

**C**ell based assays offer a biologically relevant substitute to predict the response of a drug on an organism and while initially used mostly for secondary screening, are now progressively being used for primary screens. In addition, they are used in many research areas, providing knowledge about biological targets and pathways in the whole cell.

The following are 10 Tips describing some of the important factors to consider when choosing a cell-based assay and the best ways to ensure success:

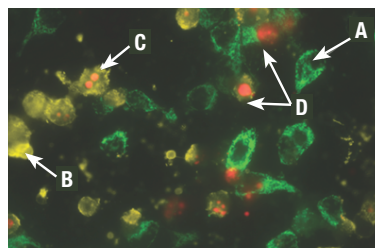
## 1. Choosing Cell Types

Choosing the right cell type to study is a key factor to achieving a successful research outcome. Different cell types (Primary cells vs. Cell lines) have been known to generate different biomarkers, which can influence the results of the assay. Primary cells (HUVECs, hepatocytes, etc.) are perfect options when performing in vitro studies, however they are difficult to grow and transfect. When performing cell viability assays that use a metabolite, it is important to know that cell lines generally have a higher metabolic rate than primary cells. Concurrently, primary cells have a higher spontaneous rate of death, which can cause background when running a cytotoxicity assay. Therefore the cell type of choice will dictate the assay conditions.

## 2. Understanding Cell Apoptosis

Understanding of the cell death process and mechanism is very important when studying a specific cell type and deciding on a suitable assay. During apoptosis the cells shut down metabolism, lose membrane integrity, release cytoplasmic material into the surrounding medium, and eventually die. Certain biomarkers of apoptosis, such as caspase activity, are transiently expressed, have very short half-lives, and therefore are only detectable within a limited range of time. Ultimately understanding the cell death process in the specific cell type could help define the experimental design and endpoint selection.

### Analyze More Stages Than Simple Annexin V-Based Kits



- A. Viable Cells
- B. Early apoptotic cells
- C. Late apoptotic cells
- D. Necrotic cells

**Figure 1:** GFP-CERTIFIED® Apoptosis/Necrosis Detection Kit (ENZ-51002) detects four distinct cell states. Mitochondrial GFP-expressing HeLa cells were treated with 2µM Staurosporine for 4 hours. The Apoptosis Detection Reagent (Gold) and Necrosis Detection Reagent (Red) specifically detect cell states with clear spectral separation from mitochondria-associated GFP signal. Healthy cells (A), cells undergoing apoptosis (B), cells undergoing late-stage apoptosis (C), and necrotic cells (D).

## 3. The Edge Effect

Edge effects are known as factors that contribute to the deterioration of assay performance. The causes of edge effect are complex and can generate high plate rejection rate in screening runs. Cells in the wells around the edge of the plate may react differently than the inner wells due to a temperature differential, which is created when the plate is placed in the incubator (1). Stacking plates on top of each other and evaporation during long incubation

times also produce the edge effect. In order to avoid this effect, it is recommended to duplicate or triplicate the experimental samples, monitor the timing, plate order, and plate position in the incubator during the assay, and making sure that the incubator temperature is evenly distributed (1). Another approach to significantly reduce the edge effect is to incubate newly seeded plates at room temperature before placing them in an incubator (2).

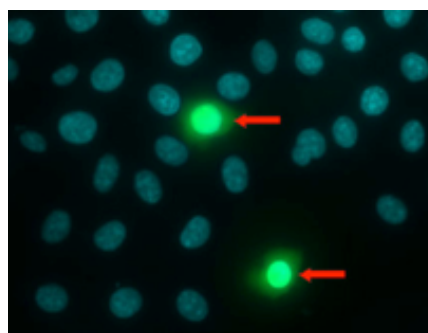
## 4. Avoid Contamination

Biological contaminants such as mold, bacteria, and other cells can cause inconsistent and irreproducible results. Assess the lab environment and be sure to cover the plates during incubation. Even the slightest contamination can ruin weeks of research. Moreover, wipe clean media bottles, pipettes, and other pertinent materials with ethanol prior to running the assay. Carry over is another problem caused by improper washing of old pipette tips. Washing old tips with EtOH and DMSO is one common way laboratories cut cost. However this can lead to carryover of DMSO into the wells resulting in much lower signal. Therefore it is recommended to use new tips.

## 5. Regulate Temperature and CO<sub>2</sub>

Controlling temperature and CO<sub>2</sub> is crucial to maintaining cell viability in cell-based assays. Cells left at room temperature before incubation can lead to increased background, resulting in reduced signal to background ratios (1). This can be corrected by stirring the cells on ice while adding them to small batches of assay plates at a time. This will minimize the amount of time the cells are exposed to room temperature and improve the overall results of the assay. Many cell lines cannot tolerate exposure to room temperature, whereas other cells require lengthy room temperature treatments for the assay. Ultimately every cell line is different and therefore optimal experimental conditions need to be determined according to each cell line. CO<sub>2</sub> is also a factor when performing cell-based assays. If the CO<sub>2</sub> concentration is too high during incubation, the activity of the cells can be adversely affected. Small variations in environmental factors such as temperature and CO<sub>2</sub> can have a very large impact on the outcome of the assay (1).

### Distinguish between live vs dead cells in a single reagent



**Figure 2:** The NUCLEAR-ID® Blue/Green dye (ENZ-53004) is detected as blue-stained nuclei in live cells and fluorescent-green nuclei in dead cells (arrows).

## 6. Pipetting Errors

Pipetting inaccuracies are very common mistakes, which can lead to variable results due to poor viability, cell death, and false cytotoxicity. Cells should be gently and evenly distributed from well to well or when preparing frozen aliquots. Pipette calibrations should be performed to prevent inaccuracies in the experimental data.

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# 10 Tips for Successful Cell Based Assays

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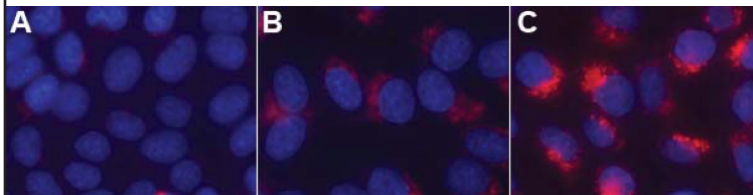
## 7. Optimize Cell Density

Consistent cell density in samples allows for accurate determination of sensitivity, range of the assay, and most importantly trustworthy data. Wells with sparse cell cultures can lead to higher detection sensitivity. Conversely high cell density cultures can result in higher background signals and may demand longer exposure times with the compound being tested, thus leading to inaccurate results. To optimize cell density, a standard curve of differing cell numbers should be generated to assay for viability and cytotoxicity. This will provide an accurate idea of the cell density needed before introducing test compounds into the assay.

## 8. Assay Controls

Using a control is a very important step in the experimental design. It determines if the assay is functioning properly. In addition, control samples will provide reference points to compare the data, in order to study any variables in the samples. Several controls need to be considered including positive controls, negative controls (untreated cells), and no cells. Ultimately these controls will provide a reference point for data analysis and give greater confidence in the biomarker being studied.

### Monitor Abnormal Lysosome Accumulation Arising from Drug-induced Phospholipidosis



**Figure 3:** Drug-induced lysosome accumulation in U-2 OS cells was evaluated using LYSO-ID® Red dye (ENZ-51015). (A) Untreated cells (B) Chlorpromazine, 28 µM (C) Verapamil, 200 µM.

## 9. Instrumentation and Detection Parameters

The detection instrument being used may also affect the result of the assay. Filter sets, instrument gain, and microplate types are all important variables to keep in mind when running a successful cell-based assay. If there is no change in the fluorescent signal, while increasing the number of treated vs. untreated cells, this can be due to an incorrect filter setting. It is recommended to confirm the Ex/Em maxima of a given fluorophore with the appropriate filter. Large error bars between treated and untreated samples can be the result of the instrument gain being set too low. Clear bottomed plates allow for cell morphology to be analyzed prior to the assay; however, these plates can lead to increased crosstalk. Black-sided well plates should be used for fluorescent assays to reduce scatter, overlap of signal, and crosstalk.

$$Z' = 1 - \frac{(3 \text{ SD of Positive Control} + 3 \text{ SD of Negative Control})}{\text{Mean of Positive Control} - \text{Mean of Negative Control}}$$

## 10. Data Analysis

Assay performance can be analyzed in a number of different ways. The signal-to-background ratio or signal window ( $S/B = \text{mean signal} / \text{mean background}$ ) and the signal-to-noise ratio ( $S/N = \text{mean signal} - \text{mean background} / \text{Standard Deviation of background}$ ) are often used as measures of assay performance. Unfortunately though, these ratios do not take assay variability into consideration. Calculating the  $Z'$  statistic, or score, provides a measure of assay quality, while taking into account both the signal-to-noise ratio and assay variability. A higher  $Z'$  value conveys greater reproducibility and precision of the assay. A perfect assay has a  $Z'$  value of 1. For a high throughput screening assay a  $Z'$  value of  $> 0.5$  is generally considered acceptable. Although the  $Z'$  statistic was originally derived for evaluating HTS assays, it is a general measure of assay quality and can be applied to any assay (3).

### References:

1. Maddox C, Rasmussen L, White EL. Adapting Cell-Based Assays to the High Throughput Screening Platform: Problems Encountered and Lessons Learned. *JALA* Charlottesville. Va. 2008; 13: 168-173.
2. Lundholt B, Pagliaro L, Scudder K. A Simple Technique for Reducing Edge Effect in Cell-Based Assays. *J. Biomol. Screen.* 2003; 8: 566-70.
3. Broadway N. How to Develop Assays: 5 Considerations and 8 Fundamentals. *Mater Methods.* 2012; 2: 121.



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