Ringers solution (Oxoid) and spiked to obtain a final cell density of approximately 1, 10, 50 or 100 cells per 100ml beer. Ten replicates were analysed for the original validation exercise and four replicates were analysed for the different beer styles.

Spiked and control beer (not spiked) samples were filtered through a 0.45µm membrane filter (Catalogue number MXHAWG124) using the Milliflex PLUS pump (MXPPLUS).

Control beers that were observed to foam greatly were washed with 75ml of sterile 1% (v/v) Tween solution (Sigma) and 75ml sterile deionised water.

The membranes were transferred to a solid media cassette pre-filled with 10ml Raka Ray (Oxoid) or NBB agar (Döhler GMBH) and the funnel part removed by snapping manually or by using the Milliflex Millisnap (MILSNAP01).

The cassettes were then placed in an anaerobic jar with an anaerobic gas pack (Oxoid) and incubated at 25°C for a specified incubation time (between 40 hours to 72 hours).

After the incubation time the cassettes were removed from the incubator for staining. The staining was done by transferring the membrane onto liquid media cassettes (MXLMC0120) containing an absorbent pad. The liquid cassette was placed on the membrane transfer tool and wetted with 2ml of Milliflex Quantum Reagent. The membrane was then placed on the wetted pad and re-incubated for 30 minutes before reading.

The reading was done though the Milliflex Quantum reader's window and pictures were captured with the camera and saved in an electronic form. The computer mouse was used to count the number of fluorescent colonies.

After counting the membrane was re-incubated for a second reading at the traditional time. In order to do this the cassette was placed in the Removal Rack to remove the membrane from the liquid media cassette. It was placed manually onto a fresh agar cassette and re-incubated an-aerobically for the remainder of the traditional incubation time. Agar cassettes for traditional analysis were incubated an-aerobically for 5 or 7 days before counting by eye.

4. Results

4.1 Equipment evaluation

A comprehensive two day training course was given by Millipore at the start of the instrument evaluation. The trainers were very friendly and helpful. A brief lecture was given to introduce the system. The instrument was set up in the laboratory by Millipore and the trainees were given ample time to practice filtering and staining the cassettes for themselves whilst being given tips on best practice. All questions regarding use of the system were answered. The trainees were shown how to clean and decontaminate the system and advice was given on how to maintain it. A comprehensive instruction manual was left on site and technical support was provided from France (over the telephone) and through a local representative in the UK.

The microbiologists found that the system was easy to use and adaptable so that the filtering, incubation and staining steps could be modified to fit in with their working day. The time until result was reduced from 5 days (traditional analysis) to 3 days (Milliflex Quantum) for most beer styles. The quick time to result allowed the microbiologist to report results in the same working week. This is not normally the case for traditional analysis. The staining and image capture process made the colonies easier to visualise and assisted the counting process. The images were captured as JPEG files and were easily copied into the project report.

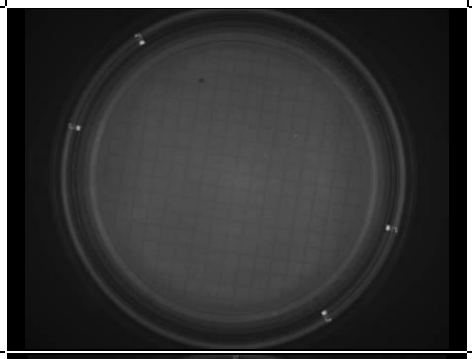
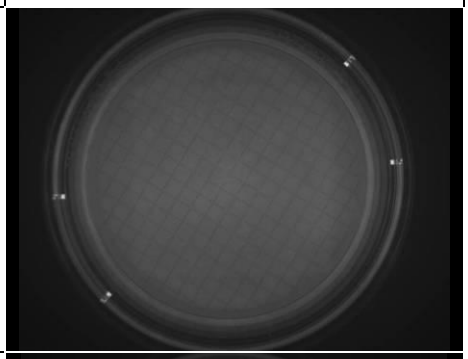
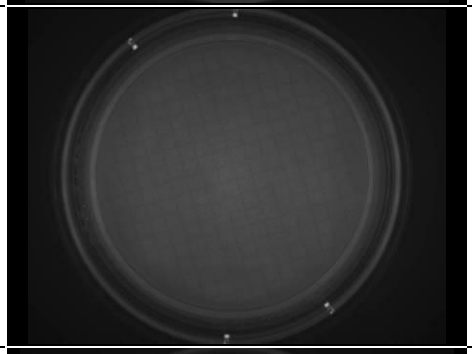
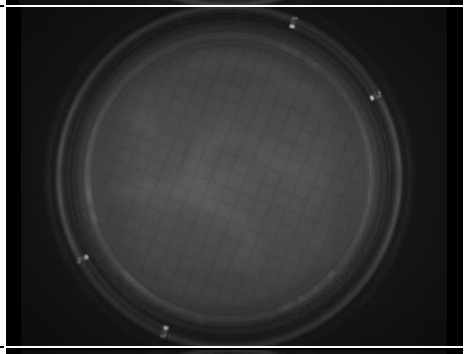
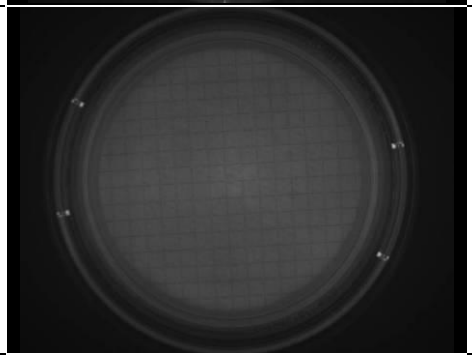
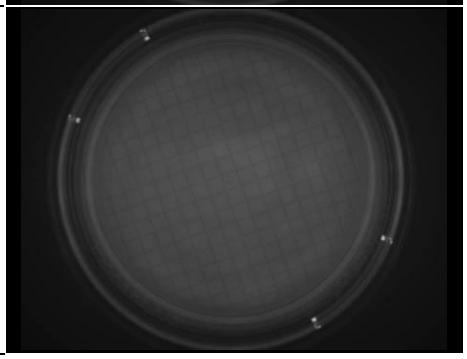
4.2 Validation of the equipment

4.2.1 Assessment of sample filterability and fluorescent background

In order to assess the product compatibility with Milliflex Quantum system; some preliminary basic tests were needed. First the filterability of various beer styles was assessed and then the absence of fluorescent background from these beers. A selection of ales and lagers, a bottle conditioned ale and a cider were tested. Some of the beers tested, particularly the bottle conditioned beer, were observed to form foam when poured in the filter funnel, although degassed beers did not have this problem. Where foaming was observed a rinsing protocol was set up using 75ml of sterile 1% (v/v) Tween solution (Sigma) and 75ml sterile deionised water to reduce the foam.

There was no fluorescent background in any of the beers tested after filtration using the Milliflex incubation at 25°C and staining with Quantum reagent (Figure 1). All beers tested were compatible with Quantum's fluorescence technology and were free from microbial contamination.

Figure 1: Quantum pictures of different sterile beer types

Beer types	Quantum pictures of sterile beer	Beer types	Quantum pictures of sterile beer
Lager 1		Lager 2	
Ale 1		Ale 2	
Bottle conditioned		Cider	

4.2.1 Determination of the time-to-result of L. brevis in lager on Raka Ray at 25°C

After assessing the filterability of the beers and confirming the absence of fluorescence background, the operators had to determine the time-to-results using the Quantum technology.

A lager beer was inoculated at a range of cell densities between 1 to 50 cells per 100ml beer and incubated for 40 to 64h (approximately one third to one half of the traditional incubation time). The tests were conducted on Raka Ray and NBB media. There were problems with the NBB plates becoming wet. When colonies were observed, they tended to spread and it was not possible to accurately determine fluorescent recoveries. Some microbes grew more slowly on NBB and were not detected in the Milliflex test period. The results on NBB were inconsistent and further tests were not conducted on this medium. The tests were then conducted on Raka Ray, incubated under an-aerobic conditions at 25°C. Short incubation times of less than 64h were not suitable for the detection of beer spoilage organisms in this experiment. Cell recovery was not good before this time. The best recovery rates were observed for *L. brevis* at an inoculation density of around 25 cells/100ml of beer and incubation at 25°C for 64h (Table 1). From the raw data, 2 rates were calculated the fluorescence and re-incubation recoveries. According to the pharmaceutical regulation for the validation of alternative methods (ISO 16140), the criteria for an appropriate incubation time with Milliflex Quantum are defined as following:

(i) The **fluorescence recovery** [Milliflex Quantum average CFUs *versus* traditional control average CFUs] is ≥ 70%. The recovery is calculated based on the formula:

Fluorescence recovery (%) = Milliflex Quantum average CFUs x 100 / traditional control average CFUs

(ii) The **re-incubation recovery** should be ≥ 70%. The re-incubation recovery is calculated after re-growing the colonies based on the formula:

Re-incubation recovery (%) = Milliflex Quantum re-grown average CFUs x 100 / traditional control average CFUs

The fluorescent and the re-incubation recovery rates after 64 hours of incubation were calculated and did conform to the criteria (> to 70%) for this test and show that the system can reach good performance with *L. brevis* in lager after 64 hours of anaerobic incubation on Raka Ray at 25°C.

Table 1: Raw data of *L. brevis* in Lager on Raka ray at 25°C (spike around 20-30 CFU)

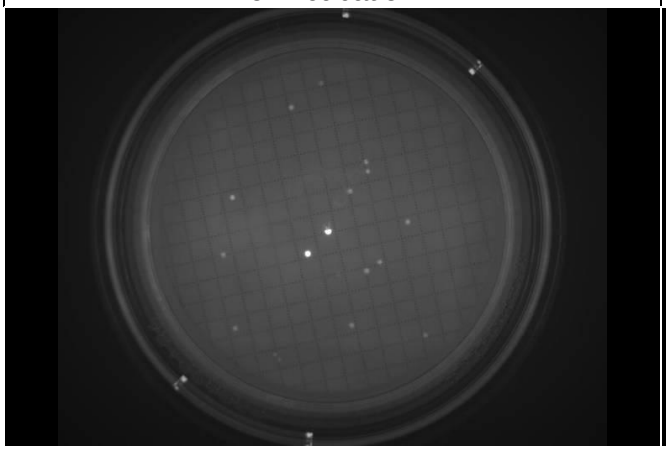
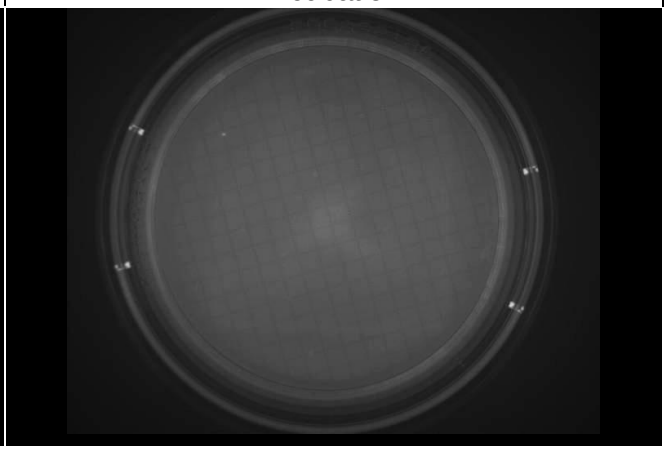
	Milliflex Quantum detection (fluorescent dots) after 64 hours	Re-incubation count (CFUs) after 5 days	Milliflex traditional controls (CFUs) after 5 days
Replicate 1	18	24	25
Replicate 2	17	24	23
Replicate 3	24	26	24
Average	**20**	**25**	**24**
Fluorescence recovery (%)	*81.9*		
Re-incubation recovery (%)		*102.8*	

Recovery rates were also good for *L. brevis* in lager at a lower inoculum level of *ca.* 5 cfu/100ml beer and showed that the system did detect low levels of *L. brevis* in lager. Figure 2 shows two quantum pictures taken from the detection of *L. brevis* in lager at different inoculum levels. The picture on the right illustrates the detection of only 1 cfu on the membrane.

Table 2: Raw data of *L. brevis* in lager on Raka ray at 25°C (spike around 5-10 CFU/100ml beer)

	Milliflex Quantum detection (fluorescent dots) after 64 hours	Re-incubation count (CFUs) after 5 days	Milliflex traditional controls (CFUs) after 5 days
Replicate 1	4	8	6
Replicate 2	9	10	8
Replicate 3	1	5	5
Average	**5**	**8**	**6**
Fluorescence recovery (%)	*73.7*		
Re-incubation recovery (%)		*121.1*	

Figure 2: Quantum pictures of *L. brevis* in lager

Replicate 1 of L. brevis in lager on Raka ray at 25°C (spike around 20-30 CFU) after 64 hours of incubation	Replicate 3 of L. brevis in lager on Raka ray at 25°C (spike around 5-10 CFU) after 64 hours of incubation
	

4.2.2 Determination of the time-to-result of L. brevis, P. damnosus and L. lindneri in lager on Raka Ray at 25°C

The microbes chosen in this study were known to have different growth rates in standard microbiological media and in beer (unpublished observations). Early experimental observations suggested that the 64h incubation time would be too short to detect the slowest growing beer spoilage microbes like *P. damnosus* and consequently the Quantum incubation time was extended to 72h. Duplicate samples were taken for each experiment. The raw data obtained with the 3 microbes after 72 hours of incubation using Quantum detection is shown in Tables 3-5. The fluorescent and re-incubation recoveries were greater than 70% for all 3 microbes in this lager. Figure 3 illustrates the Quantum pictures obtained after 72 hours of incubation. The pictures show a good fluorescent signal for each microbe.

Table 3: Raw data of *L. brevis* in lager on Raka Ray at 25°C

	Milliflex Quantum detection (fluorescent dots) after 72 hours	Re-incubation count (CFUs) after 5 days	Milliflex traditional controls (CFUs) after 5 days
Replicate 1	51	23	32
Replicate 2	49	21	27
Average	**50**	**22**	**30**
Fluorescence recovery (%)	*169.5*		
Re-incubation recovery (%)		*74.6*	

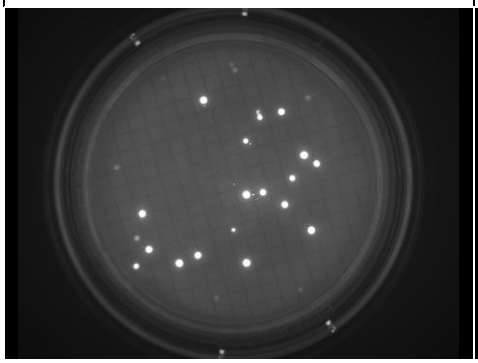
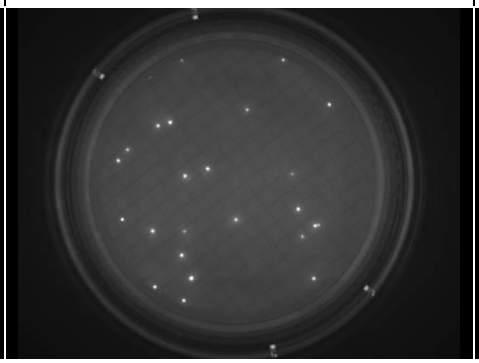
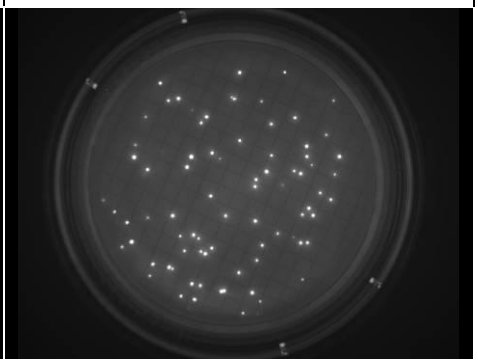
Table 4: Raw data of *P. damnosus* in lager on Raka Ray at 25°C

	Milliflex Quantum detection (fluorescent dots) after 72 hours	Re-incubation count (CFUs) after 5 days	Milliflex traditional controls (CFUs) after 5 days
Replicate 1	23	17	21
Replicate 2	28	20	22
Average	**26**	**19**	**22**
Fluorescence recovery (%)	*118.6*		
Re-incubation recovery (%)		*86.0*	

Table 5: Raw data of *L. lindneri* in lager on Raka Ray at 25°C

	Milliflex Quantum detection (fluorescent dots) after 72 hours	Re-incubation count (CFUs) after 5 days	Milliflex traditional controls (CFUs) after 5 days
Replicate 1	65	73	67
Replicate 2	62	63	60
Average	**64**	**68**	**64**
Fluorescence recovery (%)	*100*		
Re-incubation recovery (%)		*107.1*	

Figure 3: Quantum pictures of L. brevis, *P. damnosus* and *L. lindneri* in lager

Replicate 1 of L. brevis in lager on Raka ray at 25°C after 72 hours of incubation	Replicate 1 of P. damnosus in lager on Raka ray at 25°C after 72 hours of incubation	Replicate 1 of L. lindneri in lager on Raka ray at 25°C after 72 hours of incubation
		

4.2.3 Determination of the time-to-result of a mixed population of microbes in Lager on Raka Ray at 25°C

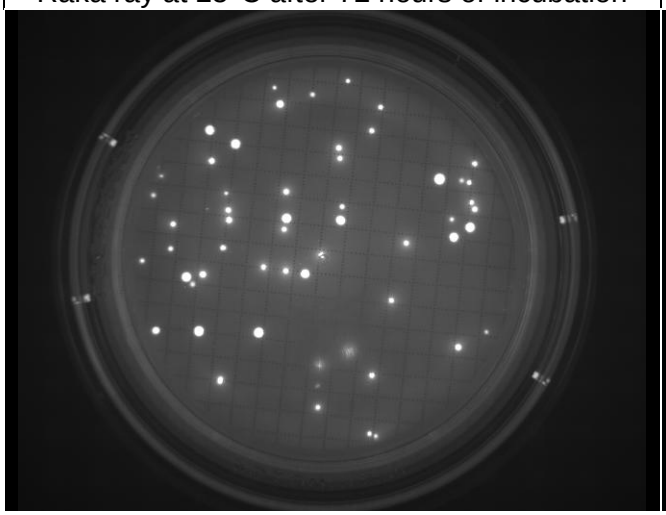
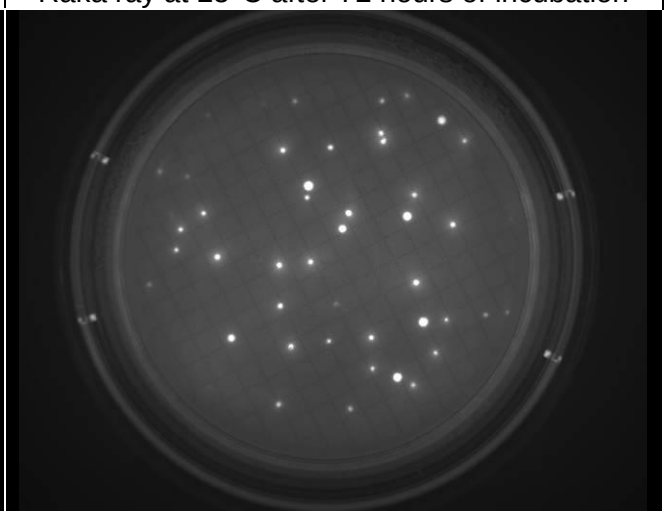
A lager was inoculated with a mixture of test micro-organisms at a cell density of approximately 50 cells per 100ml and incubated anaerobically on Raka Ray agar at 25°C for 72h. Table 6 shows the results obtained from the incubation of a mixed population in lager. Both fluorescence and re-incubation recoveries are greater than 70%. These trials show that the Milliflex Quantum system is easily able to detect a mixed population of these microbes in lager.

Table 6: Raw data of a mixed population in Lager on Raka ray at 25°C

	Milliflex Quantum detection (fluorescent dots) after 72 hours	Re-incubation count (CFUs) after 5 days	Milliflex traditional controls (CFUs) after 5 days
Replicate 1	60	55	55
Replicate 2	45	50	43
Average	**53**	**53**	**49**
Fluorescence recovery (%)	*107.1*		
Re-incubation recovery (%)		*107.1*	

Figure 4 shows quantum pictures obtained after staining for the mixed population of microbes. The different populations of microorganisms show some variation in colony size and fluorescence intensity and are visible to the eye after staining.

Figure 4: Quantum pictures of a mixed population in Lager

Replicate 1 of a mixed population in Lager on Raka ray at 25°C after 72 hours of incubation	Replicate 2 of a mixed population in Lager on Raka ray at 25°C after 72 hours of incubation
	

4.2.3 Determination of the time-to-result of a of L. brevis, P. damnosus and L. lindneri in different beer types on Raka Ray at 25°C

In the second phase of the project different beer styles were inoculated with the test micro-organisms at a cell density of approximately 50 cells per 100ml in order to show their compatibility of Millilfex Quantum. Two lagers, two ales, one bottle conditioned ale and one cider were tested. The samples were filtered, incubated on Raka Ray agar at 25°C for 72 hours. The cassettes were stained with Quantum reagent, read though the Quantum reader and re-incubated up to the traditional microbiological time. Two replicates were used for each beer.

For both lagers *L. brevis* and *L. lindneri* were detected well with Quantum technology and both the fluorescence and re-incubation count were above the 70% threshold of detection (Table 7). *P. damnosus* reached the 70% threshold in lager sample 1, but was under the threshold for the lager sample 2. The fluorescence rate was particularly low which suggested that for this type of beer a longer incubation period would be needed. The figures in the last column show that the fluorescence signals are clearly visible for most microorganisms (except *P. damnosus* in lager style 2) and confirm that the beer samples do not interfere with the Quantum technology.

For the both ale samples *L. brevis* and *L. lindneri* were detected well with Quantum technology the two rates (fluorescence and re-incubation rates) were above the 70% threshold of detection. *P. damnosus* reached the 70% threshold in Ale 2 sample for both counts, but was under it for fluorescence recovery in Ale 1 sample. That could mean that for this type of beer, a longer incubation of a few hours would be needed. The pictures in the last column show that the fluorescence signals are clearly visible for each microorganism (except *P. damnosus* in Ale 1) and confirm that the beer sample do not interfere with the Quantum technology.

For the cider sample, the fluorescence and recovery rates were above 70% for the 3 micro-organisms. The pictures confirm a good growth of the microbes and the non interference of the cider with the Quantum technology.

For the bottle conditioned ale only the *L. brevis* showed visible to growth in the traditional analysis. It was clear that the physico-chemical properties of the beer prevented the growth of *L. lindnerii* and *P. damnosus* and that this was not thought to be related to the Quantum technology.

Table 7: Raw data of L. brevis, P. damnosus and L. lindneri in Lager on Raka ray at 25°C

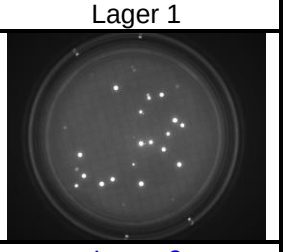
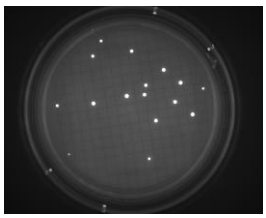
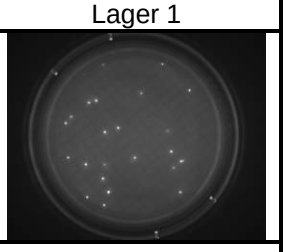
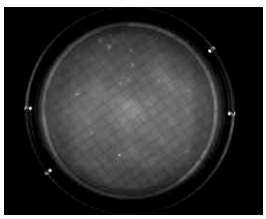
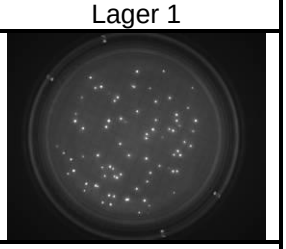
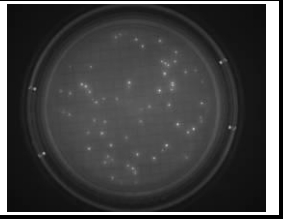
	Milliflex Quantum detection (fluorescent dots) after 72 hours		Re-incubation count (CFUs) after 5 days		Milliflex traditional controls (CFUs) after 5 days		Quantum picture
Beer type	Lager 1	*Lager 2*	Lager 1	*Lager 2*	Lager 1	*Lager 2*	Lager 1
Microbe	Lactobacillus brevis						
Average (based on 2 replicates)	50	*23*	22	*21*	30	*19*	*Lager 2*
Fluorescence recovery (%)	**169.5**	***124.3***					
Re-incubation recovery (%)			**74.6**	***110.8***			
Beer type	Lager 1	*Lager 2*	Lager 1	*Lager 2*	Lager 1	*Lager 2*	Lager 1
Microbe	Pediococcus damnosus						
Average (based on 2 replicates)	26	*16*	19	*19*	22	*29*	*Lager 2*
Fluorescence recovery (%)	**118.6**	***56.1***					
Re-incubation recovery (%)			**86.1**	***64.9***			
Beer type	Lager 1	*Lager 2*	Lager 1	*Lager 2*	Lager 1	*Lager 2*	Lager 1
Microbe	Lactobacillus lindneri						
Average (based on 2 replicates)	64	*57*	68	*68*	64	*57*	*Lager 2*
Fluorescence recovery (%)	**100**	***100***					
Re-incubation recovery (%)			**107.1**	***120.4***			

Table 2: Raw data of L. brevis, P. damnosus and L. lindneri in Ale on Raka ray at 25°C

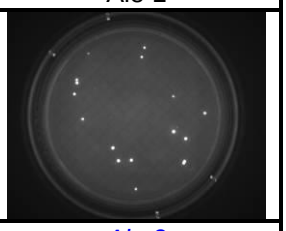
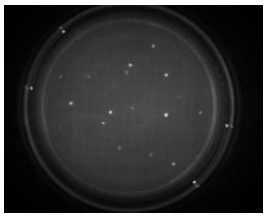
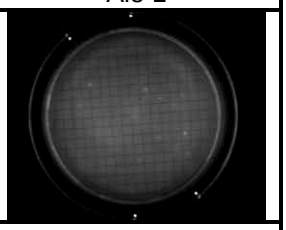
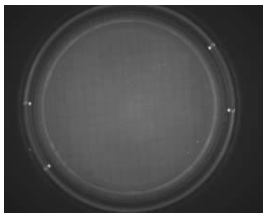
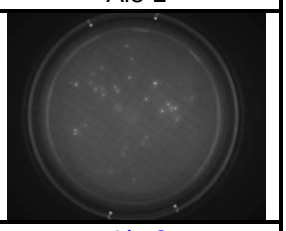
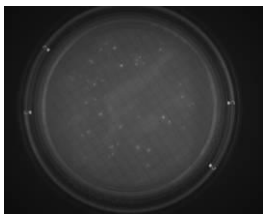
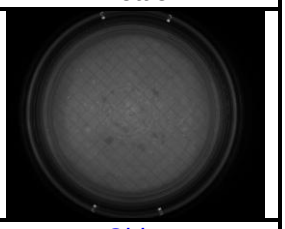
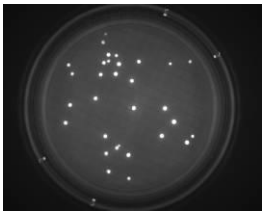
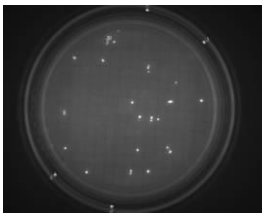
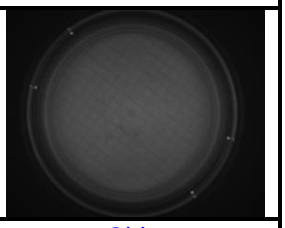
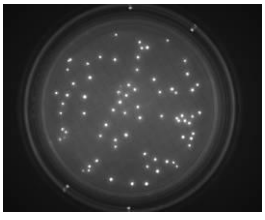
	Milliflex Quantum detection (fluorescent dots) after 72 hours		Re-incubation count (CFUs) after 5 days		Milliflex traditional controls (CFUs) after 5 days		Quantum picture
Beer type	Ale 1	*Ale 2*	Ale 1	*Ale 2*	Ale 1	*Ale 2*	Ale 1
Microbe	Lactobacillus brevis						
Average (based on 2 replicates)	29	*18*	22	*19*	27	*20*	*Ale 2*
Fluorescence recovery (%)	**107.5**	***87.5***					
Re-incubation recovery (%)			**81.1**	***95***			
Beer type	Ale 1	*Ale 2*	Ale 1	*Ale 2*	Ale 1	*Ale 2*	Ale 1
Microbe	Pediococcus damnosus						
Average (based on 2 replicates)	9	*13*	17	*21*	19	*11*	*Ale 2*
Fluorescence recovery (%)	**47.4**	***113.6***					
Re-incubation recovery (%)			**86.8**	***186.4***			
Beer type	Ale 1	*Ale 2*	Ale 1	*Ale 2*	Ale 1	*Ale 2*	Ale 1
Microbe	Lactobacillus lindneri						
Average (based on 2 replicates)	44	*36*	43	*62*	50	*52*	*Ale 2*
Fluorescence recovery (%)	**88.8**	***69.2***					
Re-incubation recovery (%)			**86.8**	***118.3***			

Table 3: Raw data of L. brevis, P. damnosus and L. lindneri in Bottle conditioned and cider on Raka ray at 25°C

	Milliflex Quantum detection (fluorescent dots) after 72 hours		Re-incubation count (CFUs) after 5 days		Milliflex traditional controls (CFUs) after 5 days		Quantum picture
Beer type	Bottle	*Cider*	Bottle	*Cider*	Bottle	*Cider*	Bottle
Microbe	Lactobacillus brevis						
Average (based on 2 replicates)	10	*26*	21	*24*	24	*20*	*Cider*
Fluorescence recovery (%)	**40.4**	***130.8***					
Re-incubation recovery (%)			**89.4**	***123.1***			

Beer type	Bottle	*Cider*	Bottle	*Cider*	Bottle	*Cider*	Bottle
Microbe	Pediococcus damnosus						
Average (based on 2 replicates)	0	*40*	16	*26*	0	*20*	*Cider*
Fluorescence recovery (%)	**NA***	***202.6***					
Re-incubation recovery (%)			**NA**	***133.3***			

NA*: Non Applicable

Beer type	Bottle	*Cider*	Bottle	*Cider*	Bottle	*Cider*	Bottle
Microbe	Lactobacillus lindneri						
Average (based on 2 replicates)	0	*79*	65	*58*	0	*61*	*Cider*
Fluorescence recovery (%)	**NA**	***129.7***					
Re-incubation recovery (%)			**NA**	***95.1***			

5. Discussion

In recent years a number of rapid microbiological detection methods have been developed to overcome the delay of traditional plate counting. ATP-bioluminescence probably is the most widely used rapid technology in the brewing industry but most ATP-based tests are non-specific and cannot be used to distinguish between ATP derived from microbes and that derived from food residues.

Molecular detection methods like PCR, ribotyping and fluorescence *in situ* hybridisation have been developed to detect lactic acid bacteria and have the potential to accelerate the detection of microbes in foods, but they are often impaired by the presence of inhibitory substances in the samples and growth media. The adoption of molecular methods has been limited in the brewing industry for a number of reasons: the tests can detect dead cells, there can be false positive or negative reactions, the sample volumes for PCR are relatively small (microlitres) compared to the volume of beer (litres) and the capital requirements for conducting these experiments can be uneconomic for product testing.

The Millipore system utilises industry standard membrane filtration techniques and combines it with a fluorescent-based staining technology to detect viable and culturable microorganisms in around one third to one half of the traditional incubation time. The main advantage of the system is the non-destructive staining. When organisms are detected there is still the possibility to continue to the traditional incubation time in order that microscopy / identification can take place to confirm the nature of the contaminant, and therefore the real risk to the final product. This new product has been adopted by the pharmaceutical and food industry to speed up microbial detection times but to our knowledge was not being used widely in the brewing industry. The objective here was to demonstrate the applications of the technology for the detection of microbes in packaged filterable beers.

L. brevis, L. lindnerii and *P. damnosus* were chosen because they are representative of the types of micro-organisms that might be found in packaged beer. It was not possible to test if the system could detect obligate anaerobic beer spoiling organisms such as *Pectinatus* and *Megasphera* because it was felt that the laboratory did not have sufficient facilities to prevent the inclusion of oxygen during the membrane filtration process. It would be possible to use the Milliflex Quantum for *Pectinatus* and *Megasphera* detection although an an-aerobic chamber would need to be used which allowed for the electrical connection and waste tubing for the Milliflex Plus Pump. This might not be practical in some laboratories.

To validate the system Millipore recommended a preliminary study testing several incubation times in order to set up the optimal incubation time for each product. Usually during this test 3 incubation times are tested for the range of one third to one half of the traditional incubation time.
The optimal incubation being the one that gives suitable fluorescence and re-incubation recovery rates determined for the slowest microorganism found in the product. A threshold of 70% of recovery for the two recovery rates was established according to the pharmaceutical regulation for the validation of alternative methods.

During this study *L. brevis* was easily detected by Quantum after 64h of incubation on Raka Ray agar, but the slower growing *L. lindnerii* and *P. damnosus* cultures were better detected after 72 hours. The 72h of incubation time was validated by a complementary test using a mixture of the test microorganisms, and by testing several beer types.

It was clear from the validation phase that the micro-organisms were growing more slowly in the experimental set up than expected. It is possible that the stresses imposed on the microbes by removing them from a nutritious broth culture to a beer environment may have resulted in a longer lag period than usually seen in a wild type culture that had been pre-adapted for survival in beer. Hop products are added to beer to impart a bitter flavour, but they also act as a preservative and

can suppress the growth of some Gram positive bacteria. Beer spoilage bacteria are able to grow in beer because they possess the hop resistance genes *horA* and *horC* (Suzuki *et al*., 2008). Some beer spoilage micro-organisms are able to increase their resistance to hops by sub-culturing in the presence of hop products. This is known as hop adaption however they can also loose some of their resistance when cultured without hops because the *horA* gene is coded by a plasmid and easily lost (Suzuki *et al*., 2008). In this experiment the lactic acid bacteria may have reduced hop resistance from repeated culturing in laboratory broth which might explain why they the incubation times until colonies could be detected were longer than expected.

The study shows that there was a clear influence of beer style on the recovery of spiked micro-organisms in both the fluorescent and traditional microbiology systems. There were differences in resistance to extrinsic stresses like ethanol, pH and hop acids between the three microbial species. In some beer styles the combined stresses were sufficient to promote cell death. In other beers the micro-organisms grow more slowly after exposure to these conditions. It is clear that a validation needs to be carried out for each product so that the incubation time to detect the slowest growing species can be accurately determined.

It is possible that some microcolonies of non lactic brewery microflora could be detected in this system. Re-incubation of stained cassettes will allow the operator to carry out further checks to identify the contaminant and to prevent false positive results.

6. Conclusions and Recommendations

The Milliflex Quantum system was found to be easy to use and completely suitable for use in brewing laboratory. It is important that each brewery validates the system with in-house beers. It is also recommended that the bacteria inoculated into these beers (for validation) are chosen carefully to reflect the differences in hop resistance and lag times between species and reflect the types of organisms typically found in their environment. The system worked well with Raka Ray agar but the trial was not successful with NBB agar and will need some further work development. The NBB agar manufacturer recommended that the agar plates should be poured as hot as possible (maximum 60°C for the cassettes) and that the plates were dried for 1h prior to utilisation. These recommendations have not been tested therefore it would be necessary to validate this and other agar recipes before use with this system.

7. References

Suzuki, K., Iijima, K., Sakamoto, K., Sami, M. And Yamashita, H. 2006. A Review of hop resistance in beer spoilage lactic acid bacteria. *J. Inst. Brew.* 112(2), 173-191.