Improved cell viability assay performance using PrestoBlue® Reagent

Detecting cell viability with the Infinite® M200 PRO + GCM™ and the Infinite M1000 PRO multimode microplate readers

Introduction

Cell proliferation and viability assays are widely used in academic and life science laboratories, and are becoming increasingly common in the pharmaceutical industry. Assessment of cellular viability markers in cell-based applications is mandatory for laboratories working with *in vitro* systems.

Resazurin is a well-established fluorescent dye that is frequently used in various commercially available assay systems. It is a redox indicator that can be added directly to cells in culture. Viable cells convert the dark blue oxidized form of the dye (resazurin) into a red-fluorescent reduced form (resorufin; λ_{Ex} = 570 nm; λ_{Em} = 590 nm). This system is specific for cell viability as non-viable cells rapidly lose metabolic capacity to reduce resazurin, and thus no fluorescent signal is detected.

Life Technologies offers an enhanced version of the commonly available resazurin-based assays, the PrestoBlue® Cell Viability Reagent. This novel dye offers the same analytical properties (fluorescence excitation and emission wavelength) as common resazurin-based assays (eg. alamarBlue®), but is characterized by a significantly shorter incubation time (minimum 15 mins vs 1 hr), increasing experimental efficiency and performance (1).

This application note describes the evaluation of Life Technologies' PrestoBlue® Cell Viability Reagent using the Infinite M200 PRO and the Infinite M1000 PRO multimode microplate readers.

Materials and methods

- Infinite M200 PRO Quad4 Monochromators™-based multimode reader with Gas Control Module (GCM) (Tecan, Austria)
- Infinite M1000 PRO Premium Quad4 Monochromators-based multimode reader (Tecan, Austria)
- PrestoBlue® Cell Viability Reagent (Life Technologies, USA)
- 96- and 384-well flat, black microplates (Greiner Bio-one, Germany)
- Human squamous epidermoid carcinoma cells (A431, ATCC # CLR-1555)
- Dulbecco's modified Eagle's medium (DMEM), high glucose (PAA Laboratories, USA)
- Heat-inactivated fetal calf serum (FCS) (PAA Laboratories, USA)
- Enhanced green fluorescent protein (EGFP)
- L-glutamine (PAA Laboratories, USA)
- Sodium pyruvate (PAA Laboratories, USA)
- Penicillin/streptomycin (PAA Laboratories, USA)
- HEPES (PAA Laboratories, USA)
- Trypsin/EDTA (PAA Laboratories, USA)
- Staurosporine (Sigma Aldrich, Austria)

Human squamous epidermoid carcinoma cells (A431) were grown to confluence in high glucose DMEM supplemented with L-glutamine, sodium pyruvate, penicillin/streptomycin, HEPES and 5 % heat-inactivated FCS at 37 °C and 5 % CO_2 in a humidified atmosphere (Binder CB incubator).

Incubation time study

Cells were harvested using trypsin/EDTA, then resuspended in fresh growth medium containing 5 % FCS and seeded as a dilution series ranging from 3,000 cells/well down to 35 cells/well (see Figure 1) into a black 384-well tissue culture plate (100 µl/well). After overnight incubation at 37 °C and 5 % CO_2 in a humidified atmosphere, 10 µl of the PrestoBlue® reagent was added directly to the sample wells. The assay was incubated at 37 °C and 5 % CO_2 inside the reader using the Infinite M200 PRO's temperature control option and ingenious Gas Control Module (GCM) which allows simultaneously control O_2 and CO_2 (2).

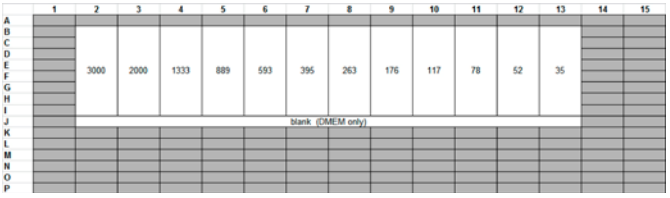

Figure 1 384-well plate layout – cell dilution series. Wells indicated in grey were filled with 100 µl ddH_2O (including wells A16-P23, not shown).

Various incubation periods were tested to identify the minimum and optimum incubation times. After each incubation period (15, 30, 45, 60 and 75 mins), cell viability was determined by measuring the fluorescent signal in FI top mode. The whole experiment (incubation and detection) was carried out inside the microplate reader, without the need for any manual interaction.

Optimized measurement settings for the Infinite M200 PRO are shown in Table 1. To provide maximum sensitivity, the z-positioning with integrated background correction function (max signal/blank ratio) was used. This innovative function helps to overcome background fluorescence resulting from phenol red in the culture medium (3).

Measurement parameter	Instrument settings
Plates	[GRE384fb.pdfx]
Shaking (prior to read)	20 sec; 1 mm amplitude; orbital
Mode	Fluorescence intensity top
Excitation wavelength	560 nm
Excitation bandwidth	9 nm
Emission wavelength	600 nm
Emission bandwidth	20 nm
Gain	Optimal
Number of flashes	25
z-position	calc. f. well B2 (signal) and J2 (blank)
Integration time	20 µs
Lag time	0 µs
Settle time	0 ms

Table 1 Measurement parameter and instrument settings for the PrestoBlue® assay using the Infinite M200 PRO.

The average value for each dilution was calculated and corrected by subtracting the average blank. The respective error bars were calculated using the Gaussian law of error propagation. Values represent corrected means from eight separate wells.

The theoretical limit of detection (LOD) for the PrestoBlue® Cell Viability assay was calculated using the following equation:

$$\text{LOD (cells/well)} = \frac{3 * \text{SD (blank)} * 3{,}000}{\text{(average sample - average blank)}}$$

For the average sample value, the result for the first dilution of the series (3,000 cells/well) was used.

Cytotoxicity study

In a second experiment, cells were harvested using trypsin/EDTA, then resuspended in fresh growth medium containing 5 % FCS and seeded into a black 96-well tissue culture plate with a density of 12,000 cells/well (200 µl/well). The supernatant was removed and 200 µl DMEM containing varying concentrations (250 - 0.015 nM) of staurosporine (STS) was pipetted into each well (Figure 2) and incubated overnight at 37 °C and 5 % CO_2.

Note: This step in the procedure can be easily automated using Tecan's HydroSpeed™ plate washer for media exchange and the HP D300 digital dispenser for compound addition.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D		blank (DMEM only)	untreated control (100 % viability)	250 nM STS	62.5 nM STS	15.62 nM STS	3.9 nM STS	0.976 nM STS	0.244 nM STS	0.061 nM STS	0.015 nM STS	
E												
F												
G												
H												

Figure 2 Plate layout – STS dose response. Wells indicated in grey were filled with 200 µl ddH_2O.

Following incubation, cells were stained by adding 20 µl PrestoBlue® reagent directly to the sample wells. The plate was then incubated for 30 mins at 37 °C, 5 % CO2 in a humidified atmosphere and cell viability was determined by measuring the resulting fluorescent signal using the Infinite M1000 PRO in FI top mode.

Optimized measurement settings for the Infinite M1000 PRO are shown in Table 2. To provide maximum sensitivity the z-positioning with integrated background correction function (max signal/blank ratio) was used (4).

Measurement parameter	Instrument settings
Plates	[GRE96fb.pdfx]
Shaking (prior to read)	20 sec; 1 mm amplitude; orbital
Mode	Fluorescence intensity top
Excitation wavelength	560 nm
Excitation bandwidth	10 nm
Emission wavelength	590 nm
Emission bandwidth	10 nm
Gain	Optimal
Number of flashes	10
z-position	calc. f. well B3 (signal) and B3 (blank)
Integration time	20 µs
Lag time	0 µs
Settle time	0 ms

Table 2 Measurement parameter and instrument settings for the Presto Blue assay using the Infinite M1000 PRO.

The average for each dilution was calculated and corrected by subtracting the average blank. This value was then related to the average of the untreated control samples, and the respective error bars were calculated using the Gaussian law of error propagation. Values represent corrected means from six separate wells.

Results and discussion

Incubation time study

Figure 3 and Table 3 show the results of the incubation time study using the Infinite M200 PRO + GCM.

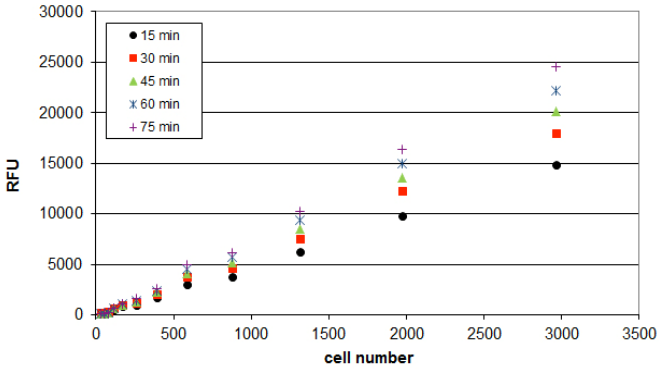

Figure 3 Cell dilution series with various incubation times measured using the PrestoBlue® assay on the Infinite M200 PRO

Detection limit in cells/well				
15 min	30 min	45 min	60 min	75 min
84	60	55	40	58

Table 3 Limit of detection (in cell/well) for the PrestoBlue® assay in 384-well plates measured on the Infinite M200 PRO.

After 15 minutes of incubation, the data shows good linearity and a LOD of around 80 cells per well. With increasing incubation periods the overall signal intensity increases, however the LOD in terms of cells/well does not significantly improve. A 30 minute incubation is therefore sufficient to achieve the maximum sensitivity of this novel viability assay.

Cytotoxicity study

The optimized incubation period of 30 minutes also proved to be sufficient for cytotoxicity analysis. The dose response curve in Figure 4 shows the cytotoxic effect of STS on A431 cells. As indicated by the very small error bars, the uniformity of the measurement was excellent, and this can be explained by the high sensitivity of the Infinite M1000 PRO for fluorescence-based assays.

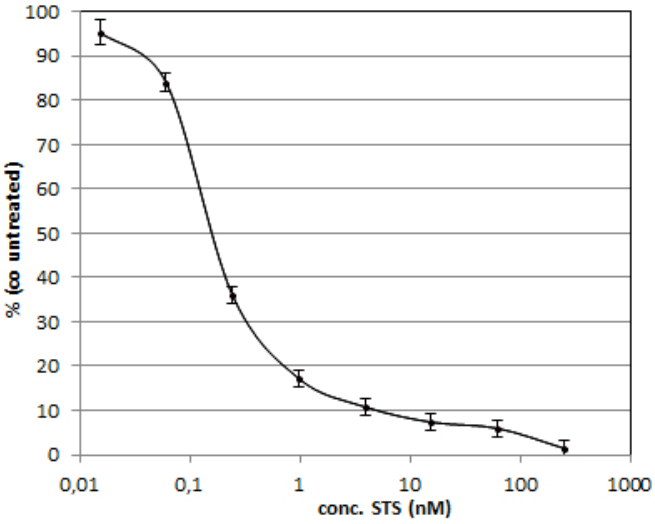

Figure 4 Staurosporine dose response curve; cytotoxic effect on A431 cells measured with the Infinite M1000 PRO.

Conclusion

This study demonstrates the suitability of the Infinite M200 PRO and the Infinite M1000 PRO multimode readers for determination of cell viability using the PrestoBlue® reagent. The dramatic decrease in incubation time for the assay (compared to common resazurin-based assays) was clearly verified in this study, which leads to increased efficiency and better assay performance (1).

The Infinite M200 PRO's incubation functions (temperature control and GCM) enable an automated assay procedure without the need for manual transfer of microplates between an incubator and the reader.

In addition, the Infinite M1000 PRO was shown to be perfectly suited to detect the fluorescent signal of the PrestoBlue® assay. The instrument's high-end fluorescence optics enable accurate signal detection, resulting in excellent assay uniformity.

The use of the PrestoBlue® viability reagent with the Infinite series multimode readers therefore offers a powerful solution for in vitro cell viability analysis in both assay development and screening applications.

References

1. www.lifetechnologies.com
2. www.tecan.com/gcm
3. Technical Note: Improved fluorescence top measurements; 396233 V.1.0, 03.2010
4. Technical Note: Maximize signal to blank intensity ratios; 396815 V.1.0, 08-2011

List of abbreviations

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
FBS	Fetal bovine serum
GCM	Gas Control Module
HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl) ethansulfonsäure

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