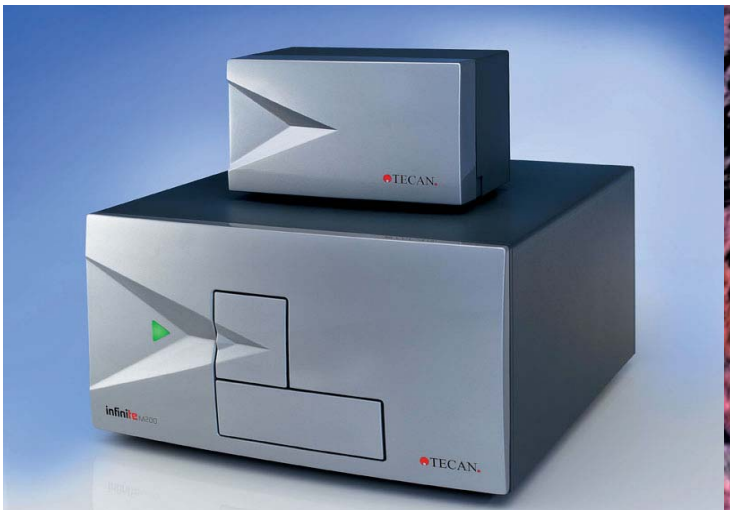
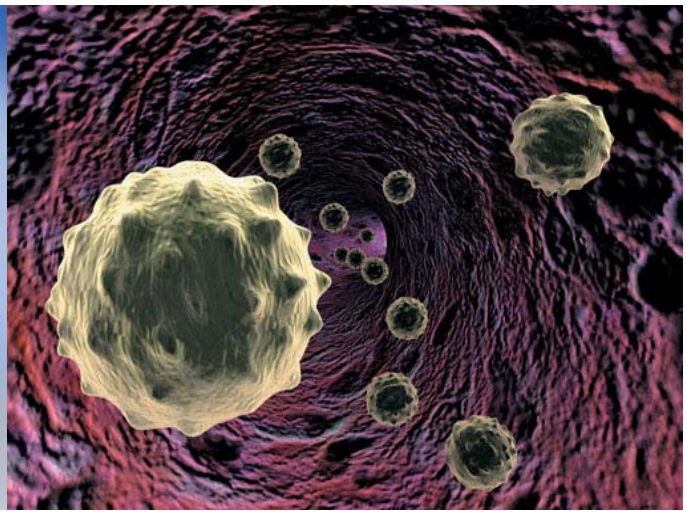
Cell Proliferation and Cell Viability Analysis in *in vitro* Systems

Cell Culture Methods on Tecan´s Infinite® 200





Introduction

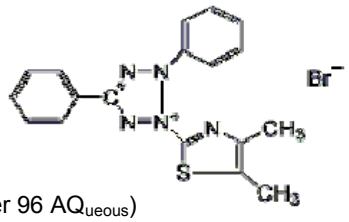
Cell proliferation and viability assays are widely spread across both, academic laboratories as well as in life science and pharmaceutical industry. Assessment of cellular viability markers in cell based applications is mandatory for labs working with *in vitro* systems.

The probably most popular method to assess cellular viability is the **MTT** assay, which is based on the reducing potential of the cell using a colorimetric reaction. In viable cells, cytoplasmatic and mitochondrial enzymes (e.g. succinate dehydrogenase) reduce the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to an insoluble, purple formazan product. A solubilization reagent (usually either dimethyl sulfoxide (DMSO) or isopropanol) is added to dissolve the non-water-soluble formazan product yielding a colored solution that can be quantified by measuring the absorbance at 565 nm [1]. However, it has been reported that the generated formazan may impair MTT assay-based viability analyses because it may have a cytotoxic effect itself, thus producing false-positive results [2].

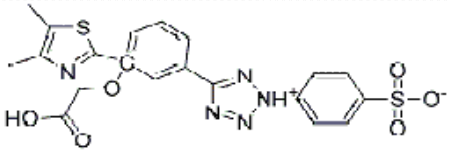
MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is an alternative to MTT. In the presence of phenazine methosulfate (PMS), MTS produces a water-soluble formazan product with an absorbance maximum at 490 nm.

The MTS assay is advantageous over MTT as its reagents are reduced more efficiently than MTT within the cell, and because the resulting product is water-soluble and less cytotoxic than the insoluble formazan used in the MTT assay [2]. The MTS assay system is commercially available from Promega Corp. under the brand name *CellTiter 96® AQueous One Solution Cell Proliferation Assay* [3].

MTT



MTS (CellTiter 96 AQ_{ueous})



Resazurin (CellTiterBlue)

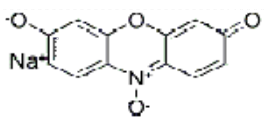


Figure 1: Molecular structures of MTT, MTS and resazurin [6].

A similar redox-based assay has been developed using the fluorescent dye **resazurin** (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide). Resazurin 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; 570 nm_{Ex}; 590 nm_{Em}). This system is specific for cell viability as nonviable cells rapidly lose metabolic capacity and do not reduce resazurin, and thus do not generate a fluorescent signal. Resazurin was initially used for bacterial studies [4], but is now available under several brand names (e.g. AlamarBlue® assay, Resazurin Fluorometric Cell Viability Assay Kit, etc.) also for eukaryotic cell-based applications [5]. Promega provides this technology under the brand name *CellTiter-Blue® Cell Viability Assay* [3].

In this Application Note the three assay systems introduced above (i.e. MTT assay, CellTiter96 AQ_{ueous} One Solution Cell Proliferation assay (MTS) and CellTiter-Blue Cell Viability Assay (resazurin) are compared using the human cholangiocarcinoma cell line CCLP-1.

The compatibility of these assay systems with Tecan´s Infinite M200 Quad4 Monochromators™-based multimode reader is demonstrated.

Part I – cell dilution series:
In a first set of experiments a cell dilution series was prepared and measured, using the three different assay systems. Theoretical detection limits (cells per well) were calculated for each dye.

Part II – PKI dilution series:
In a second step cells were treated with varying concentrations of a commercially available protein kinase inhibitor (PKI). Loss in cell viability was assessed using MTT, MTS or resazurin.

All data shown in this document are exemplified measurement data and do not represent specification values

Material and methods

Instrument

- Infinite M200 equipped with Tecan's Quad4 Monochromators (Tecan Austria, Austria) including an absorbance and an FI-bottom module.

Microplates

- Greiner® 384 well black with transparent bottom, µClear; medium volume 50 µl (Greiner Bio-One, Germany)

Reagents

- DMEM (PAA Laboratories, Austria)
- DPBS (Sigma Aldrich, Austria)
- PKI (Sigma Aldrich, Austria)
- FBS (PAA Laboratories, Austria)
- MTT (Sigma Aldrich, Austria)
- CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA)
- CellTiter-Blue Cell Viability Assay (Promega, USA)

Sample Preparation

Part I – cell dilution series:
Human cholangiocarcinoma cells (CCLP-1) were kindly provided by Prof. Dr. F. Berr / Dr. T. Kiesslich (Paracelsus Medical University and SALK, Department of Internal Medicine I) and cultured in standard medium (DMEM) supplemented with L-Glutamine, Na-Pyruvate, Pen/Strep, HEPES and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in an atmosphere of 7.5% CO_2 [7]. A 22-point cell dilution series was prepared in a 384 well plate with 50 µl medium volume per well using the following plate layout:

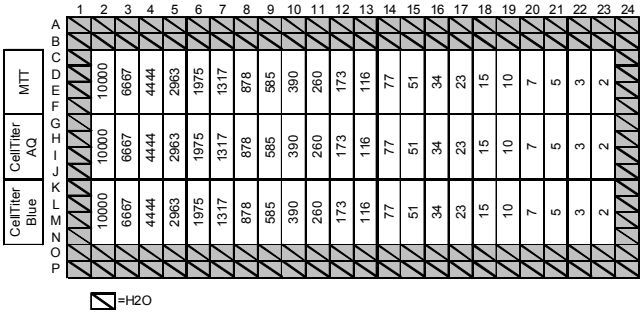


Figure 2: Plate layout for *Part I – cell dilution series*

Cells were cultivated over night before starting the assay procedure.

MTT assay protocol:
- 10 µl of a 2.5 mg/ml MTT solution was added to each well.
- Cells were incubated for 45 min at 7.5% CO_2 and 37°C.
- The supernatant was aspirated and the resulting formazan was dissolved in 50 µl/well isopropanol.
- The microplate was measured using the settings summarized in table 1.

CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay protocol:

- 10 µl of CellTiter AQ was added to each well as described in the assay package insert [3].
- Cells were incubated for 3 h at 37°C and 7.5% CO_2.
- The microplate was measured using the settings below.

CellTiter-Blue Cell Viability Assay protocol:

- 10 µl of CellTiter Blue was added as described in the assay package insert [3].
- Cells were incubated for 3 h at 37°C and 7.5% CO_2.
- The microplate was measured using the settings below.

For CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay and CellTiter-Blue Cell Viability Assay, the 3 h incubation period was done a) in a common CO_2 incubator for cell cultures or b) in the Infinite M200 using the heating function at 37°C. This was done to verify the temperature function of the Infinite M200 in the context of assay incubation.

Part II – PKI dilution series:

3240 CCLP-1 cells per well were seeded into a 384 well plate and allowed to adhere over night. The supernatant was then removed and 50 µl DMEM containing 0% FBS and varying concentrations of the PKI (µM) was pipetted into each well (figure 3) and incubated over night at 37°C and 7.5% CO_2.

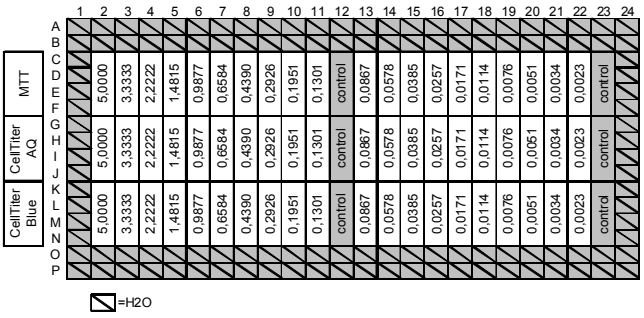


Figure 3: Plate layout for *Part II – PKI dilution series:*

Measurement settings

Measurement parameter	Instrument settings
Plates	[GRE384fb.pdfx]
Shaking (prior to read)	180 sec; 1 mm amplitude; orbital
Mode	Absorbance
Wavelength	565 nm
Bandwidth	9 nm
Number of flashes	25
Settle time	0 ms

Table 1: Measurement parameter and instrument settings for MTT assay on Infinite M200.

Measurement parameter	Instrument settings
Plates	[GRE384fb.pdfx]
Shaking (prior to read)	20 sec; 1 mm amplitude; orbital
Mode	Absorbance
Wavelength	490 nm
Bandwidth	9 nm
Number of flashes	25
Settle time	0 ms

Table 2: Measurement parameter and instrument settings for CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay.

Measurement parameter	Instrument settings
Plates	[GRE384fb.pdfx]
Shaking (prior to read)	20 sec; 1 mm amplitude; orbital
Mode	Fluorescence intensity bottom
Excitation wavelength	560 nm
Excitation bandwidth	9 nm
Emission wavelength	590 nm
Emission bandwidth	20 nm
Gain	Optimal
Number of flashes	25
Integration time	20 µs
Lag time	0 µs
Settle time	0 ms

Table 3: Measurement parameter and instrument settings for CellTiter-Blue Cell Viability Assay.

Data Analysis

Part I – cell dilution series:

The quality of the raw data was evaluated by performing the Grubbs test for outlier removal. For each dilution the average was calculated and corrected by subtracting the average blank. The respective error bars were calculated using the Gaussian law of error propagation [8]. Values represent corrected means from four separate wells. The theoretical detection limit for the MTT assay and the CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay was calculated by using the following equation:

$$\text{Sensitivity (OD)} = 3 * \text{SD (blank)} + \text{average (blank)}$$

The resulting OD value was used to calculate the corresponding concentration (cell/well) by using the equation for the respective curve-trendline (y=kx+d). The theoretical detection limit for the CellTiter-Blue Cell Viability Assay was calculated by using the following equation:

$$\text{Sensitivity (cells/well)} = \frac{3 * \text{SD (blank)} * 10000}{\text{(average sample – average blank)}}$$

For the average sample value the result for the first dilution of the series (10000 cells/well) was used.

Part II – PKI dilution series:
The quality of the raw data was evaluated by performing the Grubbs test for outlier removal. For each dilution the average was calculated and corrected by subtracting the average blank. This value was then related to the average of the untreated control samples. The respective error bars were calculated using the Gaussian law of error propagation [8]. Values represent corrected means from four separate wells.

Results and Discussion

Part I – cell dilution series:

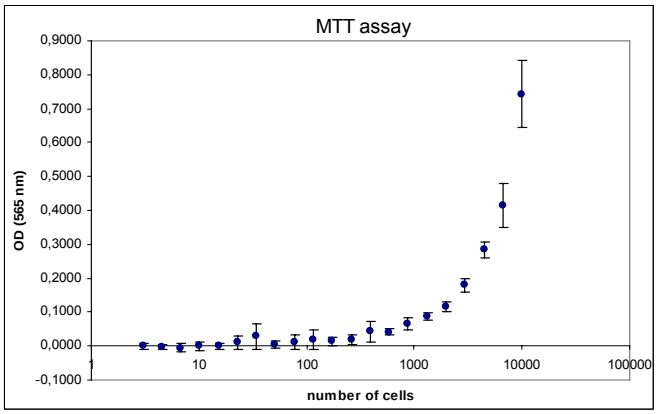


Figure 4: Cell dilution series – MTT assay.

Figure 4 shows the cell dilution curve measured with the MTT assay. The theoretical detection limit was calculated to be 475 cells / well.

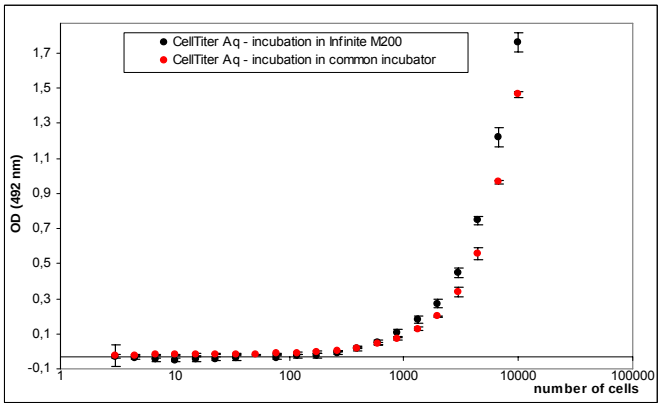


Figure 5: Cell dilution series – CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay.

The dilution curve generated with MTS-based CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay shows smaller errors especially at low concentrations and therefore, the detection limit is significantly lower than with the MTT assay. Performing the 3 h assay incubation within a common incubator results in a detection limit of approximately 143 cells/well, whereas incubation in the heated Infinite M200 results in a slightly lower sensitivity of 250 cell/well.

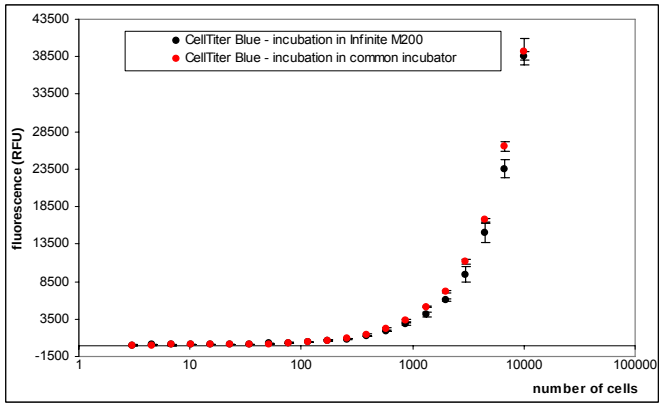


Figure 6: Cell dilution series – CellTiter-Blue Cell Viability Assay.

The resazurin-based assay CellTiter-Blue Cell Viability Assay is by far the most sensitive method tested in this study. Standard deviations are only marginal and the resulting detection limit is exceptionally low, i.e. 14 cells/well when incubating in a common incubator. Assay incubation in the reader results in a detection limit of 44 cells/well which is unsignificantly higher than in the incubator.

Part II – PKI dilution series:
To evaluate the performance of the three different viability assays in the context of cytotoxicity in CCLP-1 cells, varying concentrations of a protein kinase inhibitor were added 20 h prior to assay start. Previous preliminary experiments revealed that this substance induces cytotoxicity in CCLP-1 cells due to inhibition of a major signal transduction pathway (manuscript in preparation, Kiesslich / Berr et al.).

Data presented in Figure 7 clearly demonstrate the cytotoxic effect of the PKI on CCLP-1 cells. Depending on the assay system the lowest effective concentration differs slightly: The lowest effective concentration (92.86% of untreated control – 7.14% of the cells are affected) detected with the MTT assay is 0.658 µM PKI, whereas with the CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay, the same effect can be already detected with approximately 0.44 µM PKI. For both assays the maximal cytotoxic effect induced by 5 µM PKI ranges about 34% of untreated control (66% of the cells are affected).

Very similar results are obtained when using the CellTiter-Blue Cell Viability Assay. Again, the lowest concentration of effect is app. 0.44 µM PKI, and maximal cytotoxicity, achieved with 5 µM PKI, is approximately 30% of untreated control cells.

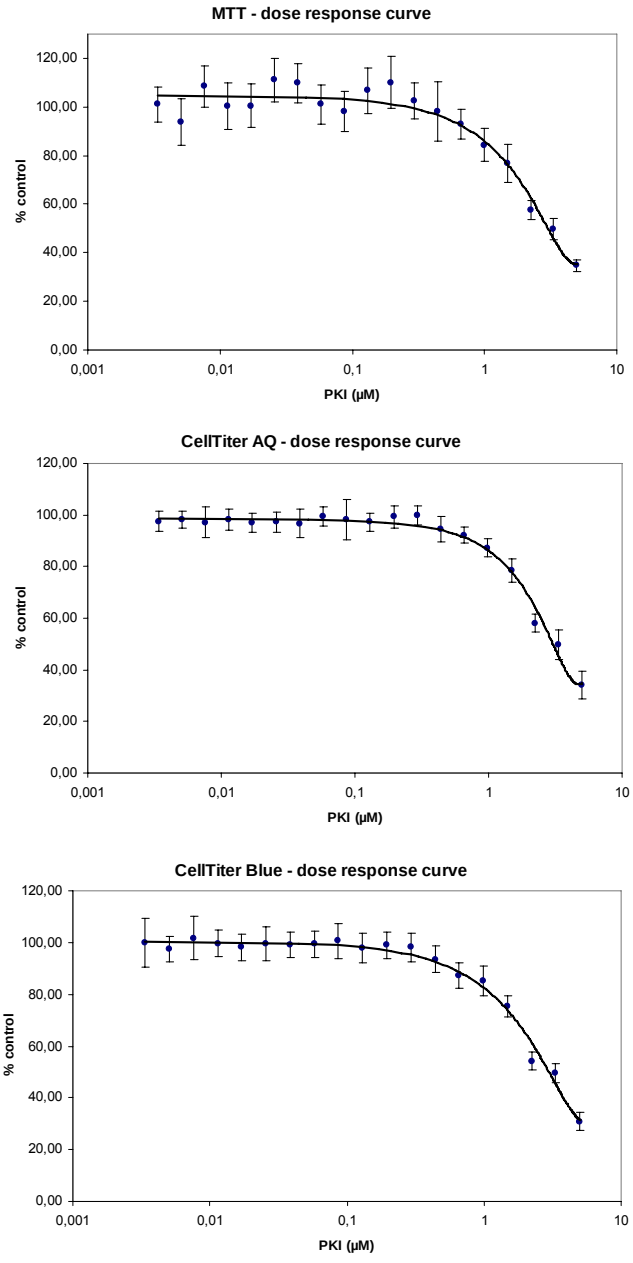


Figure 7: PKI dose response curves.

Conclusion

Cell proliferation and viability assays are commonly used to assess cell number and cytotoxic effects. In this study the performance of the Infinite M200 with respect to the MTT assay, CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay and CellTiter-Blue Cell Viability Assay, respectively, was evaluated.

The acquired data reveal that the Infinite M200 is the ideal instrument to work with such assay systems. Besides excellent sensitivity and uniformity, the temperature function of the Infinite M200 even allows for performing the whole assay inside the reader, without any loss in data quality.

The CellTiter-Blue Cell Viability Assay by Promega was by far the most sensitive method used in this study, followed by the CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay. Even though the MTT assay exhibited several drawbacks in comparison with the other two methods, it still represents a satisfactory solution for the determination of cell number and cytoxicity in the Infinite M200 multimode reader.

Literature

[1] Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods* 65 (1-2): 55–63.

[2] Cory AH, Owen TC, Barltrop JA, Cory JG (1991). Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer communications* 3 (7): 207–12.

[3] www.promega.com

[4] Hallinan FJ. (1943). Pyrex Suspensions in Turbidimetric and Colorimetric Determinations-Standards of Comparison for Bacterial Suspensions and in the Resazurin Test. *Am J Public Health Nations Health* 33(2):137-40.

[5] Ahmed SA, Gogal RM Jr, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J Immunol Methods*. 1994 Apr 15;170(2):211-24.

[6] www.chemicalbook.com

[7] Kiesslich T, Alinger B, Wolkersdörfer G, Neureiter D, Berr F: Abstract at the 33rd European-Society-for-Medical-Oncology Congress: Developmental Signalling Pathways (Hedgehog, Wnt, Notch) in Human Cholangiocarcinoma Cell Lines: Expression Analysis and Pharmacological Inhibition. Stockholm, Sweden, 12-16 September, 2008. Ann Oncol 2008; 19(S8): 53

[8] Oberdanner et al. (2005). Photodynamic treatment with fractionated light decreases production of reactive oxygen species and cytotoxicity in vitro via regeneration of glutathione. Photochem. Photobiol. 81(3), 609-613

List of Abbreviations

CCLP-1	human cholangiocarcinoma cell line
CK-2	casein kinase 2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FBS	fetal bovine serum
HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonsäure
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium),
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PKI	protein kinase inhibitor
PMS	phenazine methosulfate

Acknowledgements

Our acknowledgments are kindly expressed to Univ.-Prof. Dr. Frieder Berr (Paracelsus Medical University and SALK, Department of Internal Medicine I) and Dr. Tobias Kiesslich (Paracelsus Medical University and SALK, Department of Internal Medicine I) for collaboration in providing the cell cultures and performing liquid handling.

Groedig, Austria, July 2009

www.tecan.com, info@tecan.com

Austria +43 62 46 89 33 **Belgium** +32 15 42 13 19 **China** +86 10 5869 5936 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
Italy +39 02 92 44 790 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 174 **Portugal** +351 21 000 82 16 **Singapore** +65 644 41 886
Spain +34 93 490 01 74 **Sweden** +46 31 75 44 000 **Switzerland** +41 44 922 89 22 **UK** +44 118 9300 300 **USA** +1 919 361 5200
Other countries +41 44 922 8125

396 065 V.1.0, 08-2009