Campden BRI
food and drink innovation

Rheonix® Inc.

Evaluation of Rheonix® Generation 2 Beer SpoilerAlert™ Assay

Summary

In this study the Generation 2 Rheonix Beer SpoilerAlert™ Assay (PCR technology) using the Rheonix® Encompass Optimum™ Workstation was evaluated. The specificity of the assay was good with all the target test organisms (*Saccharomyces cerevisiae* var *diastaticus* (2 strains), 5 *Brettanomyces* spp., 11 *Lactobacillus* spp, 2 *Pediococcus* spp., 1 *Pectinatus* sp., 1 *Megasphaera* sp.) being efficiently detected in beer samples when present at or above the reported minimal limit of detection of ~ 10^4 cells/ml. Additionally, beer-spoiler associated markers were also detected. One of the non-target organisms tested (a *Bacillus* sp.) showed a cross-reaction with one of the *Lactobacillus* probes. The manufacturer reports that typically, for lactic acid bacteria (LAB) cells concentrations a pre-enrichment of 18-24 hours is required to reach detectable levels. Similar enrichments times are required for *Saccharomyces cerevisiae* var *diastaticus* while *Brettanomyces* spp. may need longer due to typically slower growth. In this study, both yeast behaved as expected with low numbers of *Saccharomyces cerevisiae* var *diastaticus* and *Brettanomyces bruxellensis* requiring 24 and 48 hours, respectively, for reliable detection. In contrast, the LAB were inoculated at <10 cfu/ml and required 48 hours to reach detectable levels. Testing of a number of common brewery sample matrices showed that good results were obtained even in the presence of brewing yeast and as such can be used for the analysis of process samples. The removal of the *Saccharomyces cerevisiae* target from the assay in Generation 2 and the inclusion of a number of *Lactobacillus* spp. as well as several *Brettanomyces*, *Megasphaera* and *Pectinatus* probes have greatly improved the usefulness of the assay.

The system was very easy to use and required minimal sample handling and hands-on time. There is a guided easy-to-follow protocol on the user interface and results are obtained within 5 hours. Results are presented in a clearly laid out table with any positive beer spoiler results being highlighted. Technical on-line and phone support were excellent.

Introduction

Rheonix® has developed a fully automated and integrated molecular testing device (Encompass workstation) which provides a one-stop solution from sample application to result. The client originally approached Campden BRI to assess their Beer SpoilerAlert™ Assay (PCR technology) using the Rheonix® Encompass Optimum™ Workstation. An evaluation was performed using the original Beer SpoilerAlert™ assay targeting a number of different genes specific for *Pediococcus claussenii*, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* var *diastaticus*, *Brettanomyces*/*Dekkera bruxellensis*, *Lactobacillus brevis*, *Pediococcus* species, Lactic acid bacteria (LAB), LAB *horA* spoiler gene, LAB *horC* spoiler gene, *P. claussenii bsrA* gene, *P. claussenii bsrB* gene. The results of this evaluation can be viewed on the Campden BRI website: (https://www.campdenbri.co.uk/compare/BeerSpoilerAlert.pdf)

Rheonix has recently expanded the target menu for the Beer SpoilerAlert™ assay (generation 2) to include a wider range of *Lactobacillus* and *Brettanomyces* species, as well as detection of two strict anaerobic genera of bacteria, *Megasphaera* and *Pectinatus*. Additionally, in response to user input, detection of the non-*diastaticus* variant strain of brewer's yeast, *Saccharomyces cerevisiae*, has been removed, and similarly, a redundant probe for *P. claussenii* has also been removed. Table 1 shows a comparison of the genes targeted in the first and second generation of the Beer SpoilerAlert assay.

Target organism/ genes	Generation 1 SpoilerAlert	Generation 2 SpoilerAlert
Lactobacillus	*L. brevis* only	**≥39 species of *Lactobacillus***
Pediococcus	*P. acidilactici, P. cellicola, P. claussenii, P. damnosus, P. inopinatus, P. parvulus, P. pentosaceus and P. stilesii*	Same (8 species)
Brewer's yeast	*Saccharomyces cerevisiae*	Removed
Wild yeast	*Saccharomyces cerevisiae* var *diastaticus*	Same
	Brettanomyces bruxellensis	**4 additional species of *Brettanomyces* (total 5 species)**
Strict anaerobes	Not included	***Pectinatus* spp (3 species) & *Megasphaera* spp (4 species)**
Hop Resistance genes	*horA, horC, bsrA, bsrB*	Same (4 marker genes)
	Total number of targets: 15	**Total number of targets: 64**

Table 1 Comparison of first and second generation Beer SpoilerAlert targets

Methods

The following micro-organisms were either retrieved from the Campden BRI microbiological collection or sourced externally: *Saccharomyces cerevisiae* var *diastaticus* (2 strains), *Brettanomyces naardensis* (2 strains), *Brettanomyces/Dekkera bruxellensis* (2 strains), *Brettanomyces custersianus*, *Brettanomyces anomalus*, *Brettanomyces acidodurans*, *Candida albicans*, *Pichia kudriavzevii*, *Saccharomyces pastorianus*, *Saccharomyces cerevisiae* (2 strains), *Bacillus* (2 species), *Lactobacillus coryniformis*, *L. delbrueckii*, *L. paucivorans, L. acidophilus*, *L. plantarum*, *L. collinoides* , *L. parabuchneri*, *L. backii*, *L. casei*, *L. paracasei*, *L. brevis* (beer spoiler, 2 species), *L. brevis* (non-beer spoiler), *Pediococcus damnosus* , *P. claussenii*, *Pectinatus cerevisiiphilus, Megasphaera cerevisiae*. The micro-organisms used in this study were primarily isolated from contaminated beverages and identified by DNA sequencing or biochemical analysis. These species identifications are correct to the best of our knowledge and the current literature, however no guarantee can be given and genetic changes due to sub-culturing cannot be ruled out.

A commercial lager beer, believed to have undergone filtration and pasteurisation, was used for the study.

The Encompass Optimum™ Workstation was supplied by Rheonix®. All Beer SpoilerAlert™ consumables were provided by Rheonix®.

Specificity of assay

The organisms were grown up in appropriate broths and then streaked onto the corresponding agar plates (except for *Pectinatus* and *Megasphaera* which were only grown in broth). Bar code stickers were attached to the Rheonix® test tubes and aliquots of 1.2 ml sterile beer were dispensed into the tubes. Single colonies were picked from the streak plates and the cells suspended in the beer in the

tubes. In the case of the strict anaerobes *Pectinatus* and *Megasphaera*, 100 µl of culture were spiked into 1.2 ml sterile beer in tubes. All samples were analysed in duplicate.

The sample tubes containing the cell suspensions (subjected to a brief vortex, ~ 2 seconds) were placed into the provided sample rack. The sample rack, Rheonix CARD® cartridges, reagent brick and pipette tip boxes were loaded onto the Encompass Optimum™ workstation following the instructions on the user interface (UI).

Sensitivity of assay

Cell numbers in the microbial stock cultures of *P. claussenii*, *L. brevis* (spoiler), *S. cerevisiae* var *diastaticus* and *B. bruxellensis* were determined microscopically in counting chambers. A sterile commercial beer mixed (3:1) with sterile 4x concentrated broth (MRS for LAB, YM for yeast) was then spiked with the individual micro-organisms at a concentration of ~1-100 cells/ml (serially diluted stock culture). The samples were then enriched for 24 hours and 48 hours, at the appropriate growth temperature and atmosphere. After enrichment, the cultures were frozen. Once the enrichment for each organism was completed all samples were run together. They were defrosted and then 1.2 ml transferred to the sample tubes. The tubes were placed into the sample rack, loaded into the workstation along with the Rheonix CARD® cartridges, pipette tip boxes and the reagent brick following the UI instructions. All samples were analysed in duplicate.

In parallel, 100 ml of the spiked beer + broths were membrane filtered (before enrichment) and 100 µl aliquots spread plated (before and after enrichment) onto agar plates (either MRS for LAB or YM for yeast).

Sample matrix compatibility of assay

Bright beer, yeast slurry, FV sample, wort

Commercial beer, yeast slurry, fermentation sample (FV) and standard wort were individually spiked with *P. claussenii*, *L. brevis* (spoiler), *S. cerevisiae* var *diastaticus* and *B. bruxellensis* by adding one colony to 1.2 ml of matrix liquid pre-dispensed into Rheonix® sample tubes. In the case of *Pectinatus cerevisiiphilus*, 100 µl stock culture was added to 1.2 ml of the matrix liquid. Duplicate tubes were prepared for all samples. The colony containing tubes were subjected to a brief vortex (~ 2 seconds). All tubes were placed into the sample rack, loaded into the workstation along with the Rheonix CARD® cartridges, pipette tip boxes and the reagent brick following the UI instructions.

Data analysis

Following each experiment, the system produces a run report. An example for a 24-sample test is shown in Figure 1. Each row represents a sample. Positive reactions are shown with a red dot. The first two columns of the report give a quick answer whether a potential spoilage organism (PSO) and any hop resistance (HR) genes have been detected. A PSO is indicated if any of the organisms included in the assay is detected. The following columns give details as to which organisms have been detected – Lactic acid bacteria (*Lactobacillus* spp (7 groups: L1-7), *Pediococcus* spp. (PEDS)), Yeast spp (*Brettanomyces* spp: B1-5, *Saccharomyces cerevisiae* var *diastaticus* (DIA)), Anaerobic bacteria (*Pectinatus* (PEC), *Megasphaera* (MEG)). Additionally, it is reported whether there was a positive reaction for the lactic acid bacteria plasmid (LABS), which is present in some lactic acid bacteria, and whether any of the hop resistance genes (HORA, HORC, BSRA, BSRB) were detected. Indeterminate results are reported if the imaging software is unable to determine whether a spot is absent or present on the filter. This typically occurs in the absence of any target detection and a failed internal control.

However, it may also occur if there is an artefact that makes spot calling impossible. When an internal control has failed, all the targets associated with that master mix will appear indeterminate. Two distinct PCR master mixes are responsible for the amplification of individual targets therefore positive results obtained with one master mix are not negated by the failure of the other master mix. Master mix 1 contains primers for the amplification of L1-7, B1-5, DIA, MEG, PEC, and BSRB. Master mix 2 contains primers for the amplification of PEDS, LABS, HORA, HORC, and BSRA. Failure of all targets requires a repeat run. Alternatively, success of a single PCR master mix may provide enough information to the user to make a decision such that a repeat run may not be necessary.

The results are analysed in conjunction with the report key (see Figure 2). This table shows which *Lactobacillus* species are in each of the 7 groupings L1-7 and gives details of some possible cross-reactions between groups e.g. L1 bacteria may also be detected by the L7 probes. This will occur when the indicated species are present at significantly high concentrations. However, the secondary probe often will not demonstrate the same level in intensity as the primary probe but will still be greater than the threshold required by the imaging software to make a positive call. Additionally, the key explains the probe combinations for the 5 *Brettanomyces* species. Finally, the specific species detected under the PEDS, PEC and MEG headings are listed.

All the results obtained from the different tests were analysed to determine the specificity of the assay, its sensitivity of detection, the compatibility with the different sample matrices tested and the reproducibility of data (replicates).

Rhēonix®

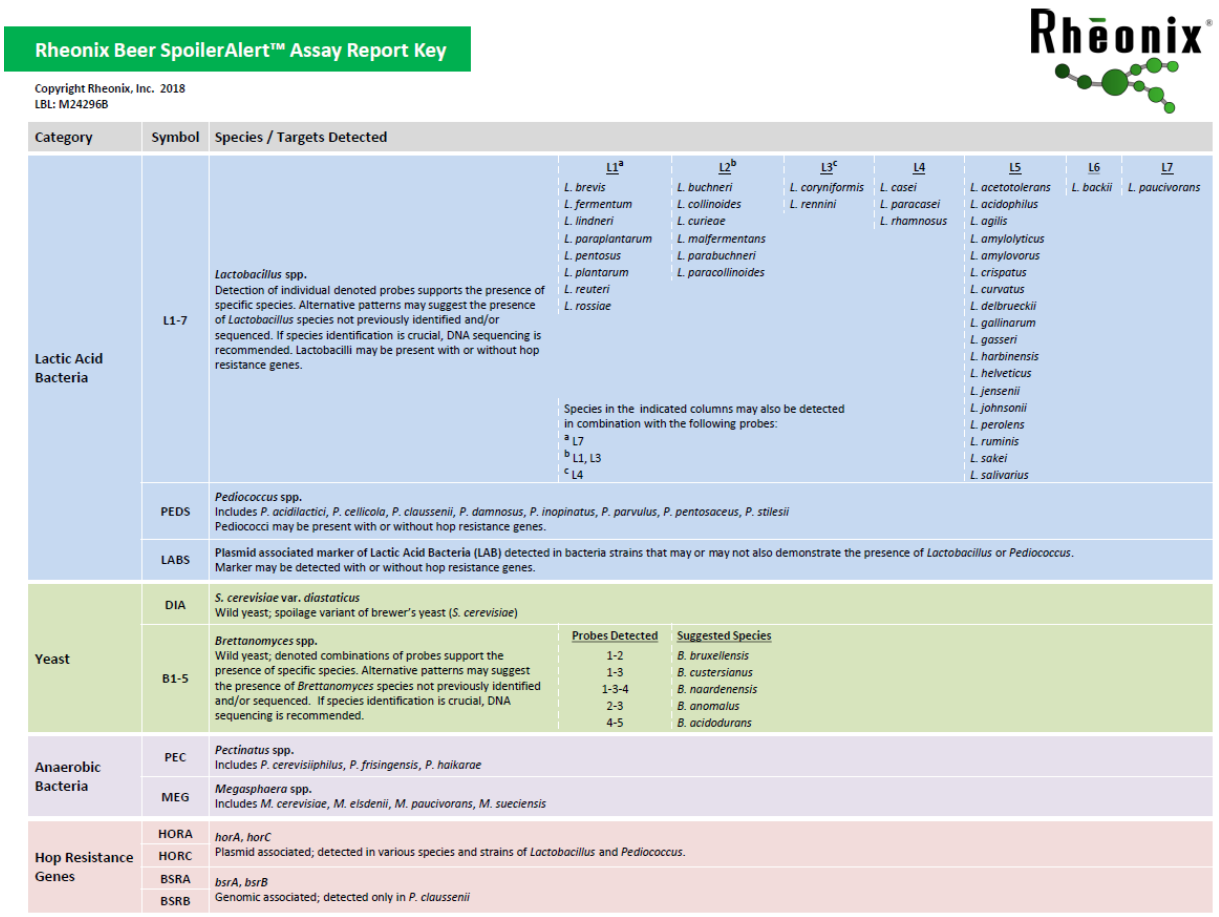
Operator: Microbiology Nutfield
Run Name: 20181026_18-30-24
Run Initiated: 10/26/2018 12:57:13
Run Completed: 10/26/2018 18:30:24

Assay: Beer SpoilerAlert™ assay
Instrument Model: Encompass Optimum™ workstation
Instrument Serial #: EncompassMDx20Jun2016-002
Reagent Lot / Expiration: 404310, 2028-09-12
CARD Lot / Expiration: 404299, 2019-03-11

		Summary		Lactic Acid Bacteria									Yeast						Anaerobes		Hop Resistance			
				Lactobacillus spp.									*Brettanomyces* spp.											
Lane	Sample ID	PSO	HR	L1	L2	L3	L4	L5	L6	L7	PEDS	LABS	B1	B2	B3	B4	B5	DIA	PEC	MEG	HORA	HORC	BSRA	BSRB
1	RHX00000015476	+			●						●													
2	RHX00000015477	+			●						●													
3	RHX00000015478	+	+	●								●									●	●		
4	RHX00000015479	+	+	●								●									●	●		
5	RHX00000015480	+	+	●																	●	I		
6	RHX00000015481		+																		●			
7	RHX00000015482	+	+		●																●			
8	RHX00000015483	+			●																			
9	RHX00000015484	+	+									●									●			
10	RHX00000015785	+	+	●								●									●			
11	RHX00000015786	+		●						●														
12	RHX00000015787	+		●																				
13	RHX00000015788	+	+	●																	●	●		
14	RHX00000015789	+	+	●																	●	●		
15	RHX00000015790	+	+	●																	●			
16	RHX00000015791	+	+	●						●											●			
17	RHX00000015792	+	+						●												●	●		
18	RHX00000015805	+	+						●												●	●		
19	RHX00000015806	+																●						
20	RHX00000015807	+																●						
21	ABC00000005997	+	+	●				●				●							●		●	●		
22	ABC00000005998	+	+					●				●							●		●	●		
23	ABC00000005999	+	+					●				●								●	●	●		
24	ABC00000006000	+																		●				

Key:
PSO Potential spoilage organism
HR Hop resistance gene
+ / ● Spoilage indicator(s) detected
(Blank) No spoilage indicator detected
I Indeterminate result
E Workstation error

Figure 1 Example 24-sample run report



Rheonix Beer SpoilerAlert™ Assay Report Key

Rheonix

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LBL: M24296B

Category	Symbol	Species / Targets Detected
Lactic Acid Bacteria	L1-7	*Lactobacillus* spp. Detection of individual denoted probes supports the presence of specific species. Alternative patterns may suggest the presence of *Lactobacillus* species not previously identified and/or sequenced. If species identification is crucial, DNA sequencing is recommended. Lactobacilli may be present with or without hop resistance genes. L1[a]: *L. brevis*, *L. fermentum*, *L. lindneri*, *L. paraplantarum*, *L. pentosus*, *L. plantarum*, *L. reuteri*, *L. rossiae* L2[b]: *L. buchneri*, *L. collinoides*, *L. curieae*, *L. malfermentans*, *L. parabuchneri*, *L. paracollinoides* L3[c]: *L. coryniformis*, *L. rennini* L4: *L. casei*, *L. paracasei*, *L. rhamnosus* L5: *L. acetotolerans*, *L. acidophilus*, *L. agilis*, *L. amylolyticus*, *L. amylovorus*, *L. crispatus*, *L. curvatus*, *L. delbrueckii*, *L. gallinarum*, *L. gasseri*, *L. harbinensis*, *L. helveticus*, *L. jensenii*, *L. johnsonii*, *L. perolens*, *L. ruminis*, *L. sakei*, *L. salivarius* L6: *L. backii* L7: *L. paucivorans* Species in the indicated columns may also be detected in combination with the following probes: [a] L7 [b] L1, L3 [c] L4
	PEDS	*Pediococcus* spp. Includes *P. acidilactici*, *P. cellicola*, *P. claussenii*, *P. damnosus*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus*, *P. stilesii* Pediococci may be present with or without hop resistance genes.
	LABS	**Plasmid associated marker of Lactic Acid Bacteria (LAB)** detected in bacteria strains that may or may not also demonstrate the presence of *Lactobacillus* or *Pediococcus*. Marker may be detected with or without hop resistance genes.
Yeast	DIA	*S. cerevisiae* var. *diastaticus* Wild yeast; spoilage variant of brewer's yeast (*S. cerevisiae*)
	B1-5	*Brettanomyces* spp. Wild yeast; denoted combinations of probes support the presence of specific species. Alternative patterns may suggest the presence of *Brettanomyces* species not previously identified and/or sequenced. If species identification is crucial, DNA sequencing is recommended. Probes Detected / Suggested Species: 1-2: *B. bruxellensis* 1-3: *B. custersianus* 1-3-4: *B. naardenensis* 2-3: *B. anomalus* 4-5: *B. acidodurans*
Anaerobic Bacteria	PEC	*Pectinatus* spp. Includes *P. cerevisiiphilus*, *P. frisingensis*, *P. haikarae*
	MEG	*Megasphaera* spp. Includes *M. cerevisiae*, *M. elsdenii*, *M. paucivorans*, *M. sueciensis*
Hop Resistance Genes	HORA HORC	*horA*, *horC* Plasmid associated; detected in various species and strains of *Lactobacillus* and *Pediococcus*.
	BSRA BSRB	*bsrA*, *bsrB* Genomic associated; detected only in *P. claussenii*

Figure 2 Report key

Results

Specificity of assay

Table 2 shows the results for the assay specificity testing. The second column lists the expected results for each organism and the last column gives the actual results. As can be seen the assay gave correct results for most test organisms.

Saccharomyces cerevisiae var *diastaticus* (2 strains), *Brettanomyces custersianus*, *Brettanomyces anomalus*, *Brettanomyces acidodurans* and one *Brettanomyces naardensis* as well as one *Brettanomyces/Dekkera bruxellensis* strain were successfully detected showing the expected results. The second *Brettanomyces naardensis* strain only showed the 'B4' response rather than the 3 probes (B1, B3, B4) suggested in the report key (see Figure 2). The manufacturer advises that B4 is the distinguishing probe for *B. naardensis* and that B1 and B3 are also detected but their signal is weaker and was therefore probably not detected in case of the second strain tested here. The report key will be amended to clarify the *B. naardensis* probe detection.

The second strain of *Brettanomyces/Dekkera bruxellensis* gave the expected B1 and B2 as per report key, but it was also positive for B3. It is unclear whether this specific strain caused a cross-reaction with the B3 probe or whether the organism was a different species. Interestingly, the manufacturer indicated that in early development of the assay the combination of B1/B2/B3 corresponded to *B.*

custersianus, but detection of B2 was inconsistent so was not included on the key. Additionally, the manufacturer indicated that over the course of this evaluation the decision was made to remove B2 from the assay as it does not provide additional discriminatory power to the speciation of *Brettanomyces.* Species confirmation of the strain of *Brettanomyces* tested here will be performed by DNA sequencing.

The non-*Brettanomyces* and brewing yeast species (*Candida albicans*, *Pichia kudriavzevii*, *Saccharomyces pastorianus*, *Saccharomyces cerevisiae* (2 strains)) were all correctly showing negative.

Two *Bacillus* species were tested. One of these was negative as expected, but the other (*Bacillus megaterium*) consistently showed positive for the L5 probe despite no *Lactobacillus* being present. The manufacturer confirmed that potential cross-reactivity of high concentrations of certain *Bacillus* species with the L5 probe is possible and that this information will be included in the product insert.

The majority of the *Lactobacillus* spp. were detected correctly, i.e. the successful test species showed the right L probes (*L. coryniformis*, *L. paucivorans, L. plantarum*, *L. collinoides*, *L. parabuchneri*, *L. backii*, *L. paracasei*, *L. casei*, *L. brevis* (beer spoiler, 2 species), *L. brevis* (non-beer spoiler)). For two of the *L. brevis* species (spoiler and non-spoiler) one of their replicates was positive for L7 as well as for L1. This combined detection is listed as a possibility for L1 species in the report key (see Figure 2). The L7 detection however appears to be weaker as it was only seen for 1 of the replicates – these replicates presumably containing a higher cell concentration. The beer spoiling and non-spoiling *L. brevis* species were correctly differentiated by the respective presence/absence of hop resistance genes (HORA, HORC). *L. parabuchneri* was successfully detected by the L2 probe however only one of the replicates showed positive for HORA. Similarly, one of the *Lactobacillus acidophilus* samples was correctly identified as belonging to the L5 group, however the second replicate sample was negative. For *L. delbrueckii* one of the duplicate samples was correctly detected as L5, but the other sample showed positive for L3 (not L5). Discrepancies between individual colonies may be due to insufficient cell concentration (e.g. *L. parabuchneri* and *L. acidophilus* results) and/or mixed colonies (e.g. *L. delbrueckii* result). Species identification of the *L. delbrueckii* sample will be verified by DNA sequencing.

Pediococcus claussenii gave positive results for PEDS and BSRA/B (hop resistance genes) as would be expected for this bacterium. *Pediococcus damnosus* also showed positive for PEDS however it was additionally consistently picked up by the L2 probe. This could be due to a cross-reaction with the L2 probe as the manufacturer has seen with some *Pediococcus* spp. Modifications to the filter key will be made to indicate this appropriately.

Both *Pectinatus cerevisiiphilus* and *Megasphaera cerevisiae* were positively detected by their respective probes.

The negative control samples (commercial beer used for the spiking of the organisms) were almost entirely negative with just once the HORA marker showing positive, possibly due to some residual genetic material in the beer (or some cross-contamination during sample preparation, although all efforts were made for this not to happen).

During these experiments it was noticed that generally a high cell concentration is required for successful detection. Especially for certain organisms/strains, a single colony was insufficient. The manufacturer recommends that all samples are enriched prior to being tested with this assay, therefore there should not be any issue with detection. For the enrichment of the organisms tested by this assay the medium needs to be carefully selected as it is common for growth media to contain DNA which is detectable with sensitive PCR assays. The presence of DNA may lead to potential false positive results and for this reason the manufacturer recommends that all media be tested prior to use with the assay. In this study MRS and YM was successfully used.

Organism	Expected detection (see key Figure 2)	Experimental test results (duplicates)
Saccharomyces cerevisiae var *diastaticus* – strain 1	DIA	DIA
Saccharomyces cerevisiae var *diastaticus* – strain 2	DIA	DIA
Brettanomyces naardensis - strain 1	B1 + B3 + B4	B1 + B3 + B4
Brettanomyces naardensis - strain 2	B1 + B3 + B4	B4
Brettanomyces bruxellensis - strain 1	B1 + B2	B1 + B2
Brettanomyces bruxellensis - strain 2	B1 + B2	B1 + B2 + B3
Brettanomyces custersianus	B1 + B3	B1 + B3
Brettanomyces anomalus	B2 + B3	B2 + B3
Brettanomyces acidodurans	B4 + B5	B4 + B5
Candida albicans	None	None
Pichia kudriavzevii	None	None
Saccharomyces pastorianus	None	None
Saccharomyces cerevisiae – strain 1	None	None
Saccharomyces cerevisiae – strain 2	None	None
Bacillus subtilis ss *subtilis*	None	None
Bacillus megaterium	None	L5
Lactobacillus brevis spoiler – strain 1	L1 (+ L7); HORA and/or HORC	Replicate 1: L1+ HORA; Replicate 2: L1+ L7+ HORA
Lactobacillus brevis spoiler – strain 2	L1 (+ L7) ; HORA and/or HORC	L1+ HORA + HORC
Lactobacillus brevis – non-spoiler	L1 (+ L7)	Replicate 1: L1 + L7; Replicate 2: L1
Lactobacillus plantarum	L1 (+ L7)	L1+ L7
Lactobacillus collinoides	L2 (+ L1 + L3)	L2
Lactobacillus parabuchneri	L2 (+ L1 + L3)	Replicate 1: L2 + HORA; Replicate 2: L2
Lactobacillus coryniformis	L3 (+L4)	L3 + LABS
Lactobacillus paracasei	L4	L4 + LABS
Lactobacillus casei	L4	L4
Lactobacillus acidophilus	L5	Replicate 1: L5; Replicate 2: negative
Lactobacillus delbrueckii	L5	Replicate 1: L3, HORA; Replicate 2: L5
Lactobacillus backii	L6	L6 + HORA + HORC
Lactobacillus paucivorans	L7	L7 + HORA + HORC
Pediococcus claussenii	PEDS + BSRA + BSRB	PEDS + LABS + BSRA + BSRB
Pediococcus damnosus	PEDS	PEDS + L2
Pectinatus cerevisiiphilus	PEC	PEC
Megasphaera cerevisiae	MEG	MEG

Table 2 Results of the assay specificity tests

Sensitivity of assay

Table 3 shows the results for the assay sensitivity testing. Four microorganisms were spiked at low level into beer/broth mixtures and incubated for 24 and 48 hours. Samples at all 3 timepoints were run with the Beer SpoilerAlert™ assay to determine how long samples need to be incubated for reliable organism detection. As can be seen all organisms were successfully detected after the 48 hour incubation. The 2 yeast species were detected after just 24 hours (one replicate only for *B. bruxellensis*), however for *Saccharomyces cerevisiae* var *diastaticus*, the cell concentration at this timepoint was already > 10^4 cells/ml. All organisms appeared to be positively detected at cell concentrations of > 10^4 cells/ml consistent with limit of detection of the assay. The manufacturer reported that LABs have been successfully detected following 24 hours enrichment of > 10 cfu/ml. The starting concentrations of the LABs in this study were 6 and 7 cfu/ml for *L. brevis* and *P. claussenii* respectively, and following 24 hours of growth only reached concentrations of 10^2 and 10^3, respectively, consistent with the lack of detection at 24 hours.

Sample	Enrichment (hrs)	Determined cell concentration (cfu/ml)	Experimental test results (duplicates)
Saccharomyces cerevisiae var *diastaticus*	0	57	Not detected
	24	3.3×10^4	**Detected (DIA)**
	48	8.9×10^7	**Detected (DIA)**
Brettanomyces bruxellensis	0	50-100	Not detected
	24	6.2×10^2	**One replicate detected (B1/2)**
	48	5.5×10^4	**Detected (B1/2)**
Lactobacillus brevis	0	6	Not detected
	24	2.6×10^2	Not detected
	48	5.9×10^5	**Detected (L1)**
Pediococcus claussenii	0	7	Not detected
	24	1.3×10^3	Not detected
	48	7.2×10^6	**Detected (PEDS)**

Table 3 Results of the assay sensitivity tests

Sample matrix compatibility of assay

Table 4 shows all the run results of the sample matrix compatibility experiments. These were carried out to establish whether the system is able to correctly pick out spoilage microorganisms in a number of process samples (wort, yeast slurry, fermentation sample, beer).

The results show that the assay correctly detected all 5 organisms in the four matrices. One workstation error occurred during these runs, the only one throughout the study. Analysis of the camera image (spot image, available in the saved results) for this sample showed the results to be good/valid, so that they could be used without having to repeat the run.

Sample	Sample matrix	Experimental test results (duplicates)
Saccharomyces cerevisiae var *diastaticus*	Wort	**Detected (DIA)**
	Yeast slurry	**Detected (DIA)**
	Fermentation sample	**Detected (DIA)**
	Beer	**Detected (DIA)**
Brettanomyces bruxellensis	Wort	**Detected (B1/2)**
	Yeast slurry	**Detected (B1/2)**
	Fermentation sample	**Detected (B1/2)**
	Beer	**Detected (B1/2)**
Lactobacillus brevis	Wort	**Detected (L1)**
	Yeast slurry	**Detected (L1)**
	Fermentation sample	**Detected (L1)**
	Beer	**Detected (L1)**
Pediococcus claussenii	Wort	**Detected (PEDS, BSRA, BSRB)**
	Yeast slurry	**Detected (PEDS, BSRA, BSRB)**
	Fermentation sample	**Detected (PEDS, BSRA, BSRB)**
	Beer	**Detected (PEDS, BSRA, BSRB)**
Pectinatus cerevisiiphilus	Wort	**Detected (PEC)**
	Yeast slurry	**Detected (PEC)**
	Fermentation sample	**Detected (PEC)**
	Beer	**Detected (PEC)**

Table 4 Results of the assay matrix compatibility tests

Conclusions

This study was conducted to evaluate the Gen 2 Beer SpoilerAlert™ Assay using the Rheonix® Encompass Optimum™ Workstation. The experiments were designed to assess the system's specificity for particular beer spoilage micro-organisms, the sensitivity of the assay and its performance when presented with different sample formats (wort, yeast slurry, fermentation sample, beer).

For the specificity testing, the system was challenged with a number of yeast and bacteria, some of which the system is setup to detect and others which were employed to check for cross-reactions. The microorganisms *Saccharomyces cerevisiae* var *diastaticus* (2 strains), *Brettanomyces naardensis*, *Brettanomyces/Dekkera bruxellensis*, *Brettanomyces custersianus*, *Brettanomyces anomalus*, *Brettanomyces acidodurans*, *Lactobacillus coryniformis*, *L. paucivorans, L. plantarum*, *L. collinoides* , *L. parabuchneri*, *L. backii*, *L. casei*, *L. paracasei*, *L. brevis* (beer spoiler, 2 species), *L. brevis* (non-beer spoiler), *Pediococcus damnosus*, *P. claussenii*, *Pectinatus cerevisiiphilus* and *Megasphaera*

cerevisiae were all successfully identified as being present in the spiked beer samples and being picked up by their respective probes. A second strain of *Brettanomyces naardensis* only showed one of the *Brettanomyces* markers listed for this species, however the significant differentiating probe was detected. On the other hand, a second strain of *Brettanomyces/Dekkera bruxellensis* gave an unexpected positive *Brettanomyces* probe result in addition to the expected two positive probes. This is currently being investigated further with DNA sequencing. For *Lactobacillus delbrueckii* and *L. acidophilus* only one of their replicates was successfully detected as expected. This could either be due to their cell concentration having been too low for accurate detection (e.g. *L. acidophilus*) or alternatively, differences in the colonies analysed (e.g. *L. delbrueckii*). Spoiler and non-spoiler strains of *L. brevis* were correctly differentiated, with the former showing positive for hop-resistance gene targets associated with beer spoilers. Most of the non-target organisms (*Candida albicans*, *Pichia kudriavzevii*, *Saccharomyces pastorianus*, *Saccharomyces cerevisiae* (2 brewing strains), *Bacillus subtilis*) did not result in any false positives. However, *Bacillus megaterium* appeared to show a cross-reaction with one of the *Lactobacillus* probes. The manufacturer has indicated a possible cross-reactivity in the presence of exceptionally high concentrations of *Bacillus*.

Generation 2 of the Beer SpoilerAlert™ assay contains seven different probes for separate *Lactobacillus* spp. groupings, five probes for five *Brettanomyces* species as well as two probes for strict anaerobic bacteria species groups (*Pectinatus* and *Megasphaera*). This allows more lactic acid bacteria, *Brettanomyces* as well as strict anaerobe beer spoilers to be detected and for these to be identified to a limited extent. If an accurate species identification is however required other techniques such as DNA sequencing would need to be employed.

The second part of the study focused on assessing the sensitivity of the assay. Four species – *Pediococcus claussenii*, *Lactobacillus brevis*, *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* var *diastaticus* – were spiked at low levels (6-100 cells/ml) into beer + enrichment broths and enriched for up to 48 hrs. All microorganisms were reliably detected at cell concentrations > 10^4 cells/ml. To ensure such concentrations are achieved 48 hours enrichment is required, when the starting concentration is less than 10 cfu/ml. The enrichment medium needs to be carefully selected as the presence of DNA in some media can lead to false positive results.

Finally, wort, yeast slurries, fermentation samples and bright beer were inoculated with *Pediococcus claussenii*, *Lactobacillus brevis*, *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* var *diastaticus* to determine any potential sample matrix effect on the test performance. All test yeast and bacteria were detected successfully in all four sample types and there were no false positive detections in the presence of brewing yeast. There was no indication of any matrix interference in the detection reactions.

In summary, the system was proven to be specific as it correctly picked up species of yeast and bacteria from the organism groups it is designed to detect as well as identifying the presence of beer spoiler hop resistance genes. No cross-reactions with other non-target organisms were found apart from one *Bacillus* species that appeared to be detected by one of the *Lactobacillus* probes. A range of process samples (wort, yeast slurry, fermentation sample, beer) could be successfully analysed with the system. The assay requires a minimum cell concentration 10^4 cells/ml to allow reliable detection. A 48-hour enrichment of samples containing < 10 cfu/ml was shown to be sufficient to reach the required level of cells and consequently positive identification. However, it is expected that starting samples with > 10 cfu/ml will only require 18-24 hours enrichment. For the enrichment it is important to select an adequate medium as some contain DNA that can lead to false positive results. Although the imaging software does work well there were instances where internal controls caused indeterminate results to be reported which would mean the sample needed a repeat analysis. However, in these cases the user has access to the camera images that can help in understanding whether these are true failures or just an imaging/threshold issue. Reproducibility was generally very good with replicates giving the same results.

The Generation 2 Beer SpoilerAlert™ assay and Encompass Optimum workstation are very easy to use with a user-friendly guided protocol. There is minimal sample handling involved (minimising any risk of cross-contamination and human error) with samples simply being dispensed into tubes which are then placed into the workstation. All other sample processing thereafter is automated and results are obtained within 5 hours, a great advantage compared to traditional microbiological analysis requiring up to 5-7 days incubation. Results are reported in a clear tabular format with positive spoiler results highlighted, making it easy to pick these out quickly. Technical support was excellent throughout the study with detailed responses and guided troubleshooting being provided rapidly.