**FINAL REPORT**

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Virginia Wine Board

# Identification and Quantification of Wine-related Microorganisms via Real-Time quantitative PCR

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## A. OBJECTIVE:

**Overall Objective:** To develop in-house methods for rapid identification and quantification of yeasts and bacteria in juice and wine in support of Enology research and extension programs by leveraging Real-Time qPCR equipment and molecular biology expertise in the Department of Food Science and Technology at Virginia Tech.

**Specific Objective 1:** Validate Real-Time qPCR method for detection of target wine microorganisms in juice and wine down to concentrations of 1-10 cfu/mL including but not limited to *Brettanomyces bruxellensis*, *Oenococcus oeni*, and *Saccharomyces cerevisiae*.

**Specific Objective 2:** Employ this technology in preliminary research related to the impact of nitrogen concentration and composition on growth of common wine microorganisms such as *Brettanomyces bruxellensis*, *Oenococcus oeni*, and *Saccharomyces cerevisiae*.

## B. SUMMARY

During wine fermentation, microbial composition and growth are crucial factors that positively or negatively impact the characteristics of wine. Therefore, studies on nitrogen content, optimization of fermentation conditions and other strategies to control the wine microbiota have been conducted. To assess the impact of these studies, not only analyzing the chemicals of end products but also monitoring the growth of microorganisms by quantification is an essential step. The Virginia Tech Enology Service lab currently utilizes traditional methods to monitor yeast and bacteria by microscopy and plate counting in a selection media. Although this method provides reliable results, it is time consuming (it takes about 2 weeks to select *B. bruxellensis*) and does not take into account the viable but not culturable state (VNC) of microorganisms. Real-time quantitative PCR is a more advanced DNA-based technique which rapidly identifies and quantifies microorganisms including VNC. Our lab recently acquired a Qiagen Rotor Gene 6000 Real-Time PCR machine previously used by Food Microbiology researchers in the Department of Food Science and Technology. Using the current funds provided by the Virginia Wine Board, supplies and materials for real-time PCR method development were purchased and set up in the new Enology and Fermentation lab to bring the real-time quantitative PCR methods online.

As reported in the progress report submitted in January 2015, we used a primer set which detects *B. bruxellensis* specifically and confirmed that the primer set only amplifies the genomic DNA of *B. bruxellensis* among the DNA samples of different wine-related reference species. Since then, species-specific PCR for *Saccharomyces cerevisiae* was also conducted in sampels containing different wine-related reference species in addition to the target species to confirm specificity of the primers. Real-time qPCR was then conducted for different dilution sets of *B. bruxellensis* and *S. cerevisiae* that were plate-counted to make a simple linear regression model. A linear regression model for *B. bruxellensis* plotting Threshold cycle (Ct value) against number of cells (Log CFU/ml) was obtained with $R^2$ value of 0.9913 and the model of *S. cerevisiae* was also obtained with an $R^2$ value of 0.9773. Further experimentation and optimization is required to set up real-time qPCR model for *O. oeni* strain. Quantification of these microorganisms from actual wine samples should also be tested. These tools will be implemented in future research dealing with the impact of juice and wine chemistry on growth of microorganisms during fermentation and wine storage.

## C. Materials & Methods

### Microorganisms and growth conditions

Five common wine yeasts including target species *Brettanomyces bruxellensis* Y1413 and *Saccharomyces cerevisiae* Y12632, and background species *Candida apicola* Y6688, *Hanseniaspora osmophila* Y1613 and *Pichia membranifaciens* were obtained from ARS Culture Collection (NRRL). All yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) medium at 30°C for 2 days.

### Extraction of genomic DNA

Yeast strains were cultured in a liquid media to the point of $O.D_{600}$=1 corresponding to 2~4 $\times 10^7$ CFU/ml and after serially diluting the culture, each sample was plate counted and the genomic DNA was extracted. To break the cell wall, lysis buffer (500 mM pH 8.0 Tris, 100 mM β-mercaptoethanol) and lyticase (3 mg/ml, Sigma) were used. To collect the genomic DNA, Amicon filter (0.5 ml, 100 k membrane) was employed according to the procedure suggested by the manufacturer.

### PCR conditions

Table 1 shows the primers for *B. bruxellensis* and *S. cerevisiae* used in both species-specific PCR and Real-Time qPCR. Each primer sets specifically amplified the target species and did not show any cross amplification. PCR reagents and reaction conditions for Real-Time qPCR are listed in Table 2 and Table 3 respectively. For Real-Time PCR, iQ-SYBR Green supermix was used to amplify the DNA and green fluorescence was obtained during amplification.

**Table 1. Primers for PCR**

| Microorganisms | Primers |
|---|---|
| *B. bruxellensis* | DBRUX-F: 5'-GGATGGGTGCACCTGGTTTACAC-3'<br>DBRUX-R: 5'-GAAGGGCCACATTCACGAACCCCG-3' |
| *S. cerevisiae* | SC1: 5'-GAAAACTCCACAGTGTGTTG-3'<br>SC2: 5'-GCT-TAAGTGCGCGGTCTTG-3' |

**Table 2. PCR reagents**

| | ***B. bruxellensis*** | ***S. cerevisiae*** |
|---|---|---|
| iQ-SYBR Green Supermix | 12.5 uL | 12.5 uL |
| Forward primer | 2.25 μL (900 nM) | 0.5 uL (0.2 uM) |
| Reverse primer | 0.75 μL (300 nM) | 0.5 uL (0.2 uM) |
| Genomic DNA | 5 μL | 2.5 uL |
| Ultrapure water | 4.5 μL | 9 uL |
| Total | 25 uL | 25 uL |

**Table 3. Reaction conditions for Real-Time qPCR**

| | *B. bruxellensis* | *S. cerevisiae* | |
|---|---|---|---|
| Initial denaturation | 95°C | 95°C | |
| Denaturation | 95°C | 95°C | 40 cycles |
| Annealing | 69°C | 63°C | |
| Extension | 72°C | 72°C | |

## D. Results

### Confirmation of primer specificity by species-specific PCR

Figure 1 shows results of *B. bruxellensis*-specific PCR (a) and *S. cerevisiae*-specific PCR (b). Each PCR only amplified the target species and did not amplify other reference species present in the sample. Therefore, it was confirmed that primers for *B. bruxellensis* and *S. cerevisiae* were specific and genomic DNA was properly extracted from the reference strains.

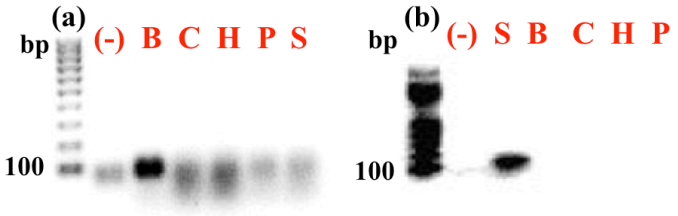


Figure 1. (a) *B. bruxellensis*-specific PCR (b) *S. cerevisiae*-specific PCR
(-): negative control, B: B. bruxellensis, C: *Candida apicola*,
H: *H. osmophilla*, P: *P. membranifaciens*, S: *S. cerevisiae*

### Real-Time qPCR

In order to set up a tool to quantify *B. bruxellensis* and *S. cerevisiae* in wine, both yeasts were cultured in a liquid media up to $2\sim4\times10^7$ CFU/ml and 10-fold serial dilution was performed. Each diluted sample was then subjected to both real-time qPCR and plate counting. By using both data sets, a simple linear regression was constructed to quantify both species from wine samples.

Figure 2 shows the result of real-time qPCR. Threshold cycle (Ct) was determined at the exponential phase of the amplification (red line) and each Ct value of diluted samples was recorded.

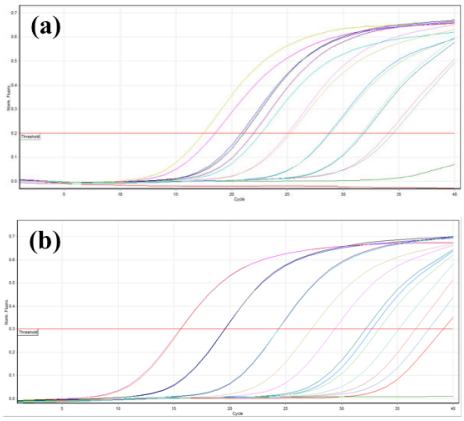


Figure 2. qPCR amplification of (a) *B. bruxellensis and* (b) *S. cerevisiae* DNA extracted from 10-fold dilution series, from (a) $1.427 \times 10^7$ to 14.27 cells/mL and (b) $3.15 \times 10^6$ to 3.15 cells/mL

Based on the Ct values determined and the CFU (colony forming unit) counted, a simple linear regression model was generated for both *B. bruxellensis* and *S. cerevisiae* by plotting the threshold cycle (Ct value) over the number of cells (Log CFU/ml) (Figure 3). The $R^2$ value of *B. bruxellensis* model was 0.9913 (Figure 3 (a)) and the model of *S. cerevisiae* was obtained $R^2$ value of 0.9773 (Figure 3 (b)).

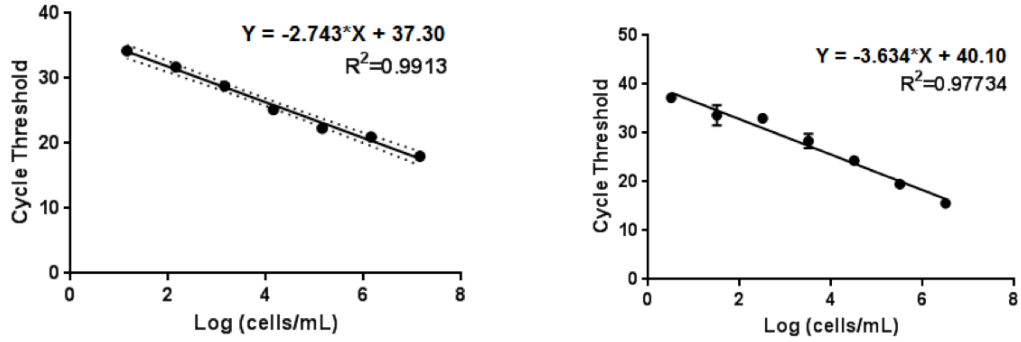


Figure 3. qPCR results of (a) *B. bruxellensis* and (b) *S. cerevisiae*. Fluorescence detection cycle threshold values are plotted versus the logarithm of cells/mL.

## E. Discussion and future work

In summary, the procedure for real-time qPCR is as follows: total genomic DNA is first extracted from the sample and real-time qPCR is then performed with the appropriate primer set to acquire the Ct value. By substituting this value into a linear regression model, CFU/ml of the target species can be determined. In this project, simple linear regression models were constructed by plotting the Ct values obtained by real-time qPCR over CFU/ml obtained via plate count. Detection limits for *B. bruxellensis* and *S. cerevisiae* were 14.3 CFU/ml and 3.2 CFU/ml, respectively. These models enable fast and accurate quantification of microorganisms compared to conventional selection and plate counting method. By applying the same approach, designing an appropriate primer set and conditions

for the real-time qPCR, models for other species can be obtained. Currently, we are conducting experiments to obtain a model for *O. oeni*. Moreover, we will continue to study the impact of nitrogen concentration and composition on growth of common wine microorganisms such as *Brettanomyces bruxellensis*, *Oenococcus oeni*, and *Saccharomyces cerevisiae* during fermentation and storage.

## F. Budget Allocation and Actuals as of 6/30/2015

| Expense | Allocation | Actual | % of category spent |
|---|---|---|---|
| GRA step 11 (33%) and Fringe | 7,912 | 7,833.80 | 99% |
| Tuition and academic fees, 2 semesters (AY) | 3,943 | 3,941.28 | 100% |
| Materials and supplies | 7,200 | 7,781.31 | 108% |
| Travel support | 800 | 298.61 | 37% |
| TOTAL | $19,855 | $19,855 | 100% |

## G. References

Cocolin, L., et al. (2004). "Molecular Detection and Identification of Brettanomyces/Dekkera bruxellensis and Brettanomyces/Dekkera anomalus in Spoiled Wines." Applied and Environmental Microbiology 70(3): 1347-1355.

Phister, T. G. and D. A. Mills (2003). "Real-Time PCR Assay for Detection and Enumeration of Dekkera bruxellensis in Wine." Applied and Environmental Microbiology 69(12): 7430-7434.

Portugal, C. and F. Ruiz-Larrea (2012). "Comparison of Specific Real-Time PCR and Conventional Culture for Detection and Enumeration of Brettanomyces in Red Wines." American Journal of Enology and Viticulture 64(1): 139-145.

Bokulich, N. A., et al. (2011). "Profiling the Yeast Communities of Wine Fermentations Using Terminal Restriction Fragment Length Polymorphism Analysis." American Journal of Enology and Viticulture 63(2): 185-194.

## APPENDIX

### Impact Statement:

Setting up real-time quantitative PCR methods in the Enology & Fermentation Lab at Virginia Tech expands research and analytical capabilities for the Enology program to support the continued growth and success of the Virginia Wine Industry. This project leverages existing equipment and expertise in the Department of Food Science & Technology, adding breadth to resources available for Enology research and extension.

### Publications and Presentations:

This project was completed in June 2015. To date, preliminary results were presented as follows:

Lee, Andrew H.; Smith, Sean; Stewart, Amanda C. Identification and Quantification of Wine Microorganisms via Real-Time PCR. 3rd Annual VT FST Poster Competition, Department of Food Science and Technology. April 23, 2015.

Future presentations on the principles behind this technique and how it can be used to benefit the Virginia Wine Industry will be provided via Extension venues including workshops organized by Dr. Molly Kelly, and feedback from these presentations will be used to gauge interest in the longer-term objective of offering this method as an analytical service through the Virginia Tech Enology Service Lab.