Creating a Multi-Functional Liquid Assay Utilizing alamarBlue REDOX Indicator

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Victor Nguyen, having been admitted to the Carl and Winifred Lee Honors College in the fall of 2008, successfully completed the Lee Honors College Thesis on April 20, 2012.

The title of the thesis is:

Creating a Multi-Functional Liquid Assay Utilizing alamarBlue REDOX Indicator

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Lee Honors College Thesis:
Creating a Multi-Functional Liquid Assay
Utilizing alamarBlue® REDOX Indicator

By Victor Nguyen
Mentored by Dr. John Geiser
Department of Biological Sciences
Abstract:

*Yersinia* outer proteins (yops) are effector proteins that have been known to cause cytolysis to host cells. Using a yeast genetic model, Yop genes from *Yersinia enterocolitica* can be inserted and expressed in *Saccharomyces cerevisiae*. With the utilization of alamarBlue® indicator, we hope to create a multi-functional liquid assay with the intent of further analysis and quantitative investigation on the effects of galactose induction of the effector protein genes. AlamarBlue® is a REDOX colorimetric/flourometric indicator specialized in detecting metabolic activity. As *Saccharomyces cerevisiae* metabolized, the indicator is added to monitor cell viability. In developing an effective assay, we determine that to properly prep the samples, sample cells must be washed and reintroduced in fresh media before proceeding with addition of sugars. Calculation of reduction percentages of alamarBlue® is utilized to analyze metabolic activity to identify viability of cells due to sugar induction. Galactose induction of several effector proteins displayed inhibition of growth consistent with YopT. Through an additional supplement of glucose, we tested for the suppression of inhibition of growth due to YopT expression. Results identified that the assay is successful in determining the threshold of glucose concentration falling between 0.05% and 0.10% in order to counteract the effects of YopT.

Experimental Preparation

Procedure:

*Saccharomyces cerevisiae* was inoculated into a 300ml Klett flask containing 25ml of YP media with 2% glucose. The flask was placed in a 30°C water bath to be shaken and let yeast grow overnight. *Saccharomyces cerevisiae* were diluted back into 50ml of fresh media at 20 Klett and allowed to grow in 30°C until 80-100 Klett. The mixture was spun down in a clinical centrifuge and resuspended in 5ml dH₂O. The mixture is spun down again and resuspended in 1.5ml of 1x LiOAc and 1x TE pH 7.5. Incubation occurred in an eppendorf tube at 30°C for 1hr in a spinning wheel incubator. 200 μl yeast suspension is aliquot into eppendMoorf tubes. 200 micrograms of heat denatured, sheared salmon sperm DNA is added and 5 micrograms of transforming
DNA; pJG485 the empty control plasmid, plasmid pLN5 containing YopO, pJG491 containing YopE, and pJG495 containing YopT; is added. Again the mixture is incubated at 30°C for 30 minutes in a spinning wheel incubator. 1.2 ml 40% PEG, 1x LiOAc, 1x TE ph 7.5 solution is added to each tube. The tubes are incubated at 30°C for 30 minutes in a spinning wheel incubator. The tubes were then heat shocked for 15 min at 42°C. The tubes were centrifuged and washed with 1ml 1x TE pH 7.5. 100 μl of each strain were plated onto S-Ura plates. Again the eppendorf tubes were spun down into a pellet. All but 100 μl were removed then pellet was resuspended and plated onto S-Ura with a quadrant streak.

**Results & Discussion:**

Using the quadrant streak, individual colonies formed after about 48 hours for each strain. New plates would have to be struck after 10-14 days to keep fresh samples of each strain. With the samples prepped, development of the assay could proceed.

**Hypothesis One: Will alamarBlue® assay yield viable data?**

**Procedure:**

A single colony from each strain is inoculated into a flask containing 25 ml S-Ura media and 2% raffinose and grown overnight at 30°C. *Saccharomyces cerevisiae* cells were diluted back into 25ml of fresh media at 20 Klett and allowed to grow in 30°C until 80-100 Klett. 2.5 ml (10% volume) of alamarBlue® is added to each flask. 200 μl aliquots of PLN5 are pipetted into the first two rows of the 96-well plate, followed by pJG485 in the next two rows, and pJG491 and pJG495 after that. Finally, glucose is added in the first 4 columns for a final percentage of 2% glucose (10 μl of 40% glucose) and galactose is added into the last 4 columns for a final 2% galactose concentration (20 μl of 20% galactose). The well-plate is placed into the spectrophotometer and Molecular Devices' SoftMax® Pro 4.7.1 is opened. Setup options are set to 30°C and to read a single wavelength at 600nm. Time points: 0hr, 1hr, 3hr, and 24hr. Data is extracted from SoftMax® Pro and calculations were made in Microsoft Excel to verify the average value and standard deviation of each strain and sugar combination. The
calculation used was absorbance at time 24hr minus absorbance at initial time to measure overall reduction of media.

Results & Discussion

Figure 1A

Figures 1A displays the first trial of absorbance reading corresponding to metabolic activity of *Saccharomyces cerevisiae* reducing alamarBlue®

![Graph showing absorbance values for different sugars](image-url)
Figures 1A displays the second trial of absorbance reading corresponding to metabolic activity of *Saccharomyces cerevisiae* reducing alamarBlue®

**Hypothesis Two: Does non-washed vs. washed cells and use of fresh media remedy initial color change and provide useable data?**

**Procedure:**

Two well-plates were tested using the previous growing methods. The first plate was the control plate (non-washed) which followed the exact same procedures. The variable plate (washed), used cells that were centrifuged down at 3000rpm into a pellet at 30°C following their growth to 80-100 Klett. The supernatant was removed and the pellet was washed by resuspension in 25 μl of dH₂O, centrifugation at 3000rpm into a pellet at 30°C, and finally resuspended in fresh media then tested. The well-plate is placed into the spectrophotometer and Softmax® Pro software is opened. Set-up is set to 30°C and to read two wavelengths 570nm and 600nm as instructed by alamarBlue® Manual. Time points: 0hr, 1hr, 3hr, and 24hr. Calculations of averages and standard deviations of absorbance were made of each sugar to verify hypothesis.
**Results and Discussion:**

**Figure 2**

<table>
<thead>
<tr>
<th></th>
<th>Washed</th>
<th></th>
<th>Unwashed</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>570nm</td>
<td>600nm</td>
<td>570nm</td>
<td>600nm</td>
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<tr>
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<td>Control</td>
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<td>0.732596</td>
<td>0.559733</td>
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<tr>
<td><strong>1hr</strong></td>
<td>Control</td>
<td></td>
<td>Control</td>
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</tr>
<tr>
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<td>0.663513</td>
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<td>Control</td>
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<tr>
<td>Glucose</td>
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<td>0.871083</td>
<td>0.917729</td>
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</tbody>
</table>

Figure 2 displays the calculated average absorbance values at two wavelengths for each sugar variable.
The control plate exhibited rapid color change at the zero and 1hr time points, a repetition of the previous result. At 24 hours, all wells were displaying a clear color. The plate containing washed cells did not alter color with the initial addition of alamarBlue®, keeping its bluish color. After 1hr, the color began to turn purple. After 24hr, the color was almost clear with the slightest tint of pink.

Through the absorbance reading, the initial absorbance values remained consistently higher with the washed cells whereas the non-washed cells displayed lower absorbance values. The data explicitly displayed higher absorbance readings in washed cells which indicate there is a greater change in metabolic activity of the cells, capturing the full spectrum of metabolic activity as opposed to the lower readings in the unwashed cells. These lower readings are representative of media reduction interference within the wells prior to the initial reading, and therefore should be addressed. The hypothesis was confirmed and the results supported washing cells to produce contrasting data which would be more accurate and useable for future calculations. Thus, for all future experiments, samples will be washed and resuspended in fresh media prior to further experimentation.

**Test One: Activation of Yop Genes via Galactose Induction**

**Procedure:**

A single colony from each strain is inoculated into a flask containing 25 ml S-Ura media and 2% raffinose and grown overnight at 30°C. Yeast cells were diluted back into 25ml of fresh media at 20 Klett and allowed to grow in 30°C until 80-100 Klett. The cells in each tube were washed and resuspended in fresh media. 2.5 ml (10% volume) of alamarBlue® is added to each flask. 200 μl of PLN5 is aliquoted into the first two rows of the 96-well plate, followed by pJG485 in the next two rows, and pJG491 and pJG495 after that. Finally, galactose is added every well for a final 2% galactose concentration (20 μl of 20% galactose). The well-plate is placed into the spectrophotometer and Softmax Pro software is opened. Setup options are set to 30°C and to read two wavelengths 570nm and 600nm. Time points in set-up are set to 24hr period with half hour intervals. Reduction data is extracted from SoftMax® Pro and further calculations
were made and graphed in Microsoft Excel to provide reduction data. REDOX calculations were cited from the alamarBlue® using the equation:

\[
\% \text{ Reduced} = \frac{C_{\text{RED}} \text{ Test Well}}{C_{\text{OX}} \text{ Negative Control Well}} = \frac{(\varepsilon_{\text{OX}})\lambda_2 A\lambda_1 - (\varepsilon_{\text{OX}})\lambda_1 A\lambda_2}{(\varepsilon_{\text{RED}})\lambda_1 A'\lambda_2 - (\varepsilon_{\text{RED}})\lambda_2 A'\lambda_1} \times 100
\]

Where
- \( C_{\text{RED}} \) = concentration of reduced form alamarBlue® (RED)
- \( C_{\text{OX}} \) = oxidized form of alamarBlue® (BLUE)
- \( \varepsilon_{\text{OX}} \) = molar extinction coefficient of alamarBlue oxidized form (BLUE)
- \( \varepsilon_{\text{RED}} \) = molar extinction coefficient of alamarBlue reduced form (RED)
- \( A \) = absorbance of test wells
- \( A' \) = absorbance of negative control well. The negative control well should contain media + alamarBlue but no cells.
- \( A'' \) = absorbance of positive growth control well
- \( \lambda_1 \) = 570nm (540nm may also be used)
- \( \lambda_2 \) = 600nm (630 may also be used)
Results & Discussion

Figure 3a

![Graph showing percentage reduction over time for PLNS trials.](image)

\[ y = 0.0087x + 0.6665 \]
\[ R^2 = 0.9365 \]

\[ y = 0.0209x + 0.2615 \]
\[ R^2 = 0.8796 \]

Figure 3b

![Graph showing percentage reduction over time for 485 trials.](image)

\[ y = 0.0059x + 0.4714 \]
\[ R^2 = 0.7105 \]

\[ y = 0.01x + 0.1983 \]
\[ R^2 = 0.9601 \]
Figures 3a,b,c,d represent two trials of Reduction Percentage trends for each transformation of Saccharomyces cerevisiae.

In each graph, the domain of the data has been restricted to include only relevant data representing metabolic activity as opposed to the static data. Static data could be due to the start-up of the instrument as well as the evaporation of media within the spectrophotometer over the elapse time.

The data results from Figures 3a, 3b and 3c, indicated that galactose induction exhibited an increased percentage reduction in PLN5, pJG485, trial one of pJG491, while trial 2 Figure 3c of pJG491 and Figure 3d representing pJG495, showed a decrease in reduction percentage. Calculations were made from the formula displayed in Figure 3. Variables have been defined, and the molar extinction coefficients were taken directly from the alamarBlue® Manual. The average absorbance values of the eight wells with the same testing variables are used as to calculate reduction percentage. As for the $A'$ variable of negative control wells, $A'_{\lambda 1}$ is 0.5377 while $A'_{\lambda 1}$ was calculated to be 0.4861 abs. These values were used as a constant in the equation from here on out as a permanent negative control values. Making the equation:

$$(117216 * A_{\lambda 1} – 80568 * A_{\lambda 2}) / (155677 * 0.4861 – 14652 * 0.5377)$$

With all the transformed yeast, PLN5 displayed data inconsistent with galactose induction with a positive growth trend. The empty plasmid pJG485 showed data matching no effect from the galactose continuing a positive reduction trend. pJG491 contained two inconsistent trials, which could’ve been an error caused by contamination or an fail galactose induction attempt. pJG495 exhibited a negative trend consistent with lethality of galactose induction of the gene. With this information in hand, pJG491 and PLN5 was omitted from the next experimental step, opting for the two strains that expressed consistent data.
**Test 2: Addition of Glucose to Suppress Lethality due to Galactose Induction**

(Procedure)

A single colony from strains 485 and 495 is inoculated each into different flasks containing 25 ml S-Ura media and 2% Raffinose and grown overnight at 30°C. Yeast cells were diluted back into 25ml of fresh media at 20 Klett and allowed to grow in 30°C until 80-100 Klett. The cells in each tube were washed and resuspended in fresh media. 2.5 ml (10% volume) of Alamar Blue and 2.8 ml of galactose (2% final) is added to each flask. 2 ml of strain 495 was aliquoted into six test tubes. Each tube was induced with glucose to form final percentages of 1, .5, .2, .1, and .05 respectfully with one control tube containing no glucose (50 μl, 25 μl, 10 μl, 5 μl, and 2.5 μl of glucose). Repeat for strain 485. 200 micro liters of each test tube is aliquoted into the 96-well plate. The well-plate is placed into the spectrophotometer and Softmax Pro software is opened. Set-up is set to 30°C and to read two wavelengths 570nm and 600nm. Time points in set-up are set to 24hr period with half hour intervals. Percentage reduction calculations were done in Softmax® Pro to provide reduction data and then extracted into Microsoft Excel for graphical display.
Results & Discussion

Figure 4a

Figure 4b
Figure 4a displays the reduction data of the control plasmid pJG485 for all variable percentages of glucose induction. Figures 4b represents the reduction data and linear trend of pJG495 galactose induction with addition supplement of variable percentages of glucose.

All the reduction data was exported from Softmax® Pro and averages were taken from each 8-well variable induction of glucose (i.e. 0.00%, 0.05%, etc) to produce one average value at each half hour interval for graphical analysis. Some data were omitted from the average calculation due to outlier values inconsistent with the linear trend, specifically 3 wells with 0.05% percent glucose induction. A Softmax Pro graph is shown below to display inconsistencies:

As seen above, there are three values which were not consistent with the trend and thus omitted from calculations. The error here again may have been due to contamination of sugars.

0.00% and 0.05% displays a negative trend indicative of the galactose induction taking precedence; thus, resulting in cell lethality. However, at 0.10% glucose the trend is positive, supporting that glucose induction has overpowered the activation of YopT. This data is evidence there may be a threshold between 0.05% and 0.10% glucose induction where the expression of YopT is nullified.
Conclusion:

Through experimentation along with trial and error analysis, the methods of preparing a useable multifunctional assay focused on alamarBlue® indicator were validated. Quantitative analysis of experimental data yielded a variety of important statistical values imperative to the research. Through development, we determined that washing the cells and use of fresh media impacts the viability of data. Also, our results suggest that galactose induction of YopT effector proteins can be nullified with the addition of glucose, with a threshold between 0.05% and 0.10% concentration. Can other effector proteins be nullified using other variable sugars, and if so, at what threshold? This is just one question that can be testing using this liquid assay. This alamarBlue® assay has proven to be effective in utilizing the yeast genetic model to quantitatively analyze. With this assay, we hope to open the door to further variable testing crucial to analysis of Yersinia effector proteins and even reaching beyond microbiology and standardizing this assay for other fields of research.
References
