TECHNICAL NOTE

Multimode Detection

Performing Kinetic Cellular Assays Using the VICTOR Nivo Equipped with CO_2 Gas Control

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Introduction

In scientific research, it is common practice to perform cellular assays over several hours or days to investigate protein expression or cell proliferation, for example. Best results are obtained if the cells are constantly kept under optimal conditions over the course of the experiment. Next to a constant temperature (e.g. 37 °C), a CO_2 enriched environment is often required for cell growth (e.g. 5% CO_2).

In reality, assay plates are often moved in and out of a cell incubator and transported to a plate reader due to the lack of a low-cost instrument that provides plate measurements in combination with temperature and gas control.

The VICTOR® Nivo™ multimode plate reader is ideal for this purpose: it is the industry's smallest filter-based multimode microplate reader (Figure 1) equipped with all major detection technologies: Absorbance, Luminescence, Fluorescence, plus optional Time-resolved Fluorescence and Fluorescence Polarization or Alpha. Also, an optional dispenser module is available with a dual injector system. Together with the available CO_2 gas control, the VICTOR Nivo allows research environments with a limited budget to run kinetic cellular assays under conditions that are comparable to a standard cell incubator as we demonstrate in this Technical Note.

Figure 1. **VICTOR Nivo equipped with its gas control unit.** The CO_2/O_2-controller (black unit at the front) for VICTOR Nivo is available separately and small enough to be placed directly on top of the instrument even if a dispenser (white unit at the back) is installed.

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Materials and Methods

Cell Culture Conditions

Stably eGFP transfected HEK293 cells were kindly provided by Dr. Kristoffer Riecken (University Medical Center Hamburg-Eppendorf, Germany). The cells were cultivated in EMEM, 10% FCS, 2 mM L-Glutamine, 100 µg/mL Streptomycin, 100 Units/mL Penicillin, 1 µg/L Puromycin. For the regular cell maintenance, DPBS was used to rinse the cells and Trypsin-EDTA (0.05%) in DPBS was used for detachment.

Assay Protocol

For this experiment, a cell suspension was prepared in culture media and eGFP-HEK293 cells were seeded at a concentration of 2000 cells/well (200 µL) in black ViewPlate-96 microplates with clear bottom (PerkinElmer, # 6005182). Column 6 and 7 of the microplate were filled with culture media without cells. Two assay plates were prepared, one to be incubated in a standard cell incubator (plate 1) and another one to be incubated in the VICTOR Nivo (plate 2).

After seeding, the assay plates were both incubated in a standard cell culture incubator at 37 °C and 5% CO_2 overnight to let the cells settle and attach. On the next day, both assay plates were placed in the Operetta CLS™ high-content analysis system (PerkinElmer) and one image per well was taken using eGFP filter settings in order to determine the initial cell confluency. For one experiment, Paclitaxel (dissolved in DMSO) was transferred to selected wells of both assay plates (final assay concentration 125 nM-0.2 nM, triplicates per concentration). This compound is known to have cytostatic effects and was therefore used as a control compound in the experiment[1].

Fluorescence Intensity (excitation 480 nm, emission 530 nm) and Absorbance (570 nm) were detected on assay plate 1, which was afterwards placed in a standard cell incubator for 72 hours. Assay plate 2 was placed in the VICTOR Nivo (37 °C +/- 5% CO_2) and a kinetic measurement (cycle time 1 h, kinetic cycles 73) was started using the same detection parameters as assay plate 1. After 3 days, assay plate 1 was taken out of the standard cell incubator and Fluorescence Intensity and Absorbance were measured again. Finally, both assay plates were placed in the Operetta CLS high-content analysis system and one image per well was recorded (eGFP channel) to determine the final cell confluence.

VICTOR Nivo Measurement Settings

The experiments were performed with VICTOR Nivo control software version 3.0.2 using the following protocol measurement settings:

Operation	Settings
Plate Kinetics	
Kinetic Cycles	73
Cycle Time (s)	1 hours 0 minutes 0.0 seconds
Shake While Waiting	No
Measurement Order	Bi-directional by rows
Fluorescence Intensity	
Measurement Type	Single label
Excitation Filter	480/30nm
Emission Filter	530/30nm
Dichroic Mirror	General (50/50)
Measurement Direction	Bottom measurement
Measurement Time (ms)	100
Z-Focus (mm)	1.5
Excitation Spot Size (mm)	0.5
Emission Spot Size (mm)	1
Flash Energy (mJ)	10

Operation	Settings
Absorbance	
Measurement Type	Single label
Excitation Filter	570/10nm
Measurement Time (ms)	100
Flash Energy (mj)	100
Delay In Kinetics*	
Delay	0 hours 58 minutes 40 seconds

*Delay in Kinetics operation must be added to the protocol for VICTOR Nivo control software version 3.0.2. It can be removed from the protocol if control software versions higher than 3.0.2 are used.

Results

Comparison of pH stability in cell culture medium incubated in the VICTOR Nivo with and without 5% CO_2

A stable pH of the cell culture medium is an important factor to maintain the health of cells and is stabilized by sufficient amounts of CO_2 in the cells' environment. The change in pH can easily be detected by the eye as the color change in cell culture media that is supplemented with the pH indicator Phenolsulfonphthalein ('Phenol red'). For a precise quantification, the change in pH correlates with an increase/decrease in absorbance at 570 nm[2]. This was used to monitor the media pH over the course of 72 hours in two experiments: one microtiter plate (200 µl medium per well) was incubated in the VICTOR Nivo in the presence of 5% CO_2 (gas controller active) and a second one in air only (gas controller inactive). A control microtiter plate was placed in a standard cell incubator (5% CO_2) in both experiments.

As shown in Figure 2, the absence of CO_2 caused a maximal increase of absorbance within the first 5 hours of the experiment. This indicates that the pH in the medium shifted significantly. In a cell-based experiment, this is likely to cause cell stress and will also lead to cell death over longer periods of time (data not shown).

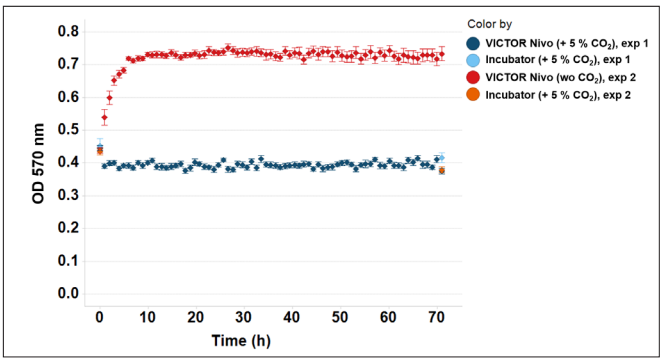

Figure 2. **Comparison of media absorbance at 570 nm ± 5% CO_2.** A microtiter plate was incubated in the VICTOR Nivo in the presence (blue symbols) and absence (red symbols) of 5% CO_2. In both experiments, a microtiter plate was placed in a standard cell incubator (5% CO_2) for comparison. The lack of CO_2 resulted in a maximal increase of absorbance within the first 5 hours of the experiment, indicating that the pH in the medium shifted. For each data point, the mean and standard deviation of 24 wells are shown.

Monitoring Cell Proliferation Over Three Days in VICTOR Nivo

Proliferation of eGFP-HEK293 cells was observed over 3 days using kinetic eGFP detection on the VICTOR Nivo (excitation filter 480/30 nm, emission filter 530/30 nm). Temperature was set to 37 °C and CO_2 to 5%. As shown in Figure 3, an increase in fluorescence intensity was detected over time that correlated with an increased confluence that was observed microscopically on the Operetta CLS high-content analysis system. Importantly, the total increase in fluorescence intensity was comparable to that of cells placed in a standard cell incubator for 72 hours.

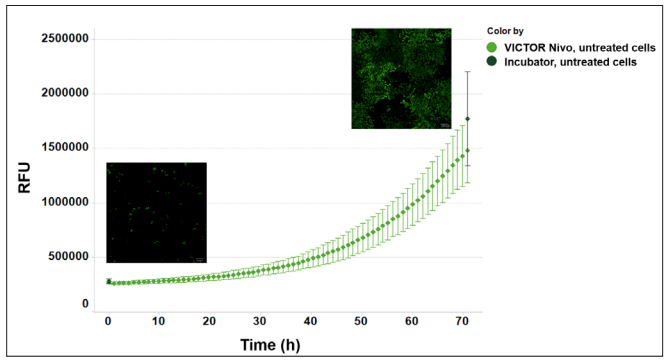

Figure 3. **Cell growth over 72 hours.** eGFP-HEK293 cells were placed for 72 hours in the VICTOR Nivo and the fluorescence intensity using an eGFP filter set was detected kinetically (light green dots). For comparison, a second microtiter plate was placed in a standard cell incubator and fluorescence intensity was detected at the beginning and at the end of the experiment (dark green dots). Over 72 hours, an increase in fluorescence was detected that correlated with an increase in confluence observed microscopically. For each data point, the mean and standard deviation of 24 wells are shown.

Evaporation of the cell culture medium is a known effect for long-term plate incubation in a low humidity environment performed at elevated temperatures. After 3 days of incubation, decreased volumes of cell culture medium were visually observed in the outer wells of the plate that was incubated in the plate reader. This observation correlates with reduced cell proliferation in the outer wells of the plate as shown in Figure 4. These wells were not evaluated and excluded from all experiments. Evaporation was not observed on the assay plate placed in a standard cell incubator with humidified air (95%).

To reduce evaporation effects in the plate's periphery, we recommend not seeding cells in the outer wells. Instead, a well-known procedure is to fill the outer wells with PBS or cell culture medium and to use only the inner wells for the study. Alternatively, also the use of plate seals could potentially be a solution to reduce evaporation, but this needs to be adjusted to the individual experimental conditions.

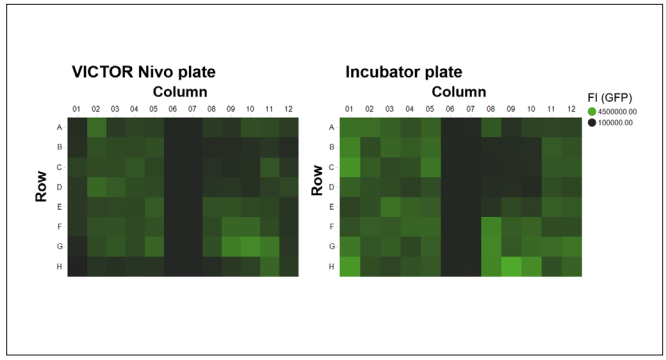

Figure 4. **Evaporation effects.** GFP signal shown after 3 days of incubation in the VICTOR Nivo equipped with CO_2 gas control (left) or a standard cell incubator (right). After incubation in the VICTOR Nivo, evaporation was observed in the outer wells of the plate, thus it is recommended to fill outer wells with PBS or media and to use only the inner wells for the experiment.

Cytotoxicity Assessment

Not only general cell proliferation was investigated but also the cellular response to a known cytotoxic compound was tested. For the experiment, Paclitaxel was selected. EGFP-HEK293 cells were incubated over 3 days with Paclitaxel in a concentration range from 125 nM to 0.2 nM. DMSO was used as a vehicle control. As shown in Figure 5A, high compound concentrations led to an arrest in cell proliferation. Data obtained from the VICTOR Nivo was normalized against the DMSO control and a dose response curve was plotted (Figure 5B). The IC_{50} values derived from the curve fit were comparable between the VICTOR Nivo (IC_{50} = 0.64 nM) and a standard cell incubator (IC_{50} = 0.75 nM).

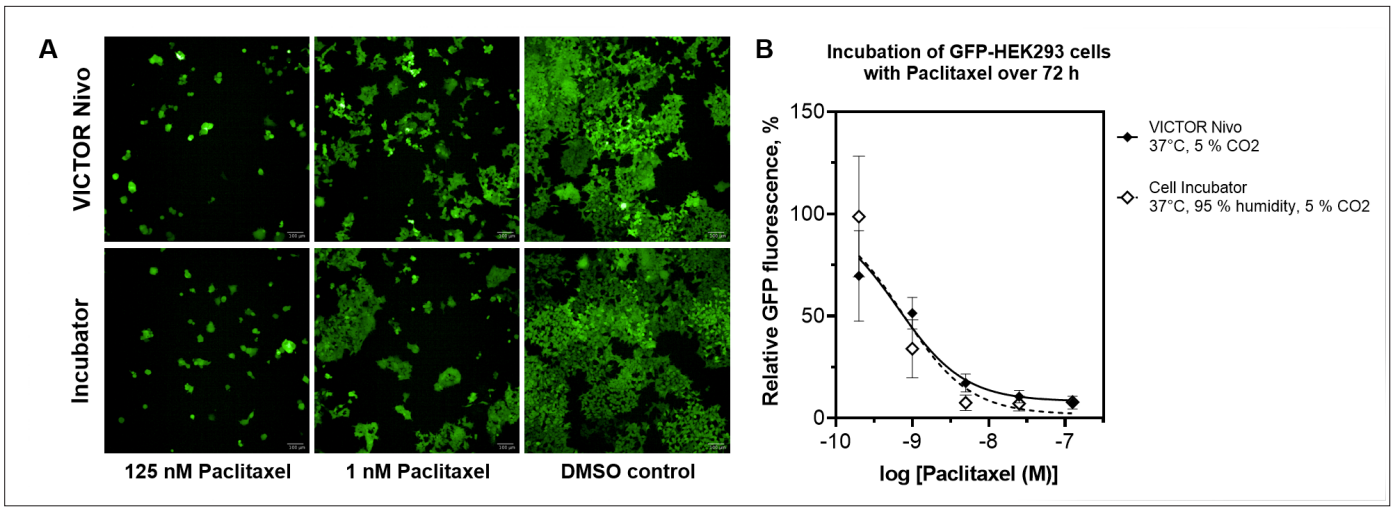

Figure 5. EGFP transfected HEK293 cells after 3 days of incubation with Paclitaxel or DMSO vehicle control (A). Fluorescence Intensity was detected on the VICTOR Nivo plate reader. The data was normalized using the DMSO control data (equals 100 % GFP fluorescence) and plotted against the Paclitaxel concentration (B). The following IC_{50} values were determined from the curve fit: IC_{50} (VICTOR Nivo) = 0.64 nM and IC_{50} (Incubator) = 0.75 nM. For each data point in (B), the mean and standard deviation of triplicates are shown.

Conclusion

We demonstrated that the CO_2 gas control is a valuable add-on to the VICTOR Nivo and provides a low budget and small footprint solution for cell-based assays. With this series of experiments, we showed that the CO_2 gas supply is of major importance for cell growth, especially in long-term kinetic assays. In addition, the lack of humidified air in VICTOR Nivo's measurement chamber was easily compensated by plate layout adjustments and equivalent assay results were achieved. In combination with all major detection technologies being available, the VICTOR Nivo offers the capability to run a variety of common cell-based assays without the need to transfer cell culture plates periodically between plate reader and incubator.

Reference

1. https://www.drugbank.ca/drugs/DB01229
2. https://www.sciencedirect.com/topics/chemistry/phenol-red

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