Primer design

Evaluation of the genesig® q16 quantitative PCR unit

Evaluation of the genesig® q16 quantitative PCR unit for the detection of three anaerobic beer spoilage bacteria.

Executive summary

The ability of the genesig® q16 qPCR unit to detect three common anaerobic beer spoilage bacteria was evaluated. Using equipment and reagents from the manufacturer, it was possible to isolate qPCR-grade DNA from *Lactobacillus brevis*, *Pediococcus damnosus* and *Pectinatus portalensis,* inoculated into beer at high (10^4-10^5 CFU/mL) and low (10^2 CFU/mL) concentrations. After DNA extraction, the sample setup and run methods were simple, and the q16 unit was able to detect all organisms within 3 hours. Based on this study of three anaerobic bacteria, the q16 unit has the potential to be used in the brewing industry to identify specific contaminants.

Background

Using standard microbiological plating and enumeration methods, the detection and quantification of anaerobic beer spoilage bacteria typically takes 5-7days. The qPCR method, in which target DNA is amplified and quantified, has the potential to accelerate this process significantly.

Primerdesign Ltd. has developed a compact, portable qPCR Machine (genesig® q16), enabling the simultaneous analysis of up to 14 samples for the rapid detection and quantification of target DNA. The client approached Campden BRI to evaluate whether the genesig® q16 unit is able to be used to detect contaminants in beer and provide a degree of quantification of the level of contamination.

Scope of Work

This assessment determined whether the q16 unit was able to detect the presence of three common anaerobic beer spoilage bacteria (*Lactobacillus brevis*, *Pediococcus damnosus* and *Pectinatus portalensis*) at two different concentrations. The suitability of the DNA extraction kit to extract qPCR-quality DNA from the organisms was evaluated, as was the ability of the primers to amplify DNA regions of each of these bacteria. The qPCR method was also compared with a standard microbiological approach for the determination of beer contaminants (spread plating on a growth medium, specific for anaerobic bacteria).

Experimental

The assessment was carried out using three bacteria: *Lactobacillus brevis*, *Pediococcus damnosus* and *Pectinatus portalensis*. The bacteria were grown in de Man, Rogosa and Sharpe (MRS) broth at 25°C and the total cell number estimated using a Helber bacterial counting chamber and microscope. The bacterial cultures were each inoculated into 10 mL of a commercial 4% ABV lager beer at two target concentrations: high (~5×10^5 cells per mL) or low (~5×10^2 cells per mL) and a 0.2 mL aliquot of this cell suspension (~10^2 cells) was taken, from which the bacterial DNA was extracted. The low concentration samples were designed to correspond to the lowest limit for detection by qPCR, guaranteed by the manufacturer: 100 DNA copies (considered to be approximately 100 cells). The DNA extraction involved a cell lysis and proteolysis step, binding of nucleic acid to charged magnetic beads suspended within a binding buffer; washing; and elution into 0.2 mL buffer. The DNA extraction protocol is reported by the manufacturer to have a >99% extraction efficiency, therefore the final 0.2 mL eluate should theoretically contain the total DNA equivalent of 100 cells (low concentration) or 100,000 cells (high concentration).

The DNA extraction step was performed using equipment and reagents from the genesig® DNA extraction kit (Figure 1).

Figure 1: DNA extraction buffers and colour-coded pipettes from the genesig® Lab-In-A-Box.

After elution, 10 µL DNA from each purified DNA sample (DNA from ~5 cells) was mixed with organism-specific primers and a 2× PCR master-mix (total volume 20 µL) and loaded into the q16 unit. In addition, for each organism a negative control (water in place of DNA) and a positive control (an organism-specific DNA fragment in place of the sample DNA) was included (Table 1). The experiment was setup using the genesig® q16 PCR software (IT-IS International Ltd; version.1.1.1), and the PCR program was run automatically on the q16 unit via USB storage device. The results were saved automatically onto the USB drive by the q16 unit as a PPF file which could then be read by the genesig® q16 PCR software.

By way of comparison, a standard microbiological screen was performed alongside the q16 DNA detection method. This involved spread plating aliquots (0.1 mL) of the inoculated beer samples onto MRS agar and incubating the plates in an anaerobic environment for 7 days at 25°C. Colonies of bacteria were counted to determine the viable cell concentration.

Table 1: Sample setup in the q16 unit

Position in q16 unit	Sample name	Extracted DNA (µL)	Positive control DNA (µL)	2×PCR Mastermix (containing primers) (µL)	Water (µL)	Total reaction volume (µL)
1	*Lactobacillus brevis* HorA negative control	0	0	10	10	**20**
2	*Lactobacillus brevis* HorA positive control	0	10	10	0	**20**
3	*Lactobacillus brevis* HorA HIGH	10	0	10	0	**20**
4	*Lactobacillus brevis* HorA LOW	10	0	10	0	**20**
5	*Lactobacillus brevis* HorC negative control	0	0	10	10	**20**
6	*Lactobacillus brevis* HorC positive control	0	10	10	0	**20**
7	*Lactobacillus brevis* HorC HIGH	10	0	10	0	**20**
8	*Lactobacillus brevis* HorC LOW	10	0	10	0	**20**
9	*Pediococcus damnosus* negative control	0	0	10	10	**20**
10	*Pediococcus damnosus* positive control	0	10	10	0	**20**
11	*Pediococcus damnosus* HIGH	10	0	10	0	**20**
12	*Pediococcus damnosus* LOW	10	0	10	0	**20**
13	*Pectinatus portalensis* negative control	0	0	10	10	**20**
14	*Pectinatus portalensis* positive control	0	10	10	0	**20**
15	*Pectinatus portalensis* HIGH	10	0	10	0	**20**
16	*Pectinatus portalensis* LOW	10	0	10	0	**20**

Results

Traditional microbiology method

The results of the microbiological agar-based screen are shown in Table 2. The target 'low level' (10^2 CFU/mL) was obtained for both *Lactobacillus brevis* and *Pediococcus damnosus*. The 'high level' sample was determined as 10^4 CFU/mL for *L. brevis* and 10^5 CFU/mL for *P. damnosus*. No colonies of *Pectinatus portalensis* could be detected by the microbiological plate growth method, even after 12 days of incubation. This bacterium is considered a strict anaerobe and therefore growth was likely inhibited by any residual oxygen present in the incubation environment.

Table 2: Enumeration of bacteria by standard plating methods.
ND = not detected.

Organism	Anaerobic bacteria (CFU/mL)	
	Low concentration	High concentration
Lactobacillus brevis	7.6×10^2	3.4×10^4
Pediococcus damnosus	1.7×10^2	1.4×10^5
Pectinatus portalensis	ND	ND

Results of the q16 PCR run

The results for the genesig® q16 analysis for each organism are shown in Table 3. The quantification cycle (Cq) number indicates the point at which fluorescence was detected by the unit. The DNA "copy number", as calculated by the analysis software, is shown for the 10 µL aliquot of extracted DNA used for the qPCR.

Table 3: Overview of the qPCR run for each organism.

Test	Sample	Status	Copy number (per10 µL aliquot DNA)	Cq
Lactobacillus HorA	Negative control	PASS		
	Positive control	PASS		16.74
	Low level	POSITIVE	1	36.10
	High level	POSITIVE	230	28.82
Lactobacillus HorC	Negative control	PASS		
	Positive control	PASS		16.66
	Low level	POSITIVE	1	39.11
	High level	POSITIVE	656	27.23
Pediococcus	Negative control	PASS		
	Positive control	PASS		17.45
	Low level	POSITIVE	1	37.64
	High level	POSITIVE	522	28.35
Pectinatus	Negative control	PASS		
	Positive control	PASS		16.61
	Low level	POSITIVE	1	37.07
	High level	POSITIVE	861	26.80

A positive result was indicated for all samples, down to a single copy number (corresponding to approximately 5 cells or 1.7-7.6 CFU per 10µL) for the low level organisms. The low level samples were detected after 36-39 of 45 PCR cycles however this was sufficient to indicate a positive result. The individual PCR curves for each successfully-run sample are shown in Figures 2-4.

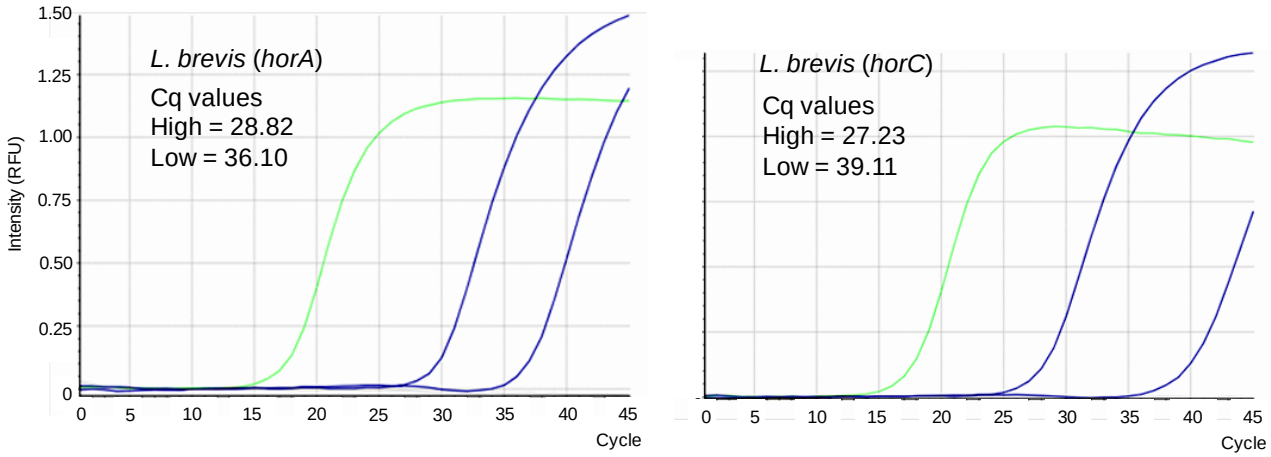

Figure 2: Detailed view of the DNA amplification curves of two concentrations of *Lactobacillus brevis* using primers specific for *horA* and *horC*. For each panel the positive control (green), the high concentration (blue, leftmost) and the low concentration (blue, rightmost) samples are shown, along with their corresponding Cq values.

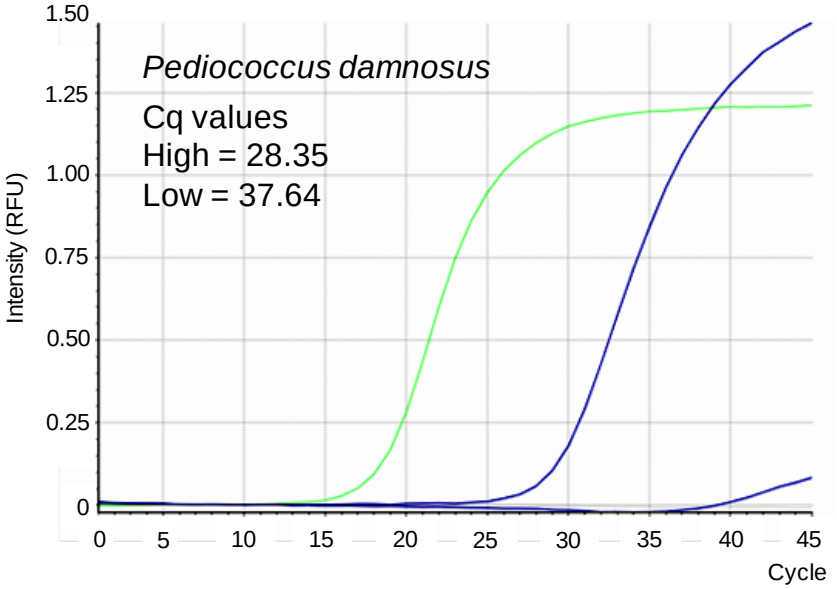

Figure 3: Detailed view of the DNA amplification curves of two concentrations of *Pediococcus damnosus*. The positive control (green), the high concentration (blue, leftmost) and the low concentration (blue, rightmost) samples are shown, along with their corresponding Cq values.

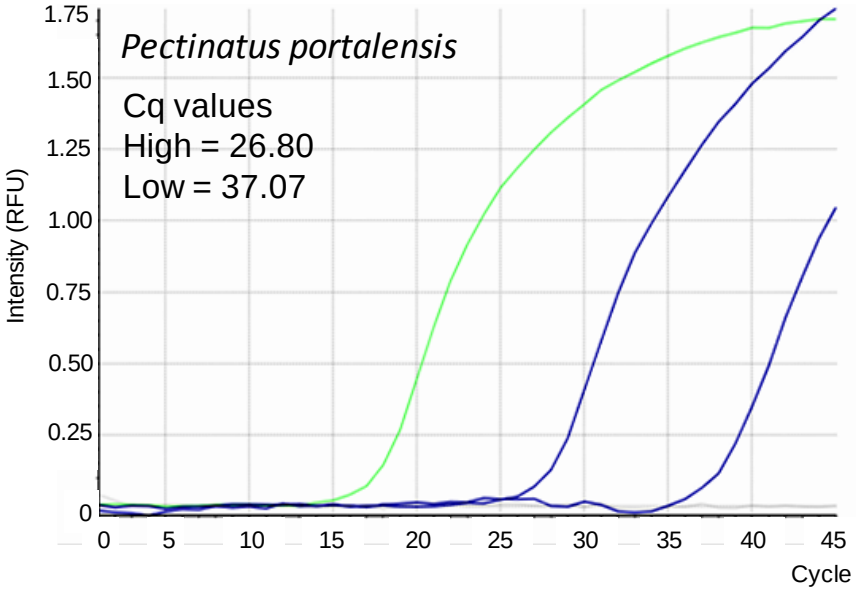

Figure 4: Detailed view of the DNA amplification curves of two concentrations of *Pectinatus portalensis*. The positive control (green), the high concentration (blue, leftmost) and the low concentration (blue, rightmost) samples are shown, along with their corresponding Cq values.

The low concentration sample for *Pediococcus damnosus* (Figure 3) showed only a very low level of fluorescence (~0.1 relative fluorescence units). This run was a repeat of an initial attempt in which the high concentration was successfully detected (Cq = 28.26; 416 DNA copies) but the low level sample could not be detected. An internal control of the initial run confirmed that the DNA extraction was successful; suggesting that the negative result was due to the low level of DNA present. The repeated test indicated a positive result after 37.6 cycles, highlighting that this sample was right at the limit of detection (equivalent to 1.7 CFU/10µL or 170 CFU/mL).

Comparison with microbiological methods

Comparisons between results from traditional microbiological plate-count-based methods and qPCR are generally difficult to make. Plate counts provide an indication of the number of viable and culturable cells, whereas the qPCR method calculates the amount of total DNA present from live or dead cells. An attempt has been made to compare the output of both methods in Table 4, based on the CFU counts per mL and the number of DNA copies detected by the q16 unit from 10 µL extracted DNA.

Table 4: Comparison of results from the traditional microbiological and qPCR methods.
ND = not detected.

Organism	**Microbiology (CFU/mL)**		**qPCR method (DNA copies per mL)**		
	Low	**High**		**Low**	**High**
Lactobacillus brevis	760	34,000	HorA	100	23,000
			HorC	100	65,600
Pediococcus damnosus	170	140,000		100	52,200
Pectinatus portalensis	ND	ND		100	86,100

Although qPCR for the rapid detection of beer spoilage organisms is becoming more widespread in laboratories, microbiological methods remain the standard technique for detecting brewery contaminants. Both approaches have their advantages and some of these are outlined in Table 5.

Table 5: Comparison of the genesig® q16 unit with conventional microbiology method for the detection of the three anaerobic bacteria.

Parameter	**Method**	
	genesig® q16	**Microbiological (spread plating)**
Analysis setup time	<1 hour (including DNA extraction)	2-3 hours (including media preparation)
Time until results	<3 hours	5-7 days
Limit of detection	1 “DNA copy” per 10µL extracted DNA*	10 CFU / mL
Specificity	Target DNA genus specific	Specific for lactic-acid bacteria
Detects viable cells	Viable and non-viable	Only viable and culturable
Allows further downstream characterisation?	No	Yes

*Based on this study, 1 DNA copy is the equivalent of 1.7-7.6 CFU per 10µL aliquot.

Conclusions

In this study the genesig® q16 quantitative PCR unit was evaluated for its ability to detect three anaerobic bacterial contaminants in beer at high and low concentrations. Standard microbiological screening methods were employed in parallel to indicate advantages and disadvantages of each method.

The manufacturer's DNA extraction method yielded qPCR-grade DNA; however no external confirmation was performed to compare DNA quality and quantity between samples. The beer containing bacteria at a high cell concentration resulted in the positive detection of DNA using the qPCR method. The low concentration of *Lactobacillus brevis* (based on primer annealing to regions within the *horA* and *horC* genes) and *Pectinatus portalensis* were also detected, with the low level of *Pediococcus damnosus* detected after the analysis was repeated.

The traditional plate-based microbiological approach used here has a lower detection limit than the qPCR method; standard filtration methods routinely used in microbiology can detect at even higher sensitivity, down to 1 CFU in a given volume (typically 100-250 mL). In addition, the microbiological approach selects for viable/culturable organisms whereas the qPCR method detects DNA, present in viable or non-viable organisms. However, the qPCR method is far more rapid than the microbiological approach and it should be noted that the microbiology culturing method for *Pectinatus portalensis* was not successful in this study. Therefore the qPCR approach is particularly useful for the detection of organisms that are difficult to culture.

Ease-of-use

Overall the q16 unit was easy to use. The machine is compact (12 centimetre footprint) and portable with the program for running the qPCR controlled offline via the accompanying computer software. The software was intuitive, and has been made freely-available on USB stick or for download from the genesig website (http://www.genesig.com/products/9696).

In this study, everything from the DNA extraction through to running the samples was performed using the equipment (pipettes, tips, reagents) provided in the manufacturer's Lab-in-a-box and DNA extraction kits. Although designed to be used by non-molecular-biologists, some steps require basic laboratory skills (e.g. accurate and aseptic pipetting of minute liquid volumes) to maximise the DNA extraction efficiency and prevent cross-contamination of samples.

Prior to the PCR run, the q16 unit indicates the machine status through coloured LEDs and provides an audio message to indicate "Power on", "Lid on" and "Run started". During the analysis, the LEDs change colour to indicate the machine is working and a 'rainbow colour' indicates the run is complete. During this trial, initial problems were encountered with starting the run from the USB, which were resolved by clearing the USB of all contents prior to use.

Final remarks

Overall the q16 unit was successful in the detection of the selected 3 anaerobic beer spoilage organisms to a detection limit of 1 "DNA copy" per 10 µL extracted DNA (corresponding to 1.7-7.6 CFU/10µL or 170-760 CFU/mL), depending on the organism tested). The small footprint of the unit is particularly appropriate for beer analysis laboratories with limited space and low sample throughput, to supplement existing microbiological methods.

Further tests for other spoilage organisms (e.g. wild yeast) would be useful and tests on non-beer matrices such as wort, culture yeast and fermentation samples would allow judgment as to whether this method is able to detect spoilers in non-beer samples.