Evaluation of a Highly Multiplexed Automated Assay for the Detection of Beer Spoilage Flora

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Introduction

Beer is a hostile environment for most bacteria, but some strains of lactic acid bacteria (LAB) have evolved survival mechanisms which may ultimately lead to product spoilage. Furthermore, certain strains of yeast can also cause spoilage despite the use of yeast during the beer-making process. Therefore, an assay that can detect both bacterial and fungal organisms at a genus, species, and genetic level is essential for detecting and characterizing beer spoilage organisms.

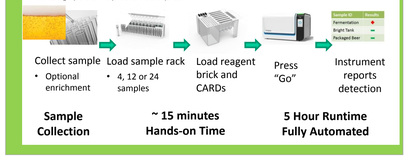
Current methods used to detect spoilage organisms may require lengthy procedures with skilled interpretation. Identification of beer spoilage organisms (BSOs) has typically been conducted using culture-based detection methods, with results obtained up to a week after sample processing begins. Furthermore, traditional methods only detect the presence of a particular species but do not necessarily indicate the presence of spoilage genes needed for these organisms to propagate in beer. In contrast, nucleic acid-based detection methods enable rapid detection of BSOs. These methods not only determine the presence of the organism, but also indicate whether or not it has the capability of persisting in the presence of antimicrobial iso-alpha acids derived from hops.

This study evaluates the sensitivity, specificity, and adaptability of the Rheonix® Beer SpoilerAlert™ assay, a fully automated sample-to-results multiplexing molecular detection kit. The sample rack, CARD® (Chemistry and Reagent Device) cartridges and reagent brick are placed in the Encompass Optimum™ workstation and all processes required for lysing organisms, extracting nucleic acids, amplifying and detecting target genes, and result analysis are automatically performed without user intervention on the Rheonix workstation. Reagents are dispensed by an onboard robot and liquid is moved via microfluidic pumps and channels within the CARDs. Amplification occurs via the onboard thermocycler and endpoint detection occurs through hybridization to a low-density capture array. Captured targets are detected and analyzed by an onboard camera and imaging software, which provides the user with a report of which genes and/or organisms are detected. Four individual samples are analyzed per CARD, with 6 CARDs per run, resulting in up to 24 independent samples analyzed in 5 hours, with minimal hands on time.

Beer SpoilerAlert™ Workflow

Simple, rapid, multiplexed microorganism detection system using the Encompass Optimum workstation

- Fully integrated sample prep and detection - True walk-away capability
- Minimal hands-on time
- Can multiplex up to 22 individual targets
- Throughput of up to 24 samples



Collect sample
- Optional enrichment

Load sample rack
- 4, 12 or 24 samples

Load reagent brick and CARDs

Press "Go"

Instrument reports detection

Sample Collection | **~ 15 minutes Hands-on Time** | **5 Hour Runtime Fully Automated**

The Rheonix Beer spoilage flora detection method targets four distinct sequences enabling rapid detection of lactic acid bacteria and four sequences detecting hop resistance genes. Furthermore, the assay also contains targets for three yeast sequences to detect the following strains: *Saccharomyces cerevisiae* (brewer's yeast), *S. cerevisiae* var. *diastaticus* and *Brettanomyces bruxellensis*. This is useful to detect cross-contamination by yeast purposefully used to make beer (*S. cerevisiae, B. bruxellensis*), while also detecting the spoilage yeast *S. cerevisiae* var. *diastaticus*. The presence of *S. cerevisiae* var. *diastaticus* is of particular concern due to its genetic homology with brewer's yeast and typically remains undetectable until spoilage occurs.

Materials & Methods

Strains, Media and Culture Conditions Microorganisms were obtained from The Beer Research Institute, ATCC, DSMZ, National Collection of Yeast Cultures, local craft breweries and the United States Department of Agriculture, maintained as stock cultures in 20% glycerol at -70˚C and propagated as per provider instructions. Lactic acid bacteria were grown using MRS supplemented with beer (B-MRS) and yeast were grown using YM (DIFCO). B-MRS was prepared with filtered clear beer. B-MRS agar plates were prepared by adding filtered beer to autoclaved MRS agar, to a final concentration of 0.5xBeer/0.5xMRS. Lactic acid bacteria were incubated at 30˚C, under a 10% CO_2 atmosphere, while yeasts were incubated aerobically at 25˚C.

Analysis of Samples on the Encompass Optimum™ Workstation To validate target specificity, overnight cultures were grown in liquid media under appropriate conditions. Cultures were counted using a Cellometer X2 (Nexcelom) confirmed with plate counts, and diluted accordingly in either media or buffer to the desired concentration. Samples were run on the Encompass Optimum™ workstation, and the results were analyzed through an automated software report.

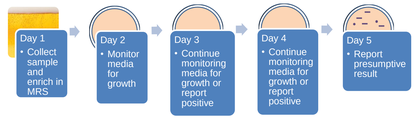
Limit of Detection (LOD) Cultures were first diluted in phosphate buffered saline (PBS), followed by dilutions in filtered beer to obtain suspensions ranging from 10^1 to 10^5 CFU/mL. Each dilution was analyzed directly on the workstation (2 mL per sample tube). Ten mL of each dilution was then filtered through a 47 mm, 0.45 µm filter. The filter was then placed in a 60 mm Petri dish with 2 mL B-MRS, and incubated for 18 and 24 hours at 30˚C under 10% CO_2 atmosphere. In parallel, 2 mL of microbial suspensions of the same concentrations were incubated directly in sample tubes under the same conditions as the filtered treatments. After 18 hours, both sets of samples were removed from the incubator. The enriched filters were scraped using cell scrapers and suspensions were transferred to sample tubes. In parallel, both sample sets were analyzed on the workstation. The samples were allowed to incubate for an additional six hours and analyzed again on the workstation.

Minimum Enrichment Inoculum (MEI) Cultures were diluted in B-MRS to obtain suspensions ranging from 10^0 to 10^3 CFU/mL. Ten mL of each dilution were filtered through a 47 mm, 0.45 µm filter. The filter was then placed in a 60 mm Petri dish with 2 mL B-MRS, and incubated for 18 and 24 hours at 30˚C under 10% CO_2 atmosphere. In parallel, 2 mL of microbial suspensions of the same concentrations were incubated directly in sample tubes under the same conditions as the filtered treatments. After 18 hours, both sets of samples were removed from the incubator. The enriched filters were scraped using cell scrapers and suspensions were transferred to sample tubes. In parallel, both sample sets were analyzed on the workstation. The samples were allowed to incubate for an additional six hours and analyzed again on the workstation.

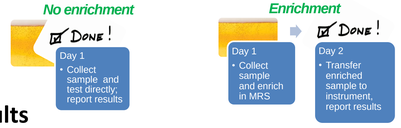
Environmental Sampling Overnight cultures of *Lactobacillus buchneri* and *Pediococcus claussenii* were diluted in PBS to obtain concentrations of 10^2, 10^3, and 10^4 CFU/mL. For each dilution, 10 x 10 µl were applied onto a sterile stainless steel surface, allowed to attach for 30 minutes, and then collected with a swab pre-wetted with MRS. Swabs were transferred immediately into MRS in sample tubes and were incubated for 24 hours at 30˚C and 10% CO_2 modified atmosphere. In addition, various locations in a brewery were swabbed, with samples taken from various locations in the plant including tanks, valves, surfaces, tubes, and drains. Swabs were treated similarly, with enrichment immediately in MRS broth.

Targets detected with the Beer SpoilerAlert™ Assay: Key for filters	
RS	Assay reference spot
Targets amplified by Master Mix 1	
MM1	Control for PCR Master Mix 1
PC	Target found specifically in *Pediococcus claussenii*
LB	*Lactobacillus brevis*
SC	*Saccharomyces cerevisiae*
DIA	*Saccharomyces cerevisiae* variant *diastaticus*
BRETT	*Brettanomyces bruxellensis*
Targets amplified by Master Mix 2	
MM2	Control for PCR Master Mix 2
PED	Target found in all currently sequenced *Pediococcus* species
LABS	Plasmid biomarker present in strains of various lactic acid bacteria (LAB)
HORA	Hop resistance gene, *horA*, found on plasmids in various LAB
HORC	Hop resistance gene, *horC*, found on plasmids in various LAB
BSRA	Hop resistance gene, *bsrA*, found in *P. claussenii*
BSRB	Hop resistance gene, *bsrB*, found in *P. claussenii*

Traditional Microbiological Plating Method Workflow



Rheonix Beer SpoilerAlert™ Assay Workflow



Results

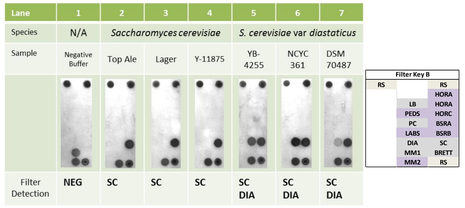
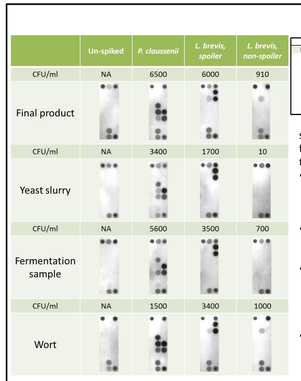


Figure 1. The test kit can distinguish *Saccharomyces cerevisiae* variant *diastaticus* from *S. cerevisiae*. Different strains of yeast were spiked into Rheonix collection buffer at a concentration approximately 1x10^7 CFU/mL and analyzed using the Encompass Optimum™ workstation. Three strains of brewers yeast (lanes 2-4) are distinguishable from three independent *S. cerevisiae* var. *diastaticus* (lanes 5-7). The key on the right of the figure indicates probe orientation on the filters.

Microorganism tested	Limit of Detection (LOD) CFU/mL		Minimum Enrichment Inoculum (MEI) Total CFU inoculum/ 2 mL			
	Direct	Filtered	Direct		Filtered	
			18 h	24 h	18h	24 h
Lactobacillus buchneri	1x10^4	5x10^4	10	10	10	1
Pediococcus claussenii	1x10^4	5x10^3	10	1	10	10
Brettanomyces bruxellensis	1x10^5	5x10^4	100	100	100	100
Saccharomyces cerevisiae var. *diastaticus*	1x10^4	5x10^4	ND	10	10	10

Figure 2. The limit of detection and minimum enrichment inoculum. The LOD and MEI are presented for *Lactobacillus buchneri* (LB), *Pediococcus claussenii* (PC), *Brettanomyces bruxellensis* (BB), and *Saccharomyces cerevisiae* var. *diastaticus* (SC/DIA). The data demonstrate LODs approximated to 1X10^4 CFU/mL for the LAB and SC/DIA, and 1X10^5 CFU/mL for BB. A minimum inoculum of 10 CFU in a final volume of 2 mL is sufficient to detect LB, PC and SC/DIA after 18 hours, while 100 CFU is required to detect BB. For LB the inoculum can be decreased to 1 CFU/2 mL when the sample is allowed to enrich for 24 hours. The LOD and MEI are defined when 100% of replicates are positive in a single experiment, however detection at lower levels has been observed in multiple experiments. (ND=not determined)

Sample Handling Procedure	Definition
Direct	Suspension of microorganisms in beer at the concentration indicated
Filtered	Sample concentrated by filtration, re-suspended in PBS
Direct/Enriched	Sample enriched in B- MRS (bacteria) or YM (yeast) in sample tubes
Filtered/Enriched	Sample concentrated by filtration and enriched in B-MRS or YM



*Actual CFU/ml indicated on figure

Figure 3. This method of BSO identification can detect contaminants at all stages of beer manufacturing. Final product (bright beer) as well as upstream process components were spiked with approximately 1x10^{4*} CFU/mL spoiler and non-spoiler lactic acid bacteria. The figure shows filter images following analysis with the assay.

- As expected, yeast slurry and fermentation samples demonstrate *S. cerevisiae* detection. *S. cerevisiae* detection in final product, likely due to the presence of residual yeast DNA.
- Expected detection for *P. claussenii* includes PC, PEDS, BSRA, BSRB, and LABS. All targets were detected in all spiked samples.
- HORA is detected in all samples spiked with the *L. brevis* spoiler. The LB marker is only detected in final product and wort. This may be due to competition by *S. cerevisiae* in the yeast and fermentation samples or due to low cell counts.
- These data demonstrate that this assay successfully detects the presence of spoiler related hop resistance genes in upstream process components.

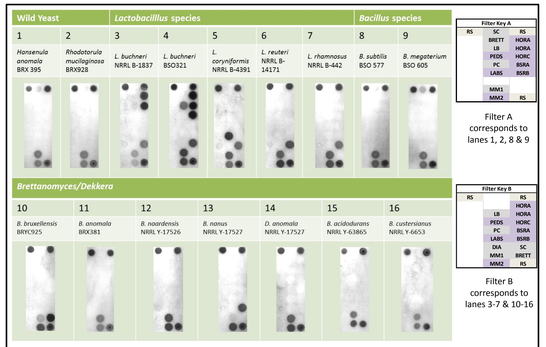


Figure 4. Assay Specificity. Assay specificity was confirmed via testing of potentially cross-reacting organisms. Two wild yeast, *Hansenula anomala*, and *Rhodotorula mucilaginosa* were tested for potential cross-reactivity with *B. bruxellensis* and *S. cerevisiae*. Two *Bacillus* species were tested for potential cross-reactivity with *Lactobacillus* and *Pediococcus*. Various non-*bruxellensis* species were tested for cross-reactivity with *B. bruxellensis*. All non-targeted species tested negative demonstrating no cross-reactivity, and thus present minimal risk for false positives to be called with these organisms. This test kit currently does not have a generic *Lactobacillus* marker, and identification of *Lactobacillus* species is dependent on the presence of the plasmid associated markers, *horA*, *horC*, and LABS. Additionally, there is a marker specific for *L. brevis* that demonstrates similarity, but not identity, with other *Lactobacillus* species. Detection of the LB probe is possible at very high concentrations of non-*brevis* species that demonstrate significant homology. The data demonstrate detection of the LB probe in *L. buchneri,* but not in *L. coryniformis, L. reuteri*, or *L. rhamnosus.* In addition, plasmid associated targets are seen in *L. buchneri* and *L. coryniformis.*

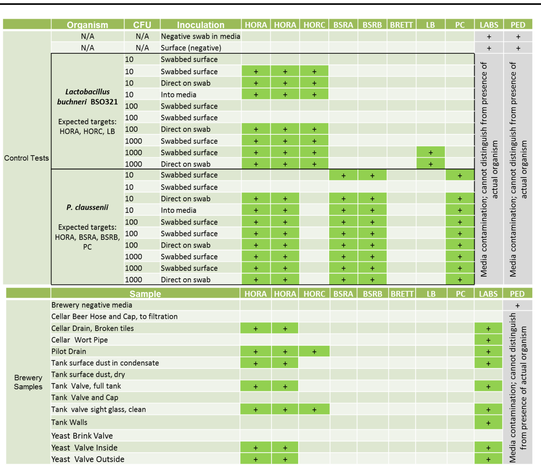


Figure 5. This method can be used to test a wide variety of surfaces for validation of sanitation procedures. Control experiments were performed with known concentrations of microorganisms. Swabs containing MRS were used to collect **Swabbed surface** samples. **Into media** samples contain a known concentration of spoilers added directly to the media, and **Direct on Swab** samples were obtained by adding microorganisms directly onto swabs. Control experiments illustrate detection of all anticipated genomic and plasmid targets. Unsurprisingly, brewery samples showed detection of SC in all samples tested (data not shown). In contrast, there was no evidence of *P. claussenii* since PC, BSRA, and BSRB were not detected; *L. brevis* was also not detected. However, the three plasmid targets including the *horA* and *horC* hop resistant genes, as well as the lactic acid bacteria marker (LABS) found in some lactic acid bacteria were detected. The presence of yeast extract in commercial MRS media can result in false positives, exhibited by PED and LABS sometimes detected in negative controls.

Summary & Conclusions

- The method described is able to detect both bacterial and fungal organisms at the genus, species, and gene level. Additionally, it detects specific genes associated with hops resistance.
- The minimum inoculum required for detection following enrichment for all target organisms is approximately ≤10 CFU/mL. The post-enrichment limit of detection is <10^4 CFU/sample before enrichment.
- The assay detects *B. bruxellensis, S. cerevisiae*, and *S. cerevisiae* var. *diastaticus*. This is useful to detect cross-contamination by yeast purposefully used to make beer (*S. cerevisiae, B. bruxellensis*), while also detecting the spoilage yeast *S. cerevisiae* var. *diastaticus*. The presence of *S. cerevisiae* var. *diastaticus* is of particular concern due to its close genetic homology with brewer's yeast.
- All *Pediococcus* species are detected, with an additional target to specify *Pediococcus claussenii*.
- The assay is adaptable in its ability to detect these microorganisms in a variety of matrices including wort, yeast slurry, fermentation, final product, and environmental samples.
- While the assay distinguishes between spoiler and non-spoiler *Lactobacillus* species, not all *Lactobacillus* species will be detected unless closely related to *L. brevis* as demonstrated by the presence of the targeted plasmid associated sequences.