

Measuring cell proliferation and cytotoxicity in microplate format using the DELFIA cell proliferation assay and VICTOR Nivo multimode plate reader.

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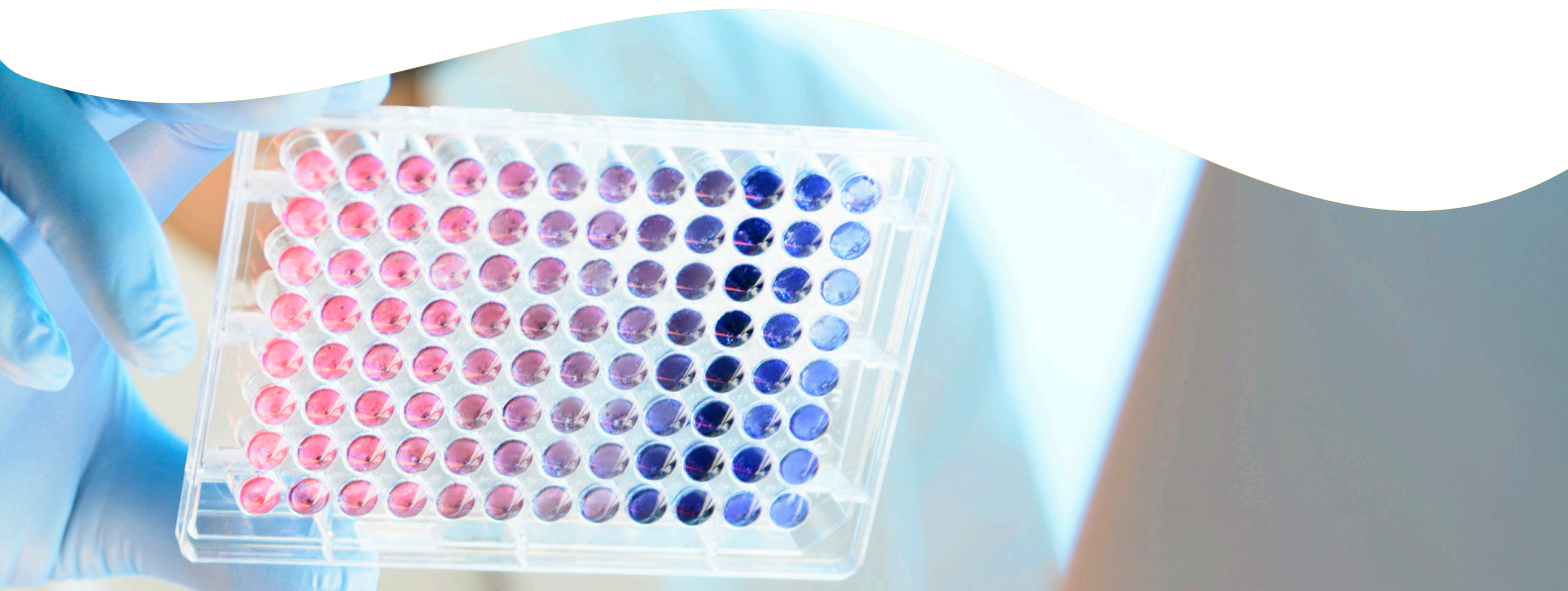
Introduction

The DELFIA® cell proliferation assay, a cell-based, non-isotopic, time-resolved fluoroimmunoassay, is based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis of growing cells. The DELFIA cell proliferation kit uses the same assay principle as the gold-standard tritiated thymidine incorporation assay, but uses a fluorescent rather than radioactive measurement. This simple assay provides rapid results of cell proliferation for both adherent and suspension cell lines cultured in micro plates. It can be used to detect cytotoxicity and effects of biomolecules capable of stimulating cellular growth such as cytokines and mitogens.

In this application note we validate the DELFIA cell proliferation assay using suspension cells and the VICTOR Nivo™ Multimode Reader to determine cell proliferation. Cell number titration, DMSO tolerance and cytotoxicity with various reference compounds were tested. VICTOR Nivo's browser based control software simplified the process of testing many different parameters. Furthermore, up to 32 filters are stored directly inside the flexible filter wheel and are automatically retrieved by the system when needed. And the direct export of results in Microsoft® Excel® format for further analysis provides an easy workflow.

Principles of the assay

The assay is based on the measurement of incorporated BrdU into newly synthesized DNA strands. During the S-phase of cellular proliferation, cells incorporate BrdU analog to thymine into the replicating DNA. Europium labeled monoclonal



antibodies attach to the incorporated BrdU and are detected using time-resolved fluorescence. Before adding the antibodies, cells have to be fixed in order to allow access to the incorporated BrdU. The Fix Solution contained in the kit denatures the proteins and fix the DNA onto the well surface. Through DELFIA Inducer, europium dissociates from the antibodies and forms a new highly fluorescent and sensitive chelate. The new europium chelate is excited at 320 or 340 nm and detected at an emission of 615 nm.

Material and method

Cell line

Jurkat cells (ATCC, no TIB-152) were cultured in Rostwell Park Memorial Institute (RPMI) 1640 Medium (Capricorn Scientific) supplemented with 10% fetal bovine serum (Capricorn Scientific, FBS-12A), 2 mM glutamine and 1% penicillin (100U/mL)/ streptomycin (100 ug/mL) at 37 °C in a humidified 5% CO₂ atmosphere.

Test compounds

For cytotoxicity testing, valinomycin (#10009152, Cayman Chemicals) diluted in dimethyl sulfoxide (DMSO, final concentration 0.1% v/v) was transferred into the plates using the Echo® 550 Liquid Handling System (Labcyte, Inc).

Assay procedure for suspension cells

For the DELFIA cell proliferation kit (Revvity, AD0200, Boston, USA) Jurkat cells were harvested by centrifugation and re-suspended in sterile culture medium. Cells were seeded at a total volume of 50 µL per well at required densities into a 384-well plate (CulturPlate-384, Revvity, #6007660) and incubated with 10 µM BrdU Labeling Solution (5 µL of diluted BrdU Labeling Solution per well) for 24 to 48 hours at 37°C with 5% CO₂. If compounds were used, cells were incubated for 48 hours with the compounds, and BrdU was added during the last 24 hours before measurement. After removing unbound BrdU by centrifugation at 300 x g for 10 minutes and aspirating the supernatant medium using the ELx405 microplate washer (#ELx405UCWS, BioTek) or Janus G3 MDT™ (#AJMDT01, Revvity), cells were dried at 60°C for approximately 2 hours. At the end of the incubation time, cells were incubated with 25 µL/well of the Fix Solution contained in the kit for 30 minutes at room temperature. The Fix Solution was removed by inverting the plate, and cells were incubated with 0.5 µg/mL Anti-BrdU-Eu antibodies (25 µL diluted Anti-BrdU-Eu

per well) for 1 hour at room temperature. After washing the plates five times with supplied washing buffer, the DELFIA Inducer (50 µL/well) was added and cells were incubated for an additional 15 minutes. The plates were measured using time-resolved fluorescence (TRF) detection on the VICTOR Nivo Multimode Reader (#HH35000500, Revvity). The europium chelate was excited at 320/75 nm and detected at an emission of 615/8 nm after a delay time of 400 µs.

All Plates, incubated at room temperature, were shaken at ~ 200 rpm. The DELFIA kit reagents were equilibrated at room temperature before addition. GraphPad Prism® (GraphPad Software) was used for data analysis and visualization (Figure1).

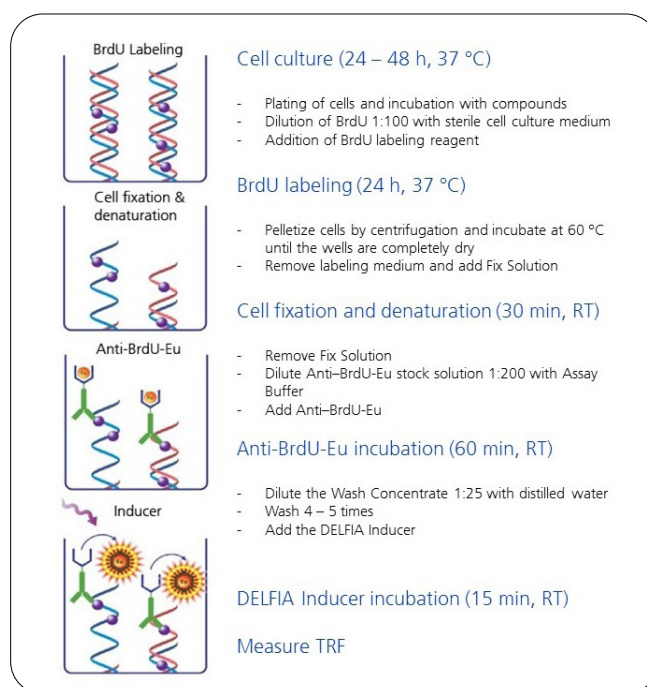


Figure 1. **Assay procedure.** The assay procedure for suspension cell lines is slightly different than the procedure for adherent cell lines. Adherent cells do not need to be dried in the plate, but suspension cells have to be centrifuged and dried at 60°C after the BrdU labeling medium removal. This procedure can be used for most cell lines but cell density and Incubation times must be optimized individually for each setup. Figure taken from Revvity application note, modified².

Results

Cell number titration

In order to evaluate linearity and dynamic range of the assay, Jurkat cells were titrated at different cell numbers in a 384-well plate. Cells were seeded in a 1:2 serial dilution, starting at a top concentration of 50,000 per well at a final volume of 50 µL. Less than 400 cells per well

were detected after an incubation of 48 hours with BrdU and a good linearity was observed for up to 6,000 cells per well (Figure 2). According to the observed linearity, a concentration of 4,000 cells per well in the CulturePlate-384 can be used to detect changes in cell proliferation caused by active substances. Note that the cell number titration should be performed for each cell line to be tested, as every cell line shows different cell proliferation activities, and the titration curve must be determined before testing compound cytotoxicity.

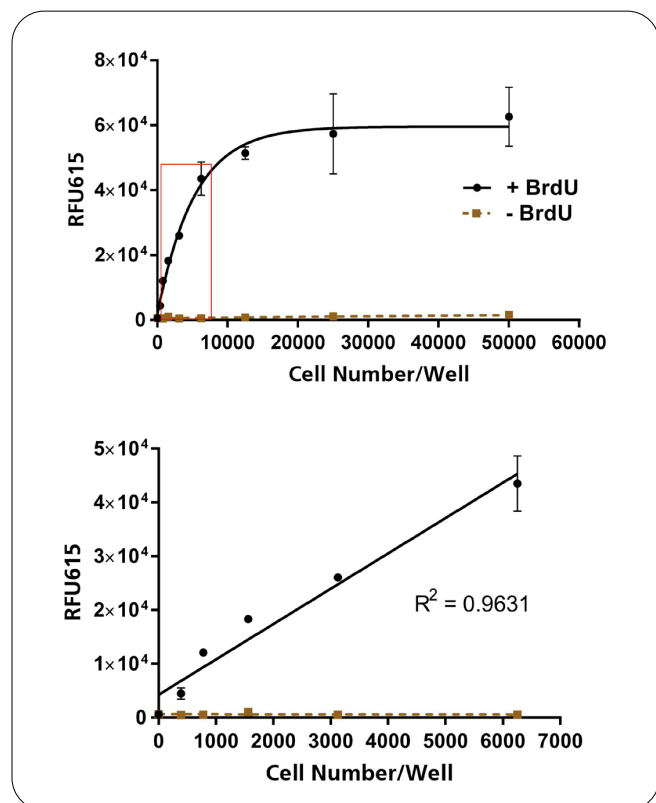


Figure 2. Cell number titration with Jurkat cells using the DELFIA Cell Proliferation assay. Jurkat cells were seeded, in a 1:2 serial dilution, starting at a top concentration of 50,000 cells per well in a 384-well plate and incubated for 48 hours with BrdU Labeling solution. The incorporation of BrdU was determinate as described in the assay protocol. Values represent means of three replicates \pm SD.

Dimethyl sulfoxide (DMSO) is commonly used to dilute compounds, but it might have a cytotoxic effect and even induce cell death at certain concentrations⁵. Therefore, it is necessary to examine the DMSO tolerance of the Jurkat cells before performing compound tests. The DMSO tolerance of Jurkat cells was tested in the range of 0.06 - 2% v/v DMSO. Jurkat cells were seeded at a concentration of 4,000 cells per well in a 384-well plate and incubated with various DMSO concentrations for 48 hours. BrdU was added during the last 24 hours of DMSO incubation. As expected, the results

show a concentration dependent decrease of RFU615 signal, revealing DMSO concentration should not exceed 0.13% v/v if using the maximum acceptance criteria of 20% inhibition of cell proliferation due to the DMSO effect (Figure 3).

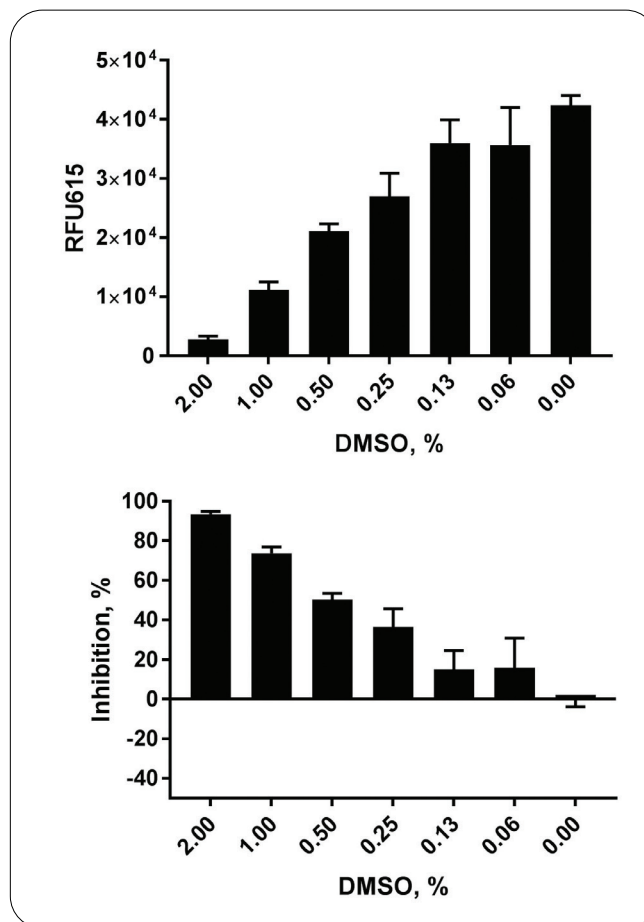


Figure 3. DMSO tolerance of Jurkat cells tested with the DELFIA Cell Proliferation assay. Jurkat cells were seeded at a concentration of 4,000 cells per well in a 384-well plate and incubated in increasing DMSO concentrations for 48 hours at 37 °C with 5% CO₂ atmosphere. BrdU was added during the last 24 hours of DMSO incubation. The incorporated BrdU was determinate as described in the assay protocol. Values represent means of three replicates \pm SD.

Cytotoxicity test

The cytotoxicity of valinomycin was tested on Jurkat cells. Valinomycin is a carrier ionophore. The peptide binds, with high affinity, a single potassium ion into its hydrophilic center and shields the positive charge of the potassium from the surrounding environment. The hydrophobic side chains on the outer structure allow the valinomycin-potassium- complex to diffuse across the cell membrane. Valinomycin disrupts the normal K⁺ gradient and essential transport mechanisms across the cell membrane, to which most cell lines react with apoptosis³.

Jurkat cells (4,000/well) were incubated with increasing concentrations of valinomycin for 48 hours. The IC₅₀ value was calculated using GraphPad® Prism 7 (non-linear regression, dose-inhibition curve, four parameter). The results in Figure 4 show that valinomycin significantly reduced the RFU615 and thus cell proliferation in Jurkat cells, reporting an IC₅₀ of 3.67 nM. Ranges between μM and nM for various human cell lines were reported by Iacobazzi¹. (Table 1) Since IC values are highly dependent on the assay and cell line, variation between values is commonly seen. Nevertheless, valinomycin can be used as a positive control for detection of cytotoxicity in Jurkat cells.

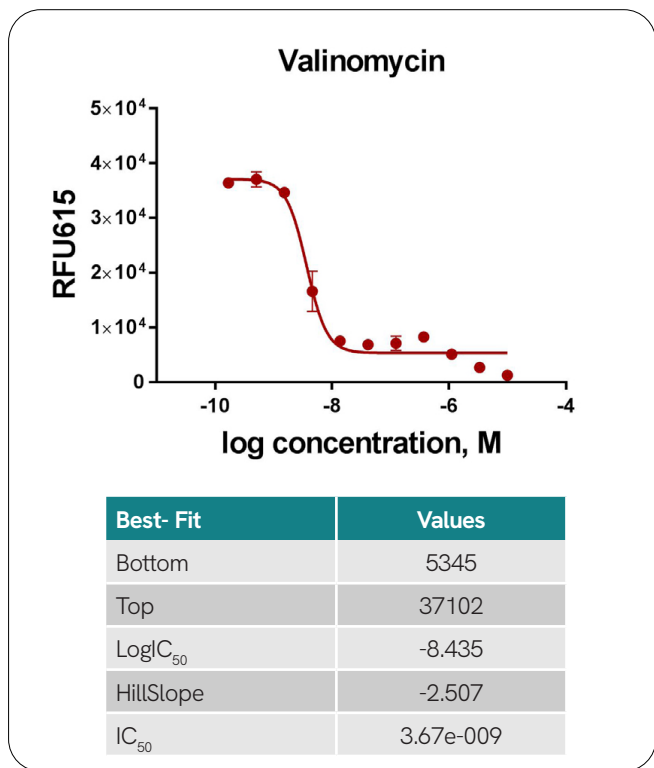


Figure 4. Cytotoxicity tested on Jurkat cells using valinomycin as a reference compound and DELFIA Cell Proliferation assay to determine the IC₅₀ value. Jurkat cells were seeded at a concentration of 4,000 cells per well in a 384-well plate and incubated with increasing compound concentrations (0.1 % v/v DMSO), starting at a top concentration of 10 μM, for 48 hours at 37°C with 5% CO₂. BrdU was added during the last 24 hours of compound incubation. BrdU concentrations were determined as described in the assay protocol. Values represent means of three replicates ±SD

Table 1. IC₅₀ Values against human cell lines¹.

	A2780	MCF-7	HepG2	C6
Valinomycin	2180 nM	1770 nM	0.8 nM	0.4 nM

Z' determination

Valinomycin was selected as a reference compound for inhibition of cell proliferation. DMSO represents the negative reference control that shows maximum 20 % inhibition of cell proliferation. Both reference controls have the same v/v % DMSO. Each control had a total of 16 measurement points. Cells were incubated with 1 μM valinomycin or 0.1 % DMSO v/v for 48 h and BrdU was added during the last 24 hours before measurement. The Z' factor was calculated using the equation⁵:

$$Z' = 1 - \frac{3 \times (SD_{\text{pos control}} + SD_{\text{neg control}})}{|AV_{\text{pos control}} - AV_{\text{neg control}}|}$$

The Z' factor is a parameter to evaluate assay quality and robustness. A good assay for high-throughput screening (HTS) should have a Z' factor ≥ 0.5. The calculated Z' factor was over 0.4 but lower than 0.5 (Figure 5). For a cell based assay a Z' factor over 0.4 is relatively good, considering the use of suspension cells and an incubation time of over 72 hours in the microplate. Evaporation effects during the incubation and depletion of nutrients in the culture medium can cause variation of the signal in the DMSO control. Furthermore, europium contamination can also cause variation. Using an automated dispenser with no plate contact can minimize europium contamination risk and give improved Z' values.

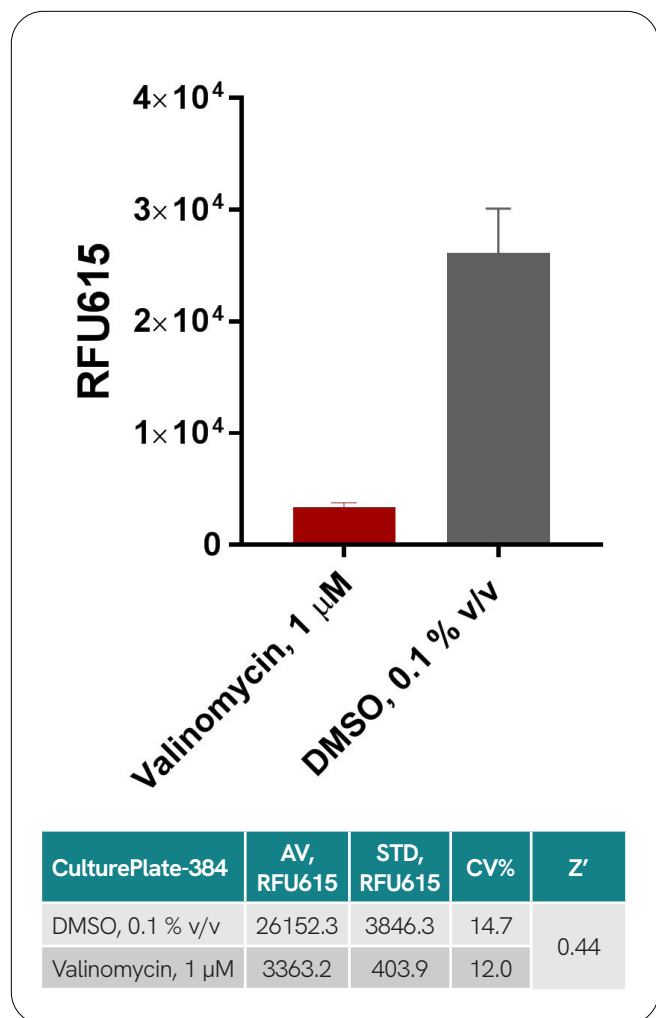


Figure 5. Z' analysis on the DELFIA Cell Proliferation assay using Jurkat cells. Jurkat cells were seeded at a concentration of 4,000 cells per well in a 384-well plate and incubated with 1 µM valinomycin (positive control) and 0.1 % v/v. DMSO (negative control) for 48 hours at 37°C in a humidified 5% CO₂ atmosphere. BrdU was added during the last 24 hours of valinomycin and DMSO incubation. Incorporated BrdU was determined according to the assay procedure.

Conclusion

This application note demonstrates a successful approach to validate the DELFIA cell proliferation assay for measurement of compound related cytotoxicity of a suspension cell line on the VICTOR Nivo Multimode Reader. The sensitive, non-isotopic assay can detect less than 400 cells per well and can be used to study cell functions in both adhered and suspension cell lines, and thus is efficient in early steps of drug discovery.

References

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