Cell viability assays with Mithras LB 940

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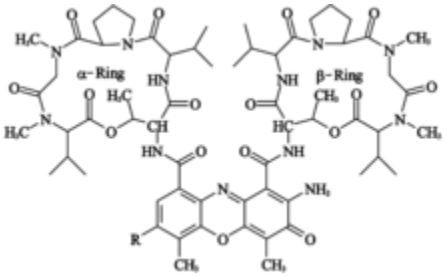
Introduction

Actinomycin D (picture 1) is a chemical compound with the formula $C_{62}H_{86}N_{12}O_{16}$ and molecular weight of 1255.41 g/mol. It is used as an anti-mitotic and an intercalating agent of DNA. It inhibits the synthesis of mRNA by intercalation into the DNA strand and thereby preventing the polymerase activity.

In this study we tested the effects of two concentrations of Actinomycin D on CHO cells. The cellular viability of those cells was assessed by different cell viability agents/assay kits like Alamar Blue (Invitrogen), UptiBlue (Interchim) and Resazurin (Interchim).



Picture 1 : Actinomycin D

The assays are easy-to-use as no cell extraction or lysis steps are required in contrast to other commonly used cell proliferation assays. All indicators are non-toxic and water soluble thus eliminating washing/fixing steps.

The systems are based on a dye (Resazurin) which is highly sensitive to the redox potential of cell growth. The dye changes by responding to the chemical reduction of growth medium from the oxidized (non-fluorescent, blue) to the reduced (fluorescent, red) form.

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The Mithras LB 940 is a multimode plate reader with an unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implied. These are

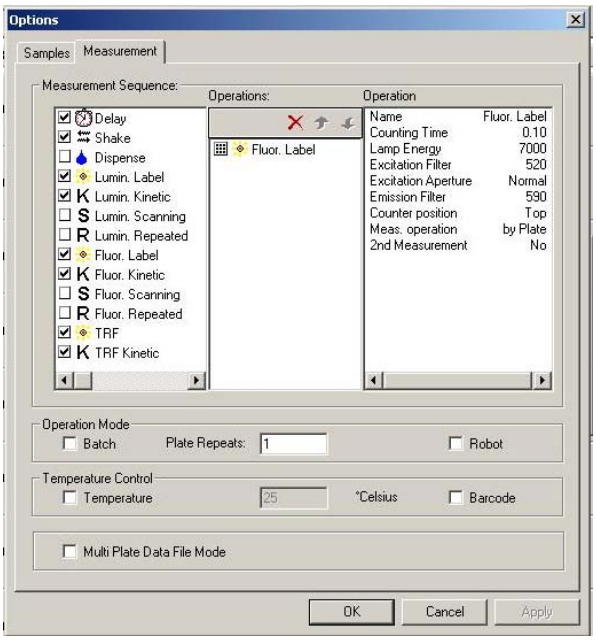
- luminescence
- BRET/BRET²
- fluorescence
- UV/VIS absorbance
- fluorescence polarization
- AlphaScreen®
- TRF
- HTRF®

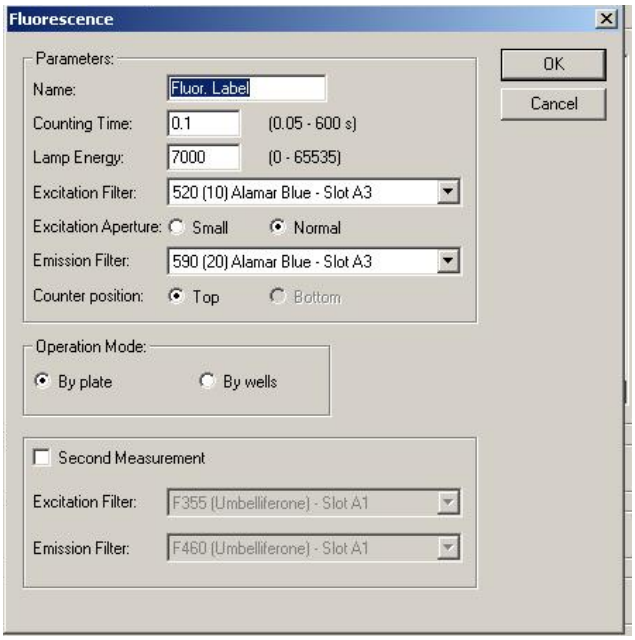
Picture 2: Mithras LB 940 multimode reader

In addition accessory options, e.g. reagent injectors, temperature control and cooled PMT detection units are available.

Instrument Settings

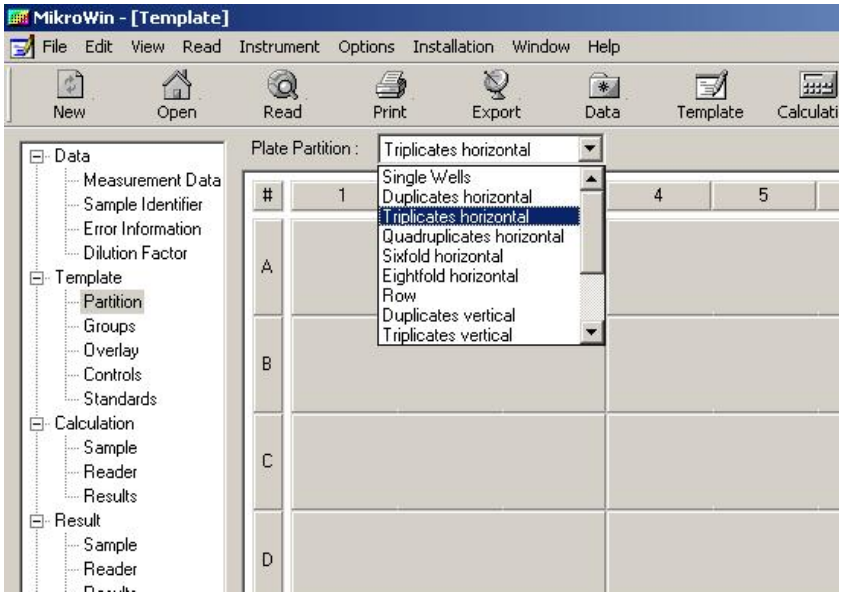
Following settings in MikroWin 2000 were used:



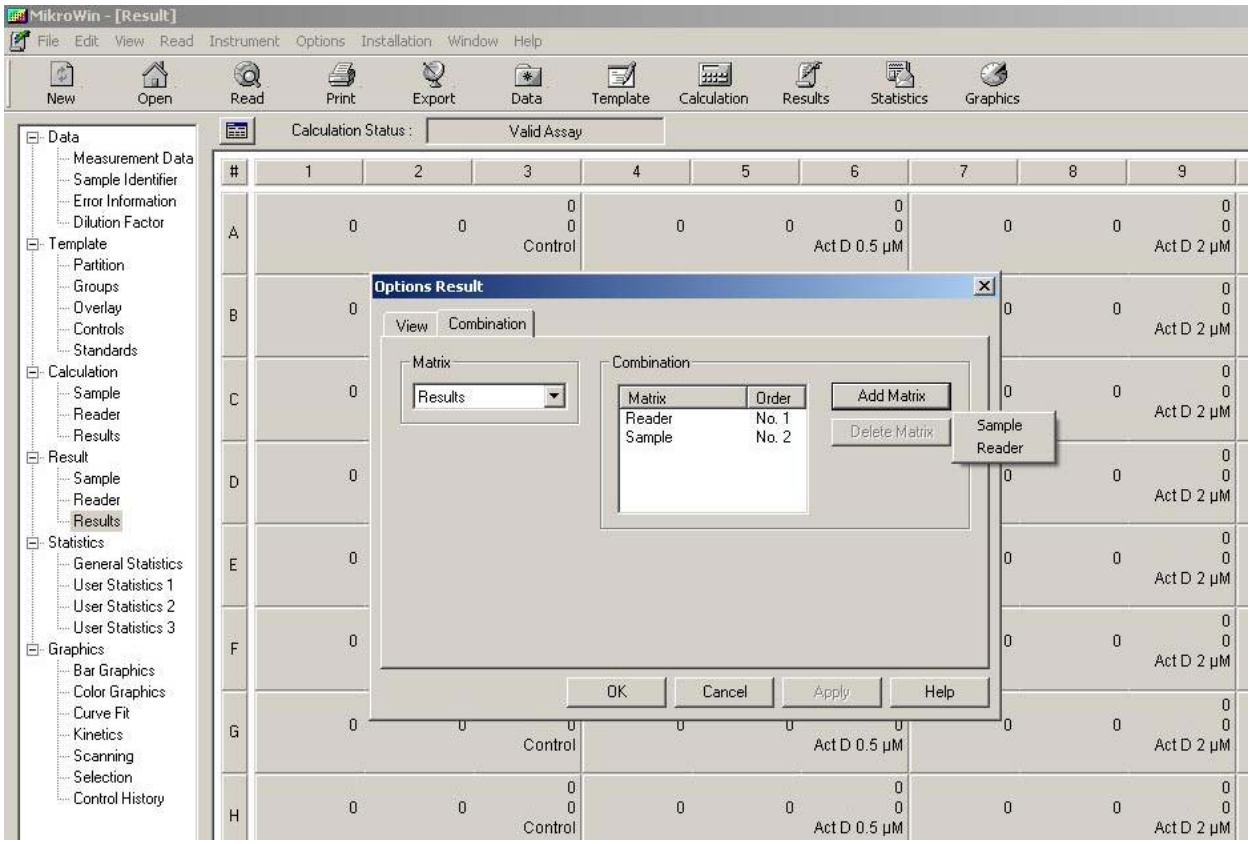


Alternatively a filter combination of 530/10nm for excitation and 600/10nm for emission can be used as well.

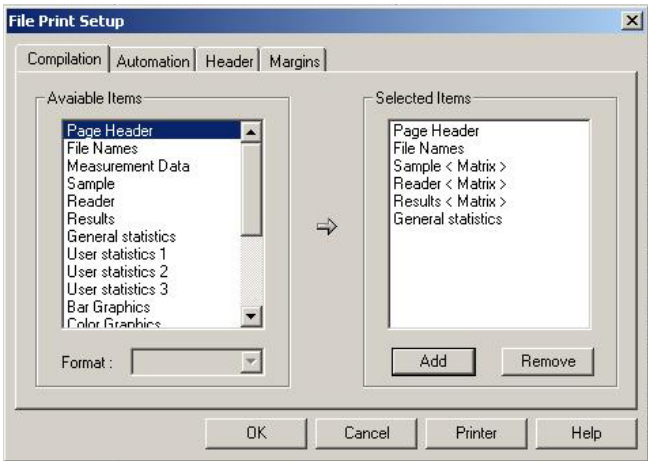
Triplicates were selected in the Plate Partition part:



Under Options/Results both the “Reader” and “Sample” matrix were added to the “Results” matrix to get the sample (name) information, single well data and average of the three wells displayed into a single screen:



The print setup was set as the following:



Measurement file : upti 120908 3h.dat — Valid Assay — Measurement date : 12/09/2008
Template file : UptiBlue Test Berthold.par — Measurement time : 13:44:15

Echantillons / Données brutes / Moyenne

	1	2	3	4	5	6	7	8	9	10	11	12
A	41160,0	38220,0	Blank control well1 38310,0 39230,0		40660,0	38380,0	Blank Act D 0.5µM well1 37440,0 38826,7		39940,0	37210,0	Blank Act D 2µM well 1 37270,0 38140,0	
B	37960,0	36620,0	Blank control well2 36610,0 37063,3		41700,0	38380,0	Blank Act D 0.5µM well2 38230,0 39436,7		39910,0	39290,0	Blank Act D 2µM well 2 37350,0 38850,0	
C	712680	690880	control well1 682510 695357		479500	448850	Act D 0.5µM well1 447000 458450		509710	482670	Act D 2µM well 1 490530 494303	
D	1,04E6	995900	control well2 997280 1,01E6		425400	393080	Act D 0.5µM well2 398810 405763		414950	379490	Act D 2µM well 2 382760 392400	
E	799860	736130	control well3 739650 758547		504740	469000	Act D 0.5µM well3 471390 481710		474460	450620	Act D 2µM well 3 445920 457000	
F	743230	749960	control well4 743980 745723		433490	410340	Act D 0.5µM well4 408300 417377		425530	401140	Act D 2µM well 4 400410 409027	
G	702100	704980	control well5 703440 703507		512470	475570	Act D 0.5µM well5 478840 488960		402190	366120	Act D 2µM well 5 367700 378670	
H	726930	727620	control well6 731460 728670		413690	384800	Act D 0.5µM well6 383600 394030		420880	387340	Act D 2µM well 6 391810 400010	

General Data

Measurement Files
EndPoint, Time of Measurement : 12/09/2008 / 13:44:15, State of Measurement : Valid Measurement
Parameter loaded during measurement time : c:\program files\mikrowin 2000\paramithras\fluoresc
Data file : upti 120908 3h - 12/09/2008 / 12:44:48
Template file : UptiBlue Test Berthold - 05/09/2008 / 10:45:20

Reader
BertholdTech Mithras, Driver Version: 1.05 , (1.0.5.0), S/N: 33-6002, Embedded Version: 1.17
Plate Type: 8x12 plate
Name ..... Fluor. Label
Counting Time [s] ..... 0.10 — Measurement Mode ..... by Plate
Excitation Filter ..... 520 — Emission Filter 590
Excitation Aperture ..... Normal
Lamp Energy ..... 7000 — Counter Position ..... Top
Second Measurement ..... No

Calculation Program
Calculation Status : Valid Assay, Calculation Time : 16/12/2008 / 09:32:44
MikroWin, Version 4.40; License No. : 2997; Assembly Code : 0066 0001 FFFB FFBF
Operating System : Windows 2000 Professional; User Name : Toxicologie; Printer Name : HP Laser.

	1	2	3
A	41160,0	Blank control well1 38220,0	38310,0 39230,0

→ Sample name
→ Three readings in triplicate
→ Average of the three readings

Methods

CHO cells were grown in IMDM medium (Invitrogen) supplemented with 10 % FCS (Fetal Calf Serum, Invitrogen), HT supplement (SIGMA) and penicillin/streptomycin (Invitrogen). The cells were cultivated in 75 cm² flasks (Falcon) in a humid atmosphere incubator at 37 °C under 5 % CO_2 to produce enough cells and then, after trypsination, seeded in 6 wells plates (Falcon) to perform the experiments. The medium was changed every 2 days until testing process.

Actinomycin D was prepared in IMDM medium and used at two concentrations (0.5 and 2 µM) versus control (IMDM medium alone).

Alamar Blue (Invitrogen), UptiBlue (Interchim) or Resazurin (Interchim) was added each 150 µL to the 6 well plates. The fluorescence readings were performed in 96 well plates to avoid toxic effects and less oxygenation. A volume of 100 µL was transferred from the 6 well plates to the 96 well black plates (Nunc) to perform the readings with the Mithras LB 940 (Berthold Technologies).

As indicated in the kit instructions, the incubation time had to be evaluated according to the cell type. The incubation of CHO cells with UptiBlue and Resazurin was done then for 3 hours.

Cell density determination

First, cell density experiments were performed to determine the optimum cell numbers.

Four 6 wells plates were seeded with different cell densities: 0, 50 000, 100 000, 300 000 and 500 000 cells per well in 1.5 mL IMDM medium. After 2 days the cell culture medium was discarded, and the cellular monolayer was washed with 1 mL Ca^{2+}/Mg^{2+} free PBS solution. Finally, 1.5 mL IMDM fresh medium and 150 µL UptiBlue, Alamar Blue or Resazurin solution (dilution 1/10e) were added to each well according to the manufacturer's instructions. Cells were kept in a humid atmosphere incubator at 37 °C and 5 % CO_2 for 3 hours. At the end of incubation, 100 µL medium was taken in duplicate from each well and transferred into 96 well black plates for reading.

The readings were performed with Mithras LB 940 multimode reader and Mikrowin 2000 software. A fluorescence parameter file with an excitation wavelength at 520 nm and an

emission wavelength at 590 nm and a counting time of 0.1 s per well was used as described before in instrument settings.

Cytotoxicity Assay

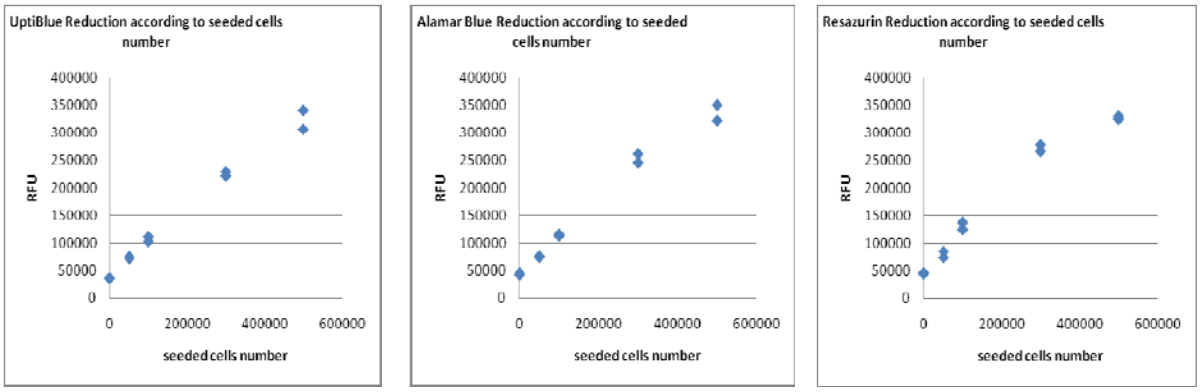
The cytotoxicity assay was performed in 6 well plates with 100 000 cells per well with the same medium and volume as used before. After 4 days cell culture, the medium was removed and each well was replaced with fresh medium containing either Actinomycin D at 0.5µM and 2µM or fresh medium without any Actinomycin D (control). Plates containing all reagents but no cells were used as a negative control to determine the potential of non-cell-related reduction of UptiBlue, Resazurin or Alamar Blue over the experimental period. After 21 hours incubation 150 µL Alamar Blue, Resazurin or UptiBlue solution was added to each well after medium removal procedure or not.

For medium removal procedure fresh medium (without any Actinomycin D) was added to each well after elimination of the old medium by aspiration. The 150 µL fluorophore solution was added into this fresh medium. Without medium removal procedure the fluorophore solution was added directly into the old medium.

Then, incubation was continued for 3 hours under the same conditions. At the end of incubation, the same reading protocol and transfer to 96 well plates was performed as used for the cell density determination.

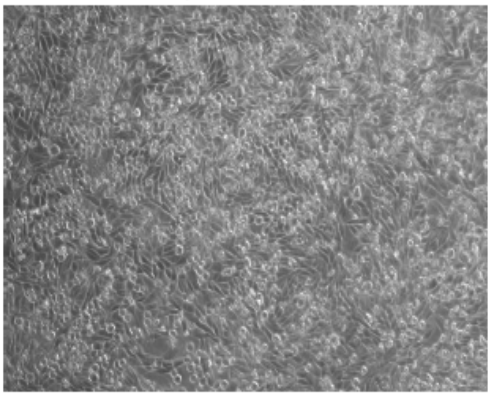
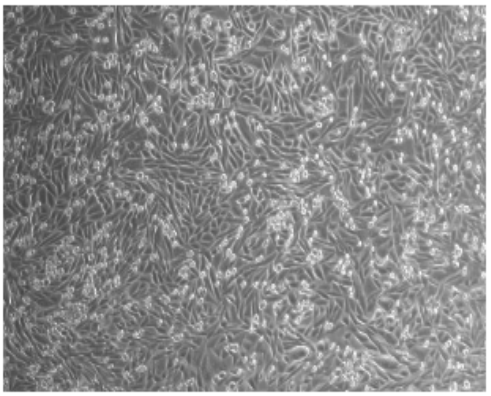
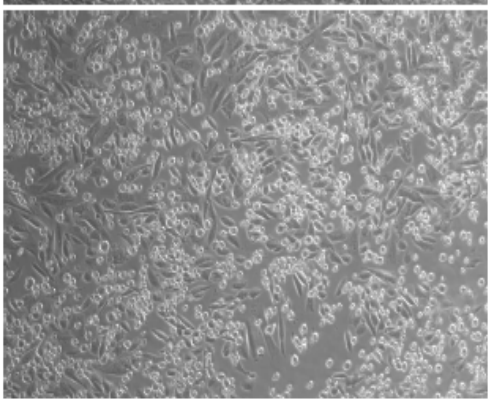
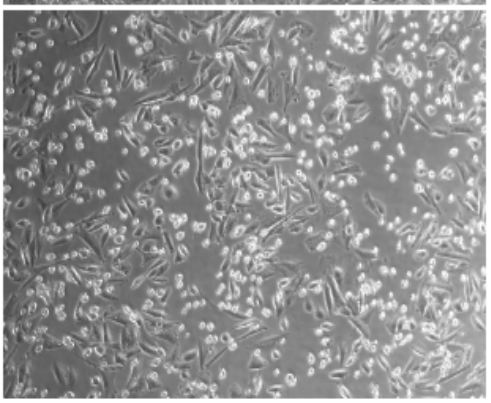
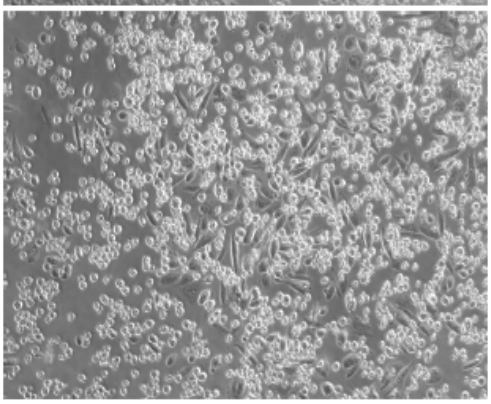
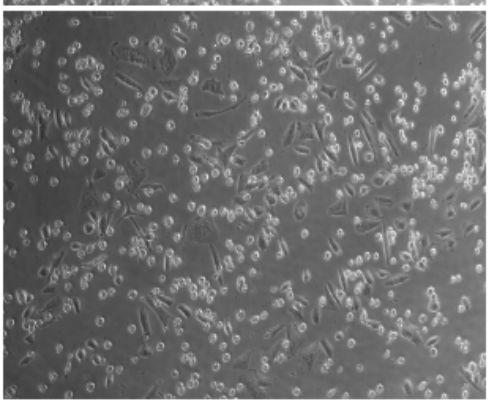
For protein quantification, the medium containing the fluorophore was discarded after the reading. The cells were washed with 2mL of Ca^{2+}/Mg^{2+} free PBS solution. Then 0.5mL of NaOH 1N solution was added to dissolve protein and to quantify protein content according to Lowry and al. (1951). The absorbance readings were performed with the Mithras at 650 nm.

Results



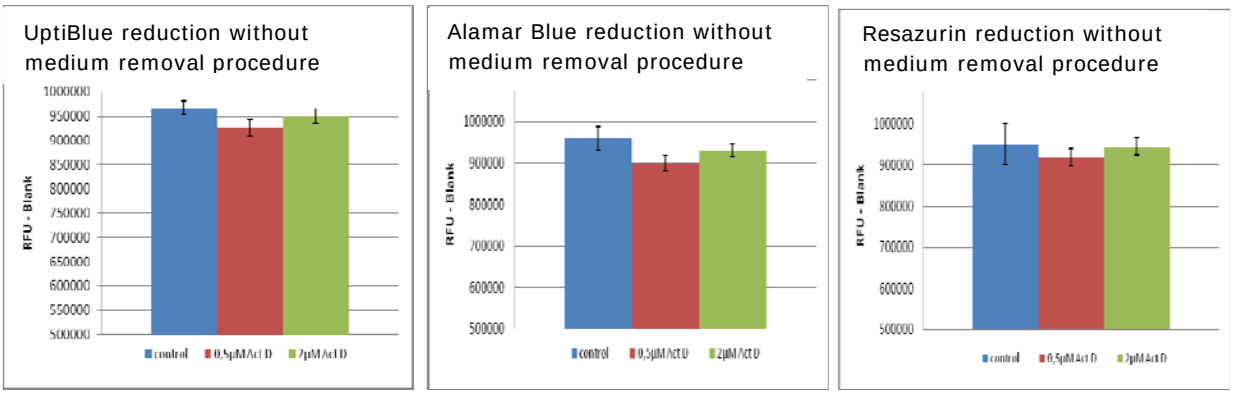
Picture 3: Results of cells density determination, relative fluorescence units according to the cell number

Picture 3 shows that the UptiBlue, AlamarBlue or Resazurin reduction was exactly proportional to the cell seeding number. With more than 300 000 cells the response approaches a saturation level. The reason may be either a depletion of fluorescent substrate and/or metabolic stress related to overcrowding.

Without medium removal procedure	With medium removal procedure	
		Control IMDM medium + 10% FCS Magnification X10
		Test 1 IMDM medium + 10% FCS + 0.5 µM Act. D Magnification X10
		Test 2 IMDM medium + 10% FCS + 2 µM Act. D Magnification X10

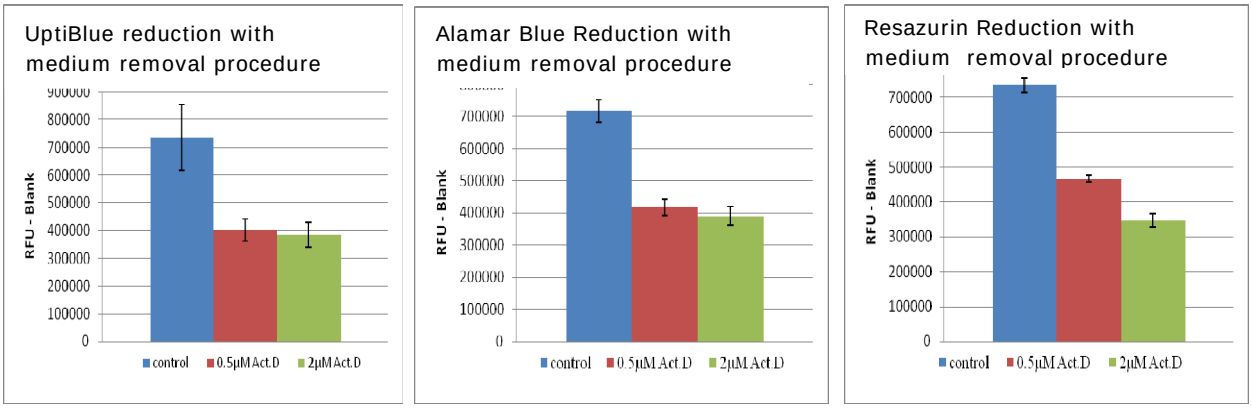
Picture 4 : Photographs of cell culture (with or without medium removal procedure)

UptiBlue	Alamar Blue	Resazurin



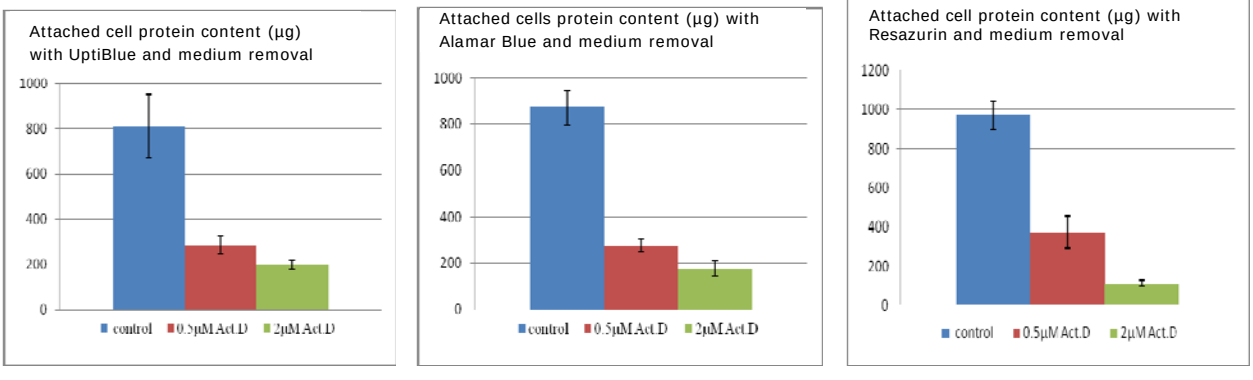
Picture 5 : Comparison of UptiBlue, Alamar Blue and Resazurin reduction without medium removal procedure

UptiBlue	Alamar Blue	Resazurin



Picture 6 : Comparison of UptiBlue, Alamar Blue and Resazurin reduction with medium removal procedure

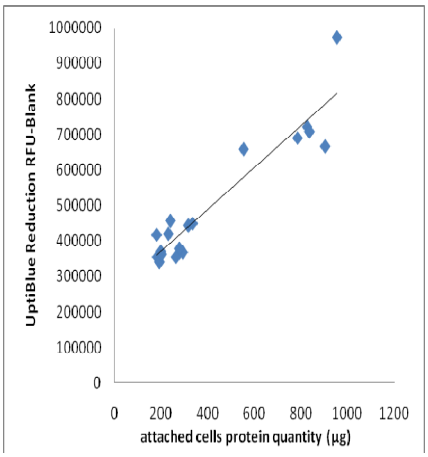
UptiBlue	Alamar Blue	Resazurin

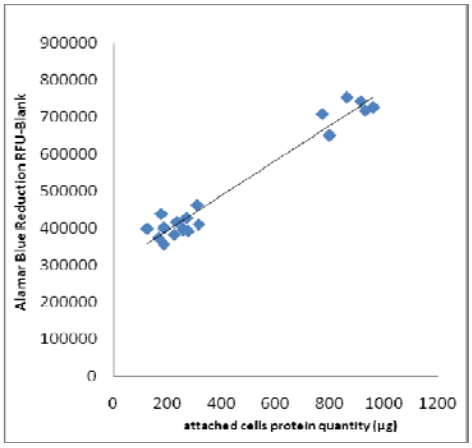


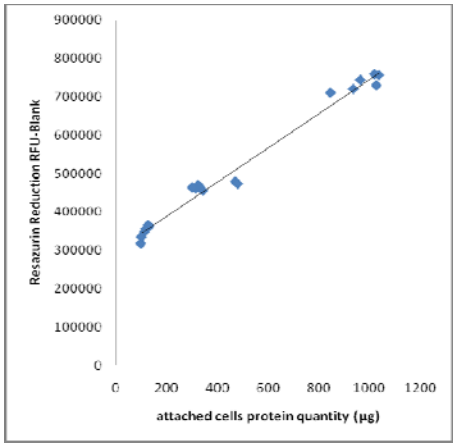
Picture 7 : Comparison of attached cell protein content

Correlation between fluorophore reduction and attached cell protein content

UptiBlue	Alamar Blue	Resazurin







Without medium removal procedure, neither by using UptiBlue, Alamar Blue or Resazurin differences between Actinomycin D solutions and control could be observed.

However, after medium change (medium removal procedure), a strong decrease of UptiBlue, Alamar Blue or Resazurin reduction in the Actinomycin D treated wells could be observed.

The CHO cells are specifically able to get suspended when they are in stress. In our case this metabolic stress was caused by the presence of Actinomycin D and the cells got suspended in culture medium.

According to the obtained results a part of the cells were still active. Finally, in our experimental conditions, it was necessary to discard medium containing suspended cells and add fresh medium before performing the cytotoxicity assay.

All different reagents used (UptiBlue, AlamarBlue and Resazurin) have shown comparable results.

Moreover, these experiments have shown showed a good correlation between the

chromophore reduction and the seeded cell numbers and the protein content of the attached cells.

Differences between the two Actinomycin D concentrations could be observed by the use of Resazurin as indicating reagent only. Additional measurements are necessary to get more detailed information.

Conclusion

Alamar Blue, UptiBlue and Resazurin are perfectly suited for performing cell viability and cytotoxicity assays in combination with the Berthold Mithras microplate reader.

The medium removal procedure before performing the cytotoxicity assay is essential to avoid influences of suspended cells and old medium.

Material:

- Mithras LB 940 (Berthold Technologies)
- Filters (Berthold Technologies):
  Excitation 520/10nm (39802) or Excitation 530/10nm (37996)
  Emission 590/20nm (37989) or Emission 600/10nm (40095)
  Absorbance filter 650/10nm (37999)
- CHO cells (Chinese Hamster Ovary)
- IMDM medium (Invitrogen)
- FCS (Fetal Calf Serum, Invitrogen),
- HT supplement (SIGMA)
- Penicillin/streptomycin (Invitrogen)
- 75 cm² flask (Falcon)
- Alamar Blue SKU# DAL1025 (Invitrogen)
- UptiBlue UP669412 (Interchim)
- Resazurin (Interchim)
- 96 wells black plates (Nunc)
- 6 wells plates (Falcon)