# Technical Note

GENERAL AUTOMATION LAB TECHNOLOGIES

# Applications of Resazurin in the Characterization of Bacterial Isolates in the Prospector System

Resazurin is a fluorogenic oxidation-reduction indicator commonly used in cell viability assays. The molecule is a key component of the GALT Prospector system where it is used to detect metabolism of bacterial isolates in the wells of the Prospector array. Here we discuss the fluorescence properties of resazurin and its reduced derivative resorufin and show how they are used to identify bacterial growth in the Prospector array.

## Background

Resazurin is a redox indicator most commonly used to assay cell viability under aerobic conditions. It is widely commercially available under the names Alamar Blue (Thermo Fisher), Reliablue (ATCC), or UptiBlue (Interchim). Resazurin changes in both color and fluorescence as it is reduced to resorufin (Fig. 1).

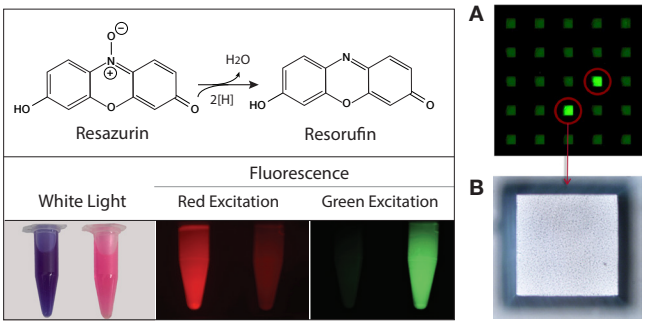


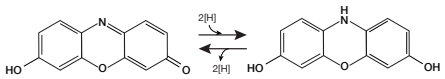

**FIGURE 1: Resazurin reduction to resorufin** in response to cellular metabolic reduction, leads to large shifts in color and fluorescence as shown in the lower left panels. **A.** Detail of GALT array showing wells with increased green fluorescence indicative of bacterial metabolism. **B**. Bacterial growth, in well marked by resorufin fluorescence, can be confirmed by microscopic observation.

## Resazurin Reduction to Resorufin

Reduction of resazurin to resorufin happens at +380mV (midpoint potential, pH 7.0 and 25°C) making it responsive to NADH, NADPH, $FADH_2$, $FMNH_2$ and cytochromes. The reduction step is completely irreversible, so assaying for this reaction represents the sum of reducing power of the sample over time. The resazurin signal is used on the Prospector array to distinguish between empty wells ("negative wells") from wells with growth ("positive wells"; Fig. 1A and B).

## Reduction of Resorufin to Dihydroresorufin

Resorufin can be further reduced to dihydroresorufin. This second step happens at -420mV (midpoint potential, pH 7.0 and 25°C) and yields a colorless, non-fluorescent product. This reduction step is completely reversible, leading to oscillatory behavior under appropriate conditions.




On a Prospector array we observe this second step in individual wells which, after indicating growth through the formation of strongly fluorescent resorufin, subsequently turn dark over a course of hours as the resorufin is further reduced (Table 1).

**TABLE 1**: Distinct fluorescent properties of resazurin and its reduced derivatives resorufin and dihydroresorufin.

| | RESAZURIN | RESORUFIN | DIHYDRO-RESORUFIN |
|---|---|---|---|
| RED EXCITATION | **Strong Red Fluorescence** | Weak Red Fluorescence | No Fluorescence |
| GREEN EXCITATION | No Fluorescence | **Strong Green Fluorescence** | No Fluorescence |

Due to this second reduction step, collecting images of an array at fixed intervals (e.g. every 24 h) and following a time-course of fluorescence is essential for properly discriminating wells. This is especially important when heterogeneously composed, unknown samples are used.

## Resazurin Behavior Varies Between Species

We have observed that different organisms behave differently and reduce resazurin at different rates; they also yield the first product (resorufin) only or reduce to the second (dihydroresorufin). Careful observation of the resazurin behavior may help discriminate different species on the array. This can be done by using the Prospector system to (1) monitor

fluorescence at multiple wavelengths, (2) record a time course at multiple wavelengths.

*Monitoring fluorescence at multiple wavelengths*

For resazurin/resorufin/dihydroresorufin, monitoring fluorescence upon the green and red excitation are appropriate. By plotting the median fluorescence of wells in both dimensions we get a more nuanced picture of the state of individual wells (Fig. 2). From this 2D plot, wells from different regions can be selected for transfer to 96-well plates.

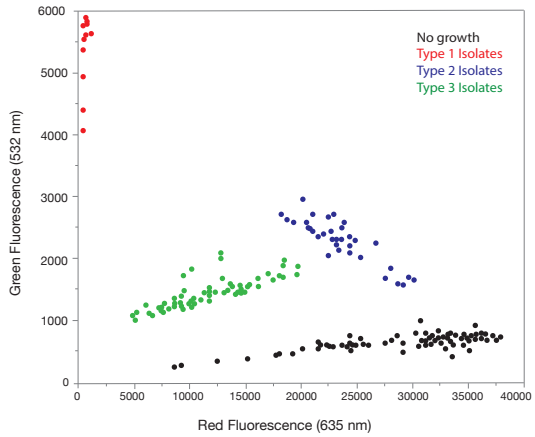



**FIGURE 2**: Scatter plot analysis of well fluorescence on Prospector array after red and green excitation shows different types of resazurin conversion.

*Time course*

Including time as a variable, in addition to multiple wavelengths, provides a very rich data set. We observed that different wells progress in time quite differently, which may reflect microbial diversity (Fig. 3). With thousands of isolates growing on a typical GALT array, it may be possible to screen based on the behavior of the indicator. Understanding how resazurin behavior is linked to growth patterns and thus to species is an area of ongoing research.

## pH Dependence

Resazurin and resorufin are sensitive to low pH: resazurin has a pKa of 6.5, and resorufin of ~6. At neutral media pH (~ pH 7), there is balance between protonated and unprotonated forms of the molecules. As the pH drops below 7, the protonated forms, which are less fluorescent and much less soluble than their unprotonated analogs, become prominent.

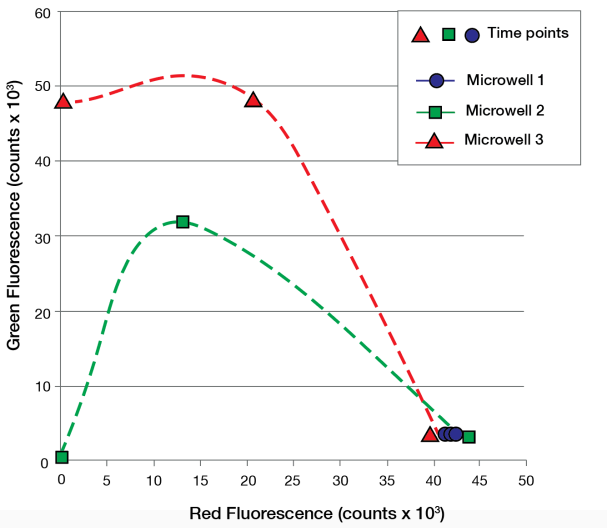



**FIGURE 3**: Time course analysis of green and red fluorescence in three representative array microwells. Fluorescence was measured after 0, 24, and, 48 hours of growth. Microwell 1 shows no growth and is not changing with time.

Protonation is another mechanism that leads to loss of signal, and must not be confused with loss of signal resulting from reduction (which indicates organismal growth). Loss of signal from low pH, when occurring in all wells, is easily compensated for by changing the camera settings. Compensating for the effect of changing pH becomes more challenging when the pH decreases (or increases) in certain wells only. Loss of signal in a well may be occurring from strong reduction potential (forming dihydroresorufin) or acid production. As either condition may indicate growth, a better understanding of the samples is required to distinguish one mechanism from the other. One strategy to mitigate this scenario is to use media with neutral or basic pH and some buffering capacity. When the experiment permits the use of good capacity buffers, consistent, comprehensible signals can be obtained .

## Summary

Resazurin is a redox indicator used in the GALT Prospector to identify bacterial metabolism. Monitoring the red and green fluorescence of cultures grown in presence of resazurin will allow conclusions about bacterial growth and the different reaction products may indicate bacterial diversity.



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