Analyzing biological processes

Long-term cell-based kinetics using Tecan's GCM™ and the Thermo Scientific Nunc Edge plate

Introduction

The investigation of biological processes requires time-dependent analysis of cellular signals, from several hours up to several days. However, long-term studies with living eukaryotic cells can only be performed for a limited time inside common microplate readers. This technical note describes the capability of Tecan's Infinite® 200 PRO multimode reader to regulate the CO_2 partial pressure inside its measurement chamber. Tecan's new patent pending Gas Control Module (GCM) is an innovative solution for cell-based experiments, offering rigorous environmental control within the detection chamber. Combining the patent pending Gas Control Module with a microplate that minimizes evaporation, such as the Thermo Scientific Nunc Edge plate, enables long-term cell-based kinetic measurements inside the reader, without negatively effecting cell proliferation and viability.
Proliferation and survival of eukaryotic cells is strictly dependent on the environmental temperature and culture medium conditions (1). The pH of cell culture media is typically maintained by a bicarbonate buffer system, using precise control of atmospheric CO_2 partial pressure to stabilize the buffer system. Common cell culture incubators are therefore equipped with sensor-based CO_2 controls, maintaining a defined atmospheric CO_2 level (ranging from 3 to 10 %) to avoid pH shifts which would prevent proliferation or cause cell death (1).

Most currently available microplate readers offer temperature control, but lack the ability to control the CO_2 level inside the measurement chamber. Therefore, long-term studies that require incubation combined with real-time kinetic measurements cannot be performed inside the reader, requiring periodical transfer of the microplate from a CO_2 incubator to the microplate reader for measurement.

Although this is possible with some automated robotic systems, it is not ideal, and manual plate transfer will result in the measurement series being distorted by overnight gaps.

The Infinite 200 PRO GCM (Figure 1) has been developed to allow control of the CO_2 partial pressure within the reader's measurement chamber, from 0 to 10 %. Using this feature in combination with a microplate that is equipped with 'evaporation control' enables long-term cell-based studies with alternating incubation and measurement steps.

The Thermo Scientific Nunc Edge plate (Figure 2) is among the few plates on the market offering a unique design that minimizes evaporation in the 96 wells. This plate is designed with a moat surrounding the microplate wells (2,3). Filling the moat with liquids such as ddH_20 or cell culture buffer minimizes evaporation when handling the plate inside a microplate reader or in a standard cell incubator. Adding a jelling agent such as agarose to the liquid eliminates the risk for spilling the liquid from the reservoir by abrupt manual or automatic plate moving (4).

When using Tecan's GCM in combination with the Nunc™ Edge™ plate, cellular survival and proliferation are no longer limited to common CO_2 incubators. This allows long-term cell-based analysis, such as cell proliferation studies, to be performed inside the reader, offering reproducible measurements without gaps in the data.

Figure 1 Tecan's patent pending Gas Control Module (GCM), compatible with the Infinite 200 PRO series.

Figure 2 ThermoScientific Nunc Edge plate with solid reservoir.

Materials and methods

Instrument

- Infinite M200 PRO Quad4 Monochromators™-based multimode reader, including Gas Control Module (GCM) equipped with CO_2 supply

Microplates

- 96-well Nunc Edge plate, transparent, cell culture treated (Thermo Scientific, USA)

Reagents

- Human squamous epidermoid carcinoma cells (A431, ATCC # CLR-1555)
- Dulbecco's modified Eagle's medium, high glucose (DMEM)
- Heat-inactivated fetal calf serum (FCS)
- Enhanced green fluorescent protein (EGFP)
- L-glutamine
- Sodium pyruvate
- Penicillin / streptomycin
- HEPES
- Trypsin
- EDTA

Unless otherwise stated, all reagents were supplied by PAA Laboratories.

Cell culture and test set-up

Human squamous epidermoid carcinoma cells (A431), stably transfected with EGFP, were grown to confluence in high glucose DMEM supplemented with L-glutamine, sodium pyruvate, penicillin / streptomycin, HEPES and 5 % heat-inactivated fetal calf serum (FCS) at 37 °C and 5 % CO2 in a humidified atmosphere (standard CO2 incubator with passive humidity control, Forma Steri-Cycle 371, Thermo).

The cells were harvested using trypsin / EDTA, then resuspended in fresh growth medium containing 5 % FCS, seeded into a 96-well Nunc Edge plate, cell culture treated (5000 cells / well in 200 µl filling volume) and covered with a standard microplate lid (Figure 3).

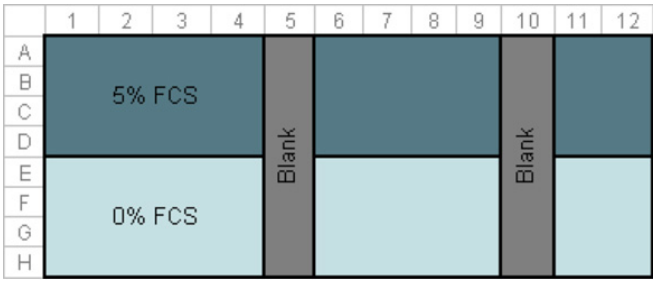

Figure 3 Plate layout; 5000 cells were seeded with and without FCS.

After overnight incubation (~14 hrs) in a standard CO_2 incubator, the culture medium was replaced (rows A-D were filled with fresh DMEM containing 5 % FCS, rows E-H were filled with fresh DMEM without FCS). In addition, the reservoir of each plate was filled with ddH_2O according to the Nunc Edge plate plate recommendations (2-4), in order to avoid evaporation during the long-term growth study.

The influence of the GCM-based CO_2 regulation on cell proliferation was determined using two experimental setups:

I. GCM Plate	
Handling	Plate left in the Infinite M200 PRO reader with active CO_2 control for incubation and measurement
Duration of analysis	75 hrs
CO_2 control (reader)	5 % (GCM controlled)
Temperature (reader)	37 °C
Kinetic (plate wise)	76 cycles (1 hr intervals)
Measurement mode	Enhanced fluorescence intensity bottom, optimal read, 28 flashes (4 x 7)
Excitation wavelength	485 (9) nm
Emission wavelength	535 (20) nm
Gain	Pre-optimized for max. cell number / well (5 x 10^4 cells) and then set manually

Table 1 Handling and measurement parameters for microplate incubation and measurement in the Infinite M200 PRO with GCM.

II. Control Plate	
Handling	Plate left in the Infinite M200 PRO reader with active CO_2 control for incubation and measurement
Duration of analysis	75 hrs
CO_2 control (reader)	N.A.
Temperature (reader)	37 °C
Kinetic (plate wise)	76 cycles (1 hr intervals)
Measurement mode	Enhanced fluorescence intensity bottom, optimal read, 28 flashes (4 x 7)
Excitation wavelength	485 (9) nm
Emission wavelength	535 (20) nm
Gain	Pre-optimized for max. cell number / well (5 x 10^4 cells) and then set manually

Table 2 Handling and measurement parameters for microplate incubation and measurement in the Infinite M200 PRO without GCM.

All plates were measured with 0 µs lag time and 20 µs integration time using the NUN96ft.pdfx plate definition file, and all recorded fluorescence signal intensities were calculated relative to the initial fluorescence signal (initial cell number at t = 0 hrs, representing 100 % GFP signal).

The remaining liquid volume in each well of the Nunc Edge plate was determined by pipetting the liquid into an Eppendorf tube and subsequent weighing.

Results and discussion

Figure 4 shows the evaporation effects in all 96 wells of a Nunc Edge plate after an incubation / measurement period of 75 hours. After such a long time inside the reader with no humidity control some edge effects became visible. However, in the majority of the wells the remaining filling volume was between 180 and 200 µl. Remarkably, all the liquid inside the reservoir was totally drained, indicating that the maximum experimental period had already been reached. However, these results demonstrate that, for a period up to 75 hours, active humidity control is not mandatory inside the microplate reader.

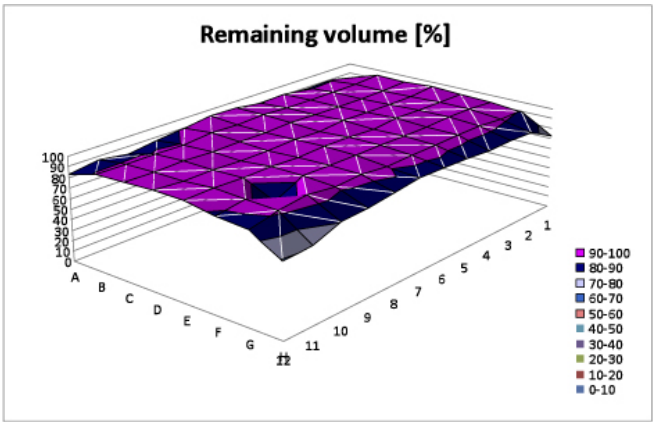

Figure 4 Remaining fill volume in each well of the Nunc Edge plate.

Figure 5 shows growth curves of cells seeded at an initial concentration of 5000 cells / well in DMEM containing 5 % FCS (A) and DMEM without FCS (B), respectively.

Cells incubated with 5 % FCS

Figure 5A shows growth curves of cells cultured in DMEM with 5 % FCS. Cells incubated and measured in an Infinite M200 PRO with CO_2 control (red triangle) show significant proliferation up to 75 hrs (~570 %, ~2.8 x 10^4 cells). Cells incubated and measured in a microplate reader without CO2 control (black circle) show limited proliferation up to 18 hrs (~130 %, ~6.5 x 10^3 cells). After this period, cells stop proliferating and the signal actually decreases down to ~60 % (~3 x 10^3 cells).

Cells incubated without FCS

Figure 5B shows growth curves of cells cultured in DMEM without FCS. This is relevant for many applications where FCS in the medium is disadvantageous. For example, FCS is regularly omitted when incubating cells with a compound that may induce cytotoxicity, as it non-specifically binds to the compound, partly inhibiting its uptake by cells.

As expected, cells grown in medium without FCS show significantly lower overall proliferation rates compared to cells grown in medium containing 5 % FCS. Cell proliferation in the Infinite 200 PRO with GCM was ~325 % (~1.75 x 10^4 cells). Again, cells incubated and measured in a reader without GCM show non-significant proliferation up to 16 hrs (114 %, ~5.7 x 10^3 cells) followed by a decline to ~57 % (~2.85 x 10^3 cells).

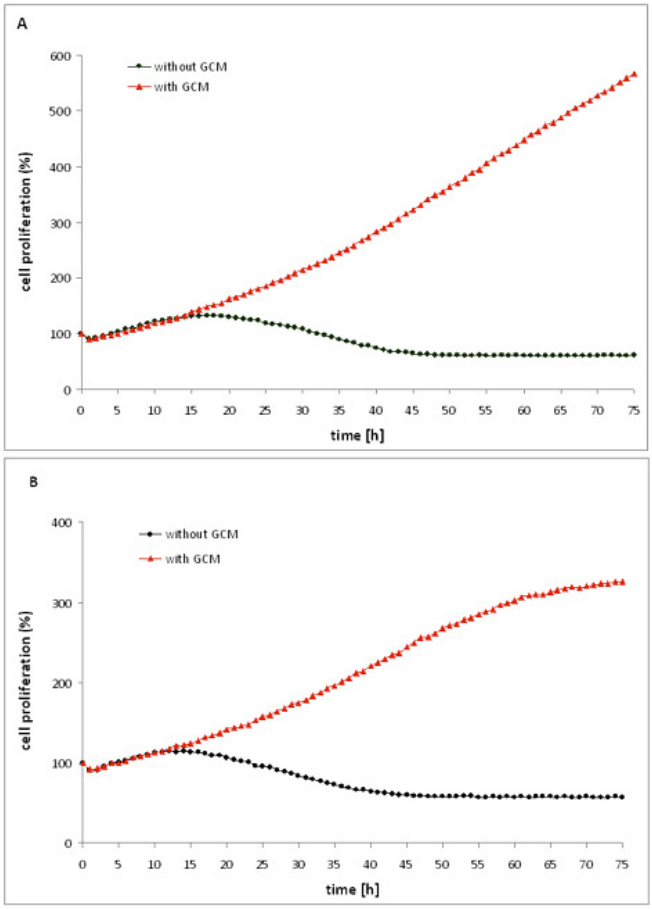

Figure 5 Proliferation of cells cultured A) with and B) without FCS for 75 hrs.

Conclusion

The results presented in this application note clearly demonstrate that the Infinite 200 PRO multimode reader, combined with Tecan's new Gas Control Module and the Thermo Scientific Nunc Edge plate, offers the capability to perform long-term cell-based studies lasting several days. Over the whole 75 hr period, cells left in a reader with GCM proliferate comparably to cells left in a common CO_2 incubator (data not shown), whereas cells left in a reader without CO_2 control stop proliferation after several hours. Growth curves resulting from experiments performed in an Infinite M200 PRO with GCM do not lack any data points (no overnight gaps), which is a significant benefit for many long-term experiments. By offering precise regulation of carbon dioxide levels within the reader, the GCM provides a more stable culture environment over time, making it ideally suited for real-time analysis of biological processes. This integrated gas inlet with external control of CO_2 stabilizes the pH value of the culture medium, helping to improve cell growth.

Literature

1) Martin Clynes, Animal Cell Culture Techniques, Springer Lab Manual, 618 pages, ISBN-10: 3540630082, 1998
2) Brochure, Thermo Scientific Nunc Edge 96-well Plate, Ref.No. BRLSPNUNCEDGE 0610
3) Peter Esser, Thermo Scientific Nunc Edge Plate – Technical Information, Intrawell cell Distribution in Micro Well Edge Plates, Thermo Scientific Application Note, Ref.No. TILSPNUNCEDGEAN 1010
4) Peter Esser, Evaporation from Thermo Scientific Nunc Edge Plate with Solid Reservoir, Thermo Scientific Application Note, Ref.No. TILSPEDGERSVOIR 0411

Abbreviations

A431	Human squamous epithelial carcinoma cells
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GCM	Gas Control Module
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acidBroth

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