

Infinite[®] 200 PRO – Gas Control Module (GCM[™])

Enabling long-term cell-based assays with eukaryotic cells via controlled CO₂ partial pressure inside the reader

Introduction

The investigation of biological processes requires time-dependent analysis of cellular signals, from several hours up to several days. However, performing long-term readouts with living eukaryotic cells inside common microplate readers has time limitations. This technical note describes the capability of Tecan's Infinite 200 PRO multimode reader to regulate the CO₂ partial pressure inside its measurement chamber. The patent pending Gas Control Module (GCM) enables long-term cell-based kinetic measurements inside the reader, without negatively affecting cell proliferation and viability.

Proliferation and survival of eukaryotic cells is strictly dependent on the environmental temperature and on the culture medium conditions (1). The pH of most cell culture media is typically controlled by a bicarbonate buffer system and a precisely controlled atmospheric CO₂ partial pressure that stabilizes the buffer system. Common cell culture incubators are therefore equipped with a sensor-based CO₂

control that maintains a certain atmospheric CO₂ level (ranging from 3 to 10 %), to avoid pH shifts which would prevent proliferation or cause cell death (1).

Most microplate readers currently available offer temperature control but lack the ability to control the CO₂ level inside the measurement chamber. Therefore, long-term studies that require cell incubation combined with in-between measurements (real-time kinetic) cannot be performed inside the reader. Periodic transfer of the microplate from the CO₂ incubator to the microplate reader can present difficulties if performed manually, and may result in the data being distorted by overnight gaps where measurements could not be taken. Automated robotic systems eliminate this problem, but are not available in all laboratories.

The Infinite 200 PRO GCM (Figure 1) has been developed to control the CO₂ partial pressure within the reader's measurement chamber, from 0 to 10 %, enabling long-term

studies with eukaryotic cells using alternating incubation and measurement steps. Cellular survival and proliferation is therefore no longer limited to common CO₂ incubators, and long-term cell-based analysis, such as cell proliferation studies, can 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.

Materials and methods

Instrument

- Infinite M200 PRO Quad4 Monochromators™-based multimode reader, including Gas Control Module (GCM) equipped with CO₂ supply

Microplates

- 96-well, Lumox™, black with transparent bottom, tissue culture treated (Sarstedt, Germany)

Reagents

- Human squamous epidermoid carcinoma cells (A431, ATCC # CLR-1555)
- Dulbecco's Modified Eagle Medium high glucose (DMEM), heat-inactivated fetal calf serum (FCS, PAA Laboratories)
- Enhanced green fluorescent protein (EGFP)
- L-glutamine (PAA Laboratories)
- sodium pyruvate (PAA Laboratories)
- penicillin / streptomycin (PAA Laboratories)
- HEPES (PAA Laboratories)

- Trypsin (PAA Laboratories)
- EDTA (PAA Laboratories)

Cell culture and test set-up

Human squamous epidermoid carcinoma cells (A431), stably transfected with EGFP, were grown to confluence in DMEM high glucose 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 black Lumox, 96-well tissue culture plates (5000 cells / well in 200 µl filling volume) and covered with a standard microplate lid (Figure 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	5% FCS				Blank	5% FCS				Blank	5% FCS	
B	5% FCS					5% FCS					5% FCS	
C	5% FCS					5% FCS					5% FCS	
D	5% FCS					5% FCS					5% FCS	
E	0% FCS				0% FCS				0% FCS		0% FCS	
F	0% FCS				0% FCS				0% FCS		0% FCS	
G	0% FCS				0% FCS				0% FCS		0% FCS	
H	0% FCS				0% FCS				0% FCS		0% FCS	

Figure 2 Plate layout; 5000 cells were seeded with and without FCS.

After overnight incubation (~14 hrs) in a standard CO₂ 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) and all plates were sealed by covering the interspace between plate and lid with Parafilm® (Brand GmbH, Germany) to avoid evaporation during the long-term growth study. Alternatively, plates can be sealed using a real-time PCR film (e.g. ThermalSeal RT2RR™, Excel Scientific, USA). CO₂ supply was still possible, as Lumox plates enable gas transfer through their fluorocarbon film base (2).

The influence of the GCM-based CO₂ regulation on cell proliferation was determined using three different 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. Positive control plate	
Handling	Plate incubated in a standard CO ₂ incubator and manually transferred to the Infinite M200 PRO reader for measurement only
Duration of analysis	75 hrs
CO ₂ control (incubator)	5 %
Temperature (incubator)	37 °C
Measurement interval	Hourly (when possible)
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 in a standard CO₂ incubator and measurement in the Infinite M200 PRO.

III. Negative control plate	
Handling	Plate left in the Infinite M200 PRO reader without CO ₂ control for incubation and measurement (75 hrs)
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 3 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 GRE96fb.pdf file, and all recorded fluorescence signal intensities were calculated relative to the initial fluorescent signal (initial cell number at t = 0 hrs, representing 100 % GFP signal).

Results and discussion

Figure 3 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 3A shows growth curves of cells cultured in DMEM with 5 % FCS. Cells incubated and measured in an Infinite M200 PRO with CO₂ control (green circle) show significant proliferation up to 75 hrs (700 %, ~ 3.5 x 10⁴ cells). This is comparable to the growth of the positive control (red triangle), with 900 % proliferation (~ 4.5 x 10⁴). Growth curves from the positive control experiment lack data points due to overnight breaks, whereas growth curves from the long-term kinetic measurement performed in the Infinite M200 PRO with GCM do not have this critical limitation.

Cells incubated and measured in a microplate reader without CO₂ control (blue square) show limited proliferation up to 18 hrs (140 %, ~7 x 10³ cells). After this period, cells stop proliferating and the signal actually decreases down to 75 % (~ 3.75 x 10³ cells).

Cells incubated without FCS

Figure 3B 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 of the positive control (330 %, 1.65 x 10⁴ cells) was comparable to proliferation of cells incubated and measured in the Infinite 200 PRO with GCM (260 %, 1.3 x 10⁴). Again, cells incubated and measured in a reader without GCM show non-

significant proliferation up to 18 hrs (135 %, $\sim 6.75 \times 10^3$ cells) which then declines to 75 % (3.75×10^3 cells).

Conclusion

Results presented in this technical note clearly demonstrate that the Infinite 200 PRO multimode reader, combined with Tecan's new Gas Control Module, 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 CO₂ incubator, whereas cells left in a reader without CO₂ 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.

Tecan's new patent pending Gas Control Module is an innovative solution for cell-based experiments, offering rigorous environmental control within the detection chamber. 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 CO₂ stabilizes the pH value of the culture medium, helping to improve cell growth.

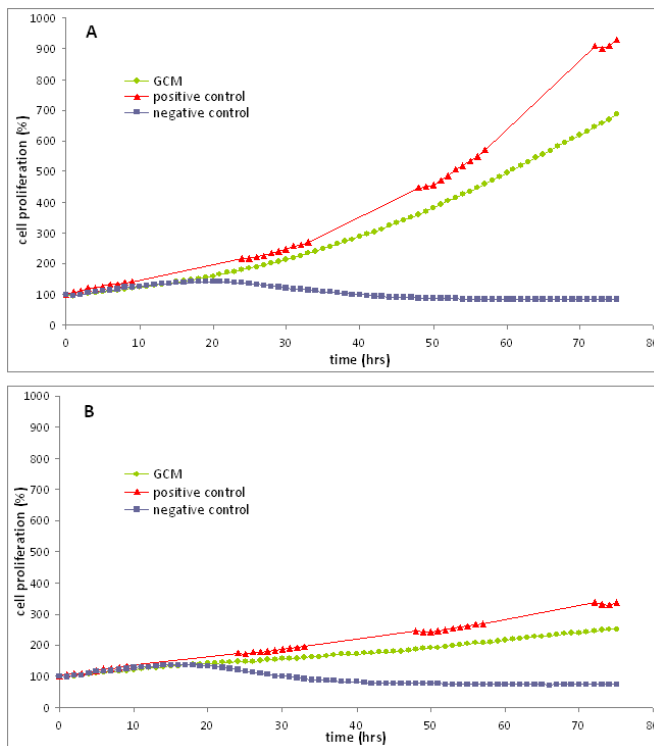


Figure 3 Proliferation of cells cultured with (A) and without FCS (B) and monitored for 75 hrs.

To investigate possible evaporation during long-term incubation, the remaining filling volume was determined. For the plate incubated in the standard CO₂ incubator, which was equipped with passive humidity control, an average volume of 190 μ l (i.e. 95 %) remained per microplate well. For the plates incubated in Infinite M200 PRO readers (with and without GCM) an average volume of 180 μ l (i.e. 90 %) remained after a period of 75 hrs. Similar growth is seen in the reader with GCM compared to the data of the standard cell incubator if sealed plates with gas permeable bottoms are used, demonstrating that humidity control is not mandatory inside the microplate reader.

Literature

- (1) Martin Clynes, *Animal Cell Culture Techniques*, Springer Lab Manual, 618 pages, ISBN-10: 3540630082, 1998
- (2) www.sarstedt.com

List of Abbreviations

A431	Human squamous epithelial carcinoma cells
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GCM	Gas Control Module
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid

Acknowledgements

We would like to express our thanks to Univ.-Doz. Dr. Kristijan Plaetzer and Verena Ziegler, Mag. Rer. Nat. (Division of Physics and Biophysics, Department of Materials Science and Physics, University of Salzburg) for providing the cell cultures and performing the experiments.

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