Monitoring cell health with alamarBlue and PrestoBlue reagents using the Varioskan LUX Multimode Microplate Reader

Abstract

In this application note, the benefits of pairing Invitrogen™ alamarBlue™ and PrestoBlue™ reagents with the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader are demonstrated. Both viability assays are simple to perform, and the generated signal (fluorescence or absorbance) typically can be read in a conventional microplate reader. However, utilizing the Varioskan LUX Multimode Microplate Reader and Thermo Scientific™ SkanIt™ Software significantly enhances the usability of these resazurin-based workflows (Figure 1), allowing fast readout of signals and immediate access to powerful data processing steps such as curve fitting and useful calculations, including cell viability percentages and cytotoxic potency.

Introduction

The assessment of cell health is a foundational method in life science research areas such as cell biology, biochemistry, molecular biology, pharmacology, and toxicology. In virtually any experiment that involves cells, researchers are interested in evaluating cell health, which typically translates into quantifying their viability. The measurement of cellular viability is particularly relevant in drug discovery projects: the viability of cultured cells is typically measured upon exposure to potential drug candidates. For that purpose, a number of cell viability assays are available in microplate format. They measure, for instance, damage to the cell membrane (e.g., lactate dehydrogenase release), cellular content of a key metabolite (e.g., luminescence determination of ATP), or cellular redox status (e.g., metabolic reduction of redox dyes).

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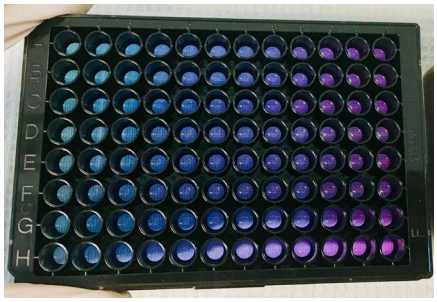

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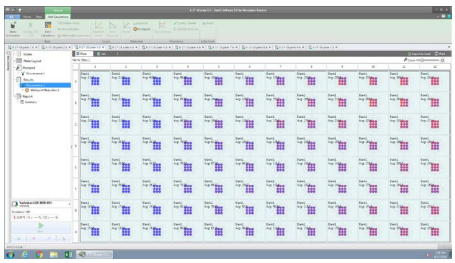

Figure 1. Cell health monitoring with alamarBlue and PrestoBlue reagents. (A) Plate and **(B)** SkanIt Software data visualization.

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Resazurin-based assays have been developed to quantify metabolic activity of viable cells (Figure 2A). Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a nonfluorescent blue dye that is reduced to a highly fluorescent pink dye, resorufin, in the presence of the redox environment of metabolically active cells [1]. Damaged and nonviable cells cannot maintain high reducing power, thus resulting in a proportionally lower resorufin signal. A number of peer-reviewed publications have demonstrated the excellent performance of resazurin-based quantitation in a variety of cellular systems [2-4]. Resazurin-based assays can be carried out in a homogeneous format (only one addition step needed) and without any cellular lysis or washing steps. Because resazurin is a noninvasive probe, stained cells can be further used in a multiplexed assay format. Additionally, resazurin-based assays have good sensitivity, wide linear ranges, and ample dynamic signal windows, which together translate to excellent assay performance.

Two of the most widely used resazurin-based products are alamarBlue and PrestoBlue reagents. The alamarBlue formulation was the first resazurin-based product, and it is still the basis for the most widely used viability assay. Compared to PrestoBlue reagent, the alamarBlue formulation can be used for longer incubations or with populations of high cell density. On the other hand, the PrestoBlue cell viability reagent is the fastest-acting resazurin-based product and is ideally suited for fast determination of mammalian cell viability. Both assays involve only one addition step, followed by an incubation and a reading step for measuring either fluorescence or absorbance (Figure 2B), which can be performed in a common microplate reader.

The simplicity and robust performance of alamarBlue and PrestoBlue assays are indisputable, and these benefits underlie their high popularity. However, data processing for viability assays remains a step in which researchers often need to use dedicated scientific graphics and mathematical programs, which are not always freely available. Here we demonstrate the benefits of performing alamarBlue and PrestoBlue assays with the Varioskan LUX multimode reader. Utilizing this instrument significantly enhances the simplicity of resazurin-based workflows by providing a quick and sensitive readout of the fluorescence (or absorbance) signal as well as enabling powerful data processing with SkanIt Software.

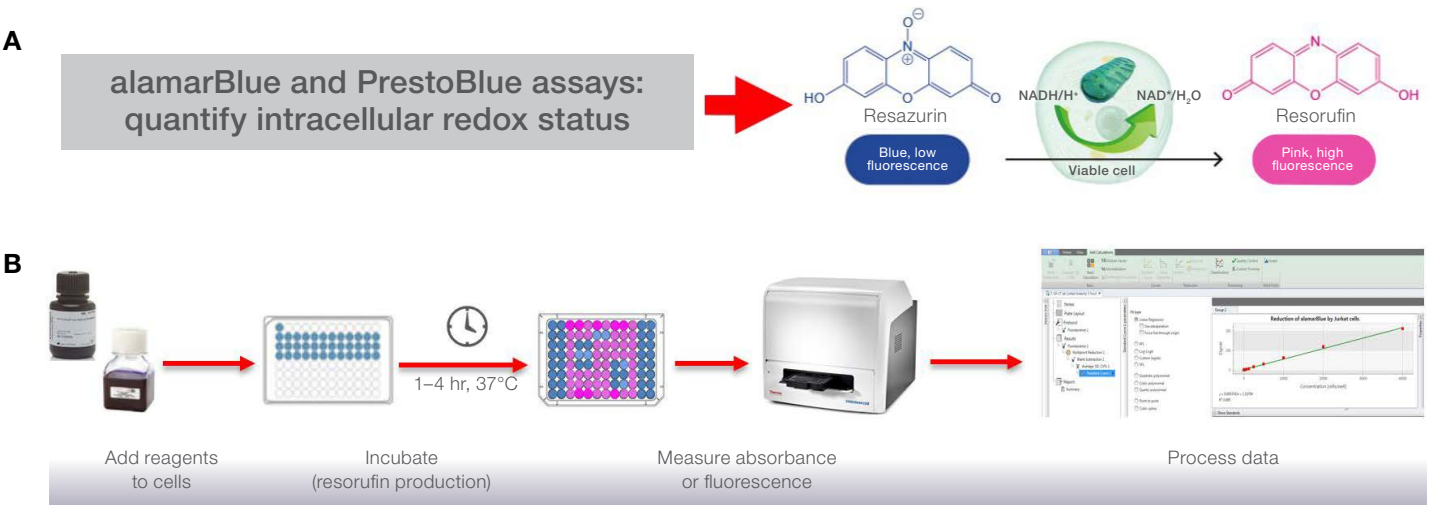

Figure 2. Resazurin-based alamarBlue and PrestoBlue assays. (A) Schematic example of intracellular reduction of resazurin by NADH-dependent enzymes. **(B)** General protocol used for measurement of alamarBlue and PrestoBlue assays using the Varioskan LUX multimode reader.

Materials and methods

Cell lines and culture conditions

For this study, one suspension (Jurkat) and two adherent (A549 and U2OS) cell lines were used. A549 basal epithelial cells (ATCC, Cat. No. CCL-185) were cultured in Gibco™ Minimum Essential Medium (Cat. No. 11095-098) supplemented with 10% Gibco™ Fetal Bovine Serum (FBS) (Cat. No. 16000-036). U2OS osteosarcoma cells (ATCC, Cat. No. HTB-96) were cultured in Gibco™ McCoy's 5A (Modified) Medium (Cat. No. 12330-031) supplemented with 10% FBS, while Jurkat lymphoma T cells (ATCC, Cat. No. TIB-152) were cultured in Gibco™ RPMI 1640 Medium (Cat. No. 22400-105) also supplemented with 10% FBS. Cells were kept in a humidified incubator at 5% CO_2 and 37°C until 90–95% confluency. Cells were then rinsed with Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS, Cat. No. 14190-250), followed by dissociation with Gibco™ TrypLE™ Express Enzyme (Cat. No. 12605-036). The Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000) was used to determine cell concentrations before plating the cells into Greiner 96-well black microplates. The cells were allowed to adhere to the bottom of the plate overnight at 37°C.

Cell titration and drug dose–response studies

To assess linearity in cell studies, cells were seeded into microplates at different concentrations and then treated with either PrestoBlue reagent (Cat. No. A13262) or alamarBlue reagent (Cat. No. DAL1025). Adherent (A549 and U2OS) and suspension (Jurkat) cell lines were seeded and assayed within a range of either 0–2 x 10^4 cells/well or 0–4 x 10^4 cells/well, respectively; four wells per cell count were included in the analysis. For the drug dose–response studies, cells were seeded and allowed to adhere to the surface of the plates overnight at 37°C; adherent cells were used at a concentration of 1 x 10^4 cells/well, while suspension cells were used at a concentration of 4 x 10^4 cells/well. After 18 hours of growth, cells were treated with increasing concentrations of a model cytotoxic compound, amsacrine (0–100 µM in DMSO), for about 18 hours before the viability assay was performed. Cells exposed to 0.14% DMSO were used as controls for 100% viability. At the end of an overnight incubation period at 37°C, alamarBlue or PrestoBlue reagent was added (to a final dilution of 1:10 in the wells), and cells were further incubated for 3 hours (for alamarBlue reagent) or 1 hour (for PrestoBlue reagent). The fluorescence of the resorufin was detected by 560 nm excitation and 590 nm emission, with an excitation bandwidth of 12 nm, using the Varioskan LUX multimode reader. The fluorescence was measured using bottom optics at 4 defined points in each well, as shown in Figure 3. Bottom reading is typically recommended for detection of fluorescence from adherent cells. The multipoint measurement can also be customized for other regions of interest (Figure 3, insert).

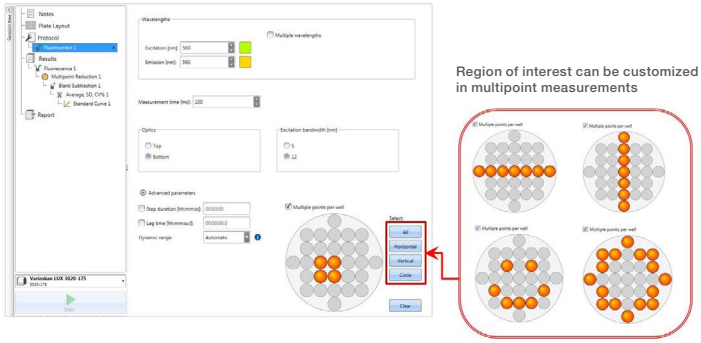

Figure 3. SkanIt Software 5.0 protocol for detection of resorufin fluorescence intensity in alamarBlue and PrestoBlue assays. Multipoint measurements can be customized to the region of interest within each well.

Data visualization and analysis

Data acquisition was followed in real time as fluorescence readouts were made by the Varioskan LUX multimode reader. The fluorescence intensity was visualized on a heat map created for every well, with an automatically adjusted scale that reflected the assay dynamic range, as defined by the lowest and highest fluorescence signals measured across a plate (Figure 1B). For data analysis, a "Multipoint reduction" step was added in the SkanIt Software protocol session, and the average of the multipoint fluorescence signal for every well was calculated. After this step, a "Blank subtraction" was carried out to account for any background reduction of resazurin occurring in medium-only wells treated with PrestoBlue or alamarBlue reagent. The following data processing steps were completed to report the cell viabilities.

To analyze the results of the cell titration experiments, a "Standard curve" step was added and a linear regression analysis was selected, which included a quantitative estimation of the quality of the fit (R^2). Experimental data originating from the three different cell lines were combined into one single graph. For that specific purpose, the results were exported from SkanIt Software as Microsoft™ Excel™ files, where all three data series were combined into one multiline graph.

To calculate the percentages of cell viability in amsacrine-treated cells, a "% Normalization" step was carried out. The data were normalized to 100% viable cells assuming that the signal obtained from the solvent-only wells corresponded to 100% viable cells. For fitting the potency curves, a "Dose response" step was then added into the "Session tree", and a 4-parameter logistic (4PL) fitting was chosen. Calculation of ED_{50} (effective dose, equal to effective concentration EC_{50}) was automatically performed by SkanIt Software 5.0. In parallel, the data were also analyzed using GraphPad Prism™ 7 Software (GraphPad Software, La Jolla, California).

Results and discussion

The addition of different cell concentrations of the three tested cell lines (A549, U2O2, and Jurkat) resulted in linear increases in fluorescence signal for both alamarBlue and PrestoBlue assays (Figure 4A and B, respectively). Of note are the differences in the fluorescence signal among the three cell lines tested. The observed differences are most likely due to the differences in the intrinsic metabolisms among the three cell lines tested. For each different cell line, we recommend optimization of experimental conditions.

Even though it cannot be assumed that results from resazurin calibration curves in one cell line can be extrapolated to a different cell line, setting new protocols for different cell lines is very straightforward because of the simplicity of alamarBlue and PrestoBlue reagents. In addition to being easy to use, these cell viability reagents are noninvasive, allowing cells to be reused for other experiments. This is an important advantage compared to other commercially available reagents that require cell lysis for the measurement of cell viability.

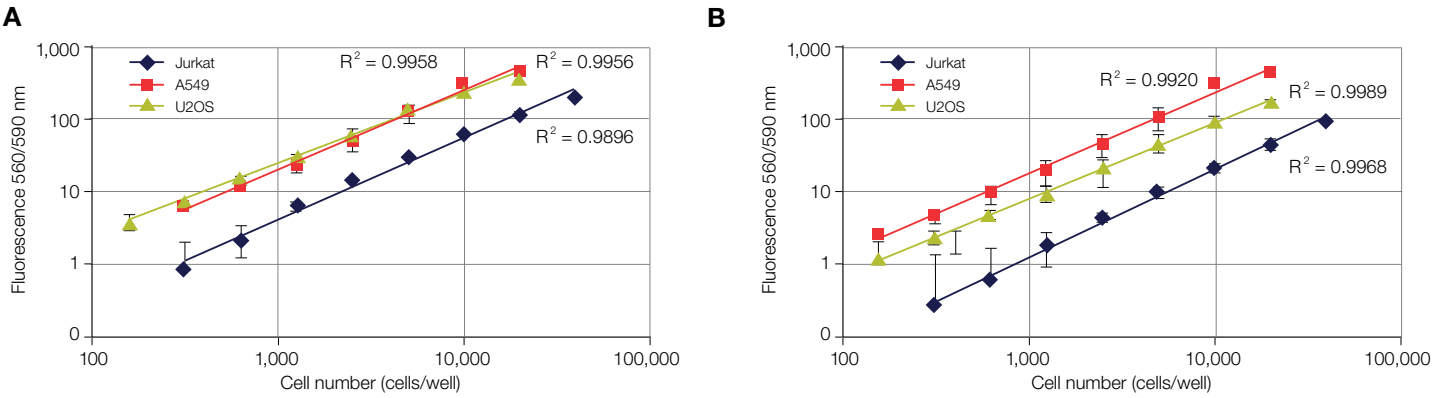

Figure 4. Relationship between resorufin fluorescence signal and cell concentration. (A) alamarBlue and **(B)** and PrestoBlue reagents were tested in three different cell lines.

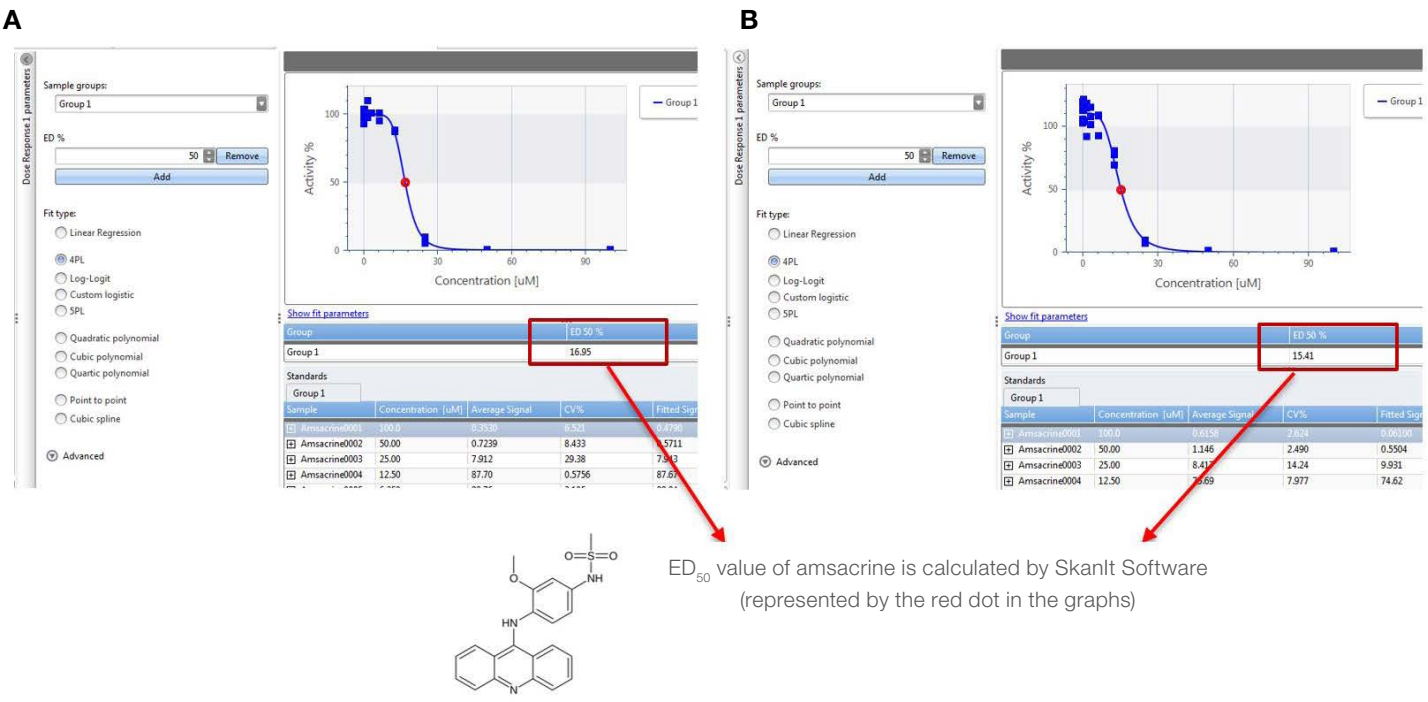

Figure 5. Representative dose–response curves obtained for the cytotoxic effect of amsacrine in A549 cells using SkanIt Software. (A) alamarBlue and **(B)** PrestoBlue reagents were used for the assays. The structure of amsacrine is shown as an insert in the figure.

Each cell line was exposed to varying concentrations of amsacrine, as a model drug, and ED_{50} values were calculated for both resazurin-based assays using SkanIt Software. An example of the data processing is shown in Figure 5 (above) for alamarBlue and PrestoBlue assays in A549 cells.

The calculated ED_{50} values for the three cell lines are shown in Table 1, and they were found to be similar between alamarBlue and PrestoBlue assays. Cytotoxicity was roughly 10 times higher in Jurkat cells compared to the other two cell lines. This higher sensitivity of Jurkat cells (leukemic T cells) may be related to the fact that amsacrine is a chemotherapeutic agent that intercalates into the DNA of tumor cells, and it is especially effective against leukemia cells.

Potency values in Table 1 were calculated in parallel with SkanIt Software and GraphPad Prism Software, which is a widely used statistical program and especially popular among life scientists. The potency values calculated with SkanIt Software differed by less than 5% from those calculated using GraphPad Prism Software, thus confirming the reliability of SkanIt Software in providing high-quality, accurate fitting for dose–response studies of investigational drugs.

Table 1. Cytotoxic effects of amsacrine in three different cell lines.

	ED_{50} for amsacrine (µM) calculated with SkanIt Software		**ED_{50} for amsacrine (µM) calculated with GraphPad Prism Software**	
Cells	alamarBlue reagent	PrestoBlue reagent	alamarBlue reagent	PrestoBlue reagent
Jurkat	1.030	1.386	1.061	1.340
A549	16.95	15.41	16.70	14.38
U2OS	14.21	14.50	14.27	14.04

An additional advantage of using SkanIt Software is that established protocols for alamarBlue and PrestoBlue assays are already available. To use them, simply go to the session browser of SkanIt Software and select “Online”. Under the preselected protocols for the Varioskan LUX multimode reader, there are protocols for alamarBlue and PrestoBlue assays (Figure 6).

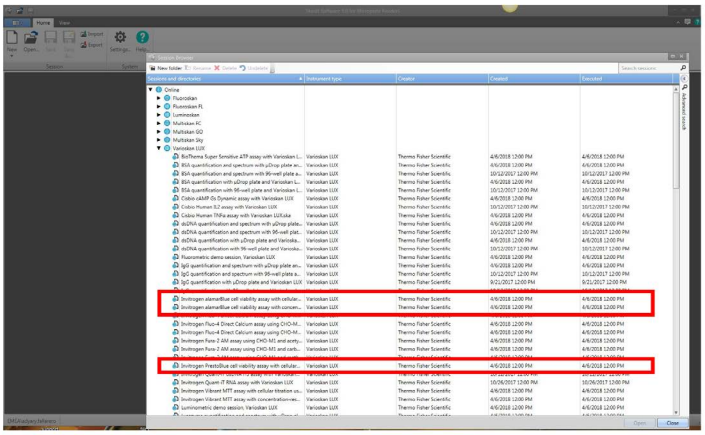

Figure 6. Examples of assays found in the online protocol library of SkanIt 5.0 Software (alamarBlue and PrestoBlue assays indicated by red rectangles).

Conclusions

The simplicity and excellent performance of alamarBlue and PrestoBlue assays have been widely documented in the scientific literature. Here we have demonstrated that the Varioskan LUX multimode reader enhances the usability of alamarBlue and PrestoBlue assays even further by allowing accurate signal readings, fast curve fitting, and reliable calculation of cell viability and cytotoxic potency.

References

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