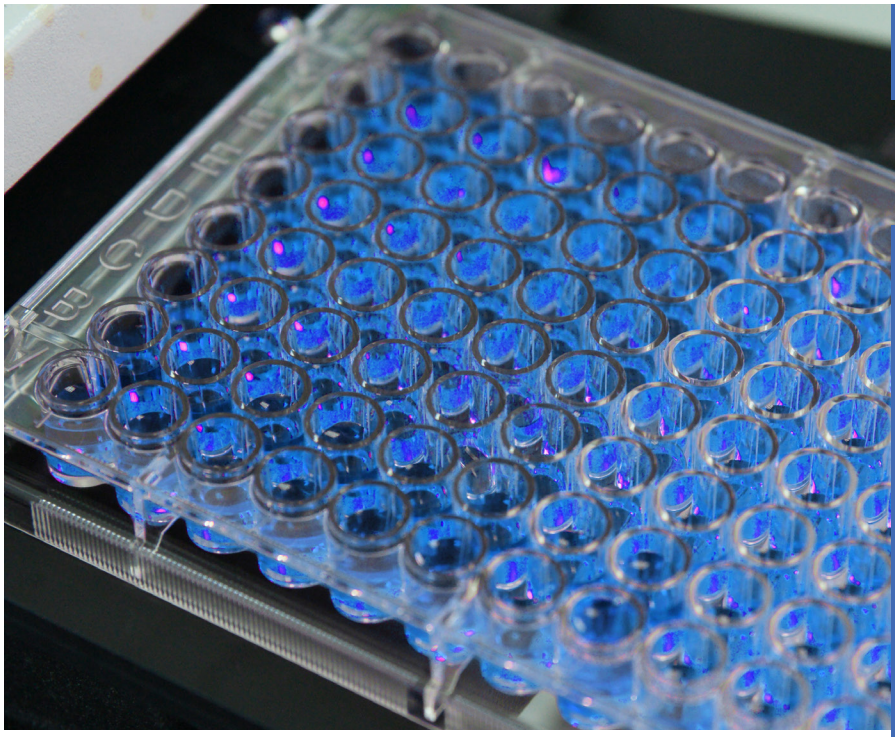

APPLICATION NOTE

Multimode Detection

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ELISA-based Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) Quantification on VICTOR Nivo Multimode Plate Reader

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known to be an important enzyme for the process of cellular glycolysis, as it catalyses the conversion of glyceraldehyde-3-phosphate to 1.3-diphosphoglycerate. However, recent studies suggest that it may also play roles in other cellular processes. For instance, it has been described as a cell apoptosis modulator because of its dysfunctional expression present in different cancer cells, or even as a novel therapeutic target, because it might take part in tumor formation.[1] Its regulation takes place at the transcriptional (GAPDH mRNA level regulation) and posttranslational levels (via various amino acids exchanges), which are undoubtedly highly contributing to the mediation of the GAPDH cellular functions that are independent of its ordinary glycolytic role: tRNA transport, DNA repair and replication, endo/exocytosis, phosphotransferase activity or even cell death.[1]

One such mediator of GAPDH expression and activity, 3-bromopyruvic acid (3-BrPA), has been recently identified as a potential antitumor halogenated analogue of the pyruvic acid. Recent studies have proved that it inhibits various enzymes involved in the glycolysis process, and it is believed to induce cancer cell death via glycolysis blocking. Furthermore, it has been shown that GAPDH expression and function levels may be altered by 3-BrPA. GAPDH might be pyruvylated by 3-BrPA, inhibiting its activity in the glycolysis process. This inhibition may lead to reduced energy production and reduced protein expression rate, or cell death.[3]

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Here we show an Enzyme-linked Immunosorbent (ELISA)-based assay for the study of the 3-BrPA influence on GAPDH concentration in $HepG_2$ cells optimized for the VICTOR Nivo multimode plate reader. Since the VICTOR Nivo is available with both filter- and spectrometer-based absorbance measurement technologies, both models were used and the results were compared.

Materials and Methods

Cell Seeding

For the study of the 3-BrPA effect on GAPDH expression the $HepG_2$ cell line (DSMZ, ACC 180™) was used. Cells were washed with PBS (Capricorn #PBS-1A), harvested with Trypsin (Capricorn #Try-1B) and re-suspended in cell culture medium MEM with Earle`s Salts (Capricorn #MEM-XA) at a cell density of 625,000 cells/mL. The cell suspension was seeded in 800 µL/well in 12-well plates (Corning #3513). In order to analyse the 3-BrPA influence on GAPDH level, a serial dilution of 3-BrPA in cell culture medium was performed in the following concentrations (in the final solution): 400 µM, 200 µM, 125 µM, 100 µM, 80 µM, 70 µM, 60 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 0 µM. The 3-BrPA solutions were titrated (200 µL) on top of the cell suspension and the plates were incubated at 37 °C for 24 hours.

Protein Extraction

By the end of the 24 hours incubation, the cells were scraped off the bottom of the wells and collected for cell number determination and centrifugation. After removing the supernatant, the cells were re-suspended in Protein Buffer Extraction (Bioo Scientific MaxDiscovery GAPDH ELISA kit #3401-01) and frozen at -80 °C for 30 minutes. After thawing at room temperature, the suspension containing lysed cells was diluted to the equivalent of 5,000 cells/mL using the Secondary Standard Diluent (Bioo Scientific MaxDiscovery GAPDH ELISA kit #3401-01).

GAPDH Standard Preparation

For the GAPDH standard assay that was used as a technical control, the GAPDH standard (Bioo Scientific MaxDiscovery GAPDH ELISA kit #3401-01) was diluted to 1.0 µg/mL in Primary Standard Diluent (Bioo Scientific MaxDiscovery GAPDH ELISA kit #3401-01) and then to 0.6 µg/mL in Secondary Standard Diluent (Bioo Scientific MaxDiscovery GAPDH ELISA kit #3401-01). A serial dilution of the GAPDH Standard was performed with a dilution factor of 1:3 in Secondary Standard Diluent starting from 0.6 µg/mL.

ELISA

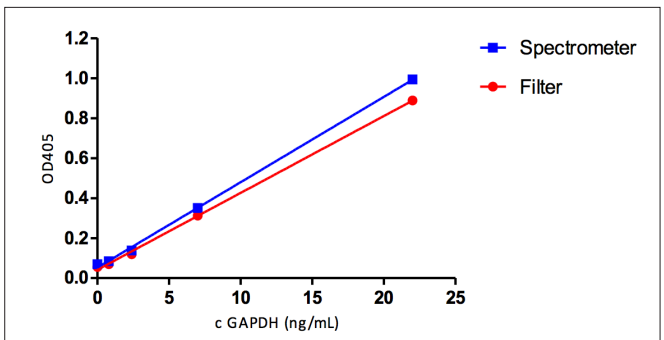
The ELISA assay was performed using the MaxDiscovery GAPDH ELISA Kit (Bioo Scientific #3401-01). In this assay, GAPDH is caught between the capture antibody (which is pre-coated onto the plate) and the Horseradish Peroxidase (HRP) conjugated detection antibody. The enzyme-specific chromophore substrate TMB (3, 3', 5, 5'- tetramethylbenzidine) is oxidized by hydrogen peroxide in the reaction catalyzed by the HRP; generating a blue color which absorbs in the range of 370-652 nm and which turns yellow in the acidic stop buffer, reaching a maximum absorbance at 450 nm. The measured absorbance signal is then proportional to the GAPDH amount in the sample, for which a calibration curve can be generated (Bioo Scientific, 2016).

All the reagents were brought to room temperature except the Standard GAPDH and the HRP Conjugate. 100 µL/well of each diluted protein extract solution/GAPDH Standard solution were plated in triplicates in the 96-well ELISA Kit plate and incubated at 37 °C for one hour. The HRP Conjugate was diluted in HRP Conjugate Diluent (5 µL HRP/1 mL Diluent) and incubated at room temperature for 15-20 min before it was added into the wells. After one hour incubation the plate was washed five times with 250 µL/well Wash Solution 1x with one minute soaking time between the washing steps. 100 µL/well of the diluted HRP Antibody were added into the sample wells and the plate was incubated at 37 °C for one hour. The plate was washed again five times with 250 µL/well Wash Solution 1x with one minute soaking time between the washing steps. 100 µL/well of TMB substrate were added in the sample wells. The solution was mixed by gentle plate shaking (800 rpm) on a small shaker for one minute. The lid was covered with an aluminium sealing foil and the plate was incubated at room temperature for 15-25 minutes. 100 µL/well of Stop Buffer were added on top of the substrate and the absorbance was measured at 405 nm with a bandwidth of 10 nm for 500 ms measurement time on VICTOR Nivo 5F and VICTOR Nivo 5S. Data analysis was performed using GraphPad Prism® software.

Results and Discussion

GAPDH Standard

In order to show the efficiency of using the GAPDH ELISA Kit for absorbance measurements performed on VICTOR Nivo 5F and VICTOR Nivo 5S, the GAPDH Standard solution was used as an analyte (Figure 1).



	Spectrometer	Filter
R^2	0.9989	0.9989

Figure 1. Linear regression generated from the optical density – GAPDH concentration relation for the spectrometer and filter mode absorbance measurements together with the R^2 values.

The results show a very good linear correlation between optical density and analyte concentration for both spectrometer and filter mode. Furthermore, the coefficients of determination (R^2) of 0.9989 are very close to the optimal value of 1, which describes that these linear regressions can be considered calibration curves for GAPDH quantification assays suitably optimized for the VICTOR Nivo multimode plate reader.

Cell Number Titration

For the cell-based assay sensitivity range determination, the GAPDH level in protein extracts from different cell numbers per mL was quantified using the filter-based absorbance measurement (Figure 2).

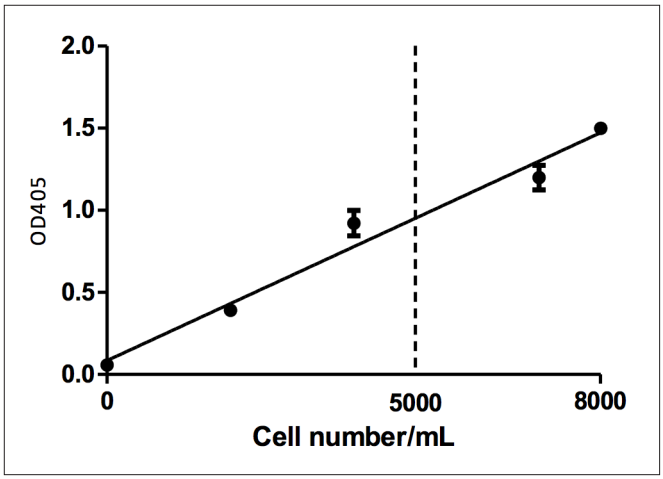


Figure 2. Optical density quantifying the amount of GAPDH in different cell numbers per mL.

As expected, the GAPDH concentration increases with the number of cells per mL. Using the regression curve generated with the supplied GAPDH standard, the cell density of 5,000 cells/mL was chosen to be used in the following experiments. This cell number corresponds to an OD_{405} nm value close to 1, which is in the optimal measurement range of absorbance measurements.

Detection of 3-BrPA influence on GAPDH cellular level

Protein extracts from cells treated with different concentrations of 3-BrPA were used as analyte solutions in the ELISA in order to detect the expected decrease in the amount of cellular GAPDH generated by the 3-BrPA activity. The absorbance was measured using the VICTOR Nivo 5F (filter-based) and VICTOR Nivo 5S (spectrometer-based) microplate readers at a wavelength setting of 405 nm (Figure 4). Absorbance measurements are based on the quantification of the amount of light that passes through a sample. The absorbance reading mode on the VICTOR Nivo 5F uses an optical filter for filtering the light at the required wavelength. In the spectrometer mode on the VICTOR Nivo 5S, the absorbance is recorded for the whole ultraviolet-visible spectrum and either the whole spectrum, parts of the spectrum or specific wavelengths are used for analysis.

A full absorbance spectrum was recorded in the spectrometer mode (on VICTOR Nivo 5S) for the sample with no 3-BrPA treatment in order to show that 405 nm is a suitable wavelength for the optical density measurement of this assay (Figure 3).

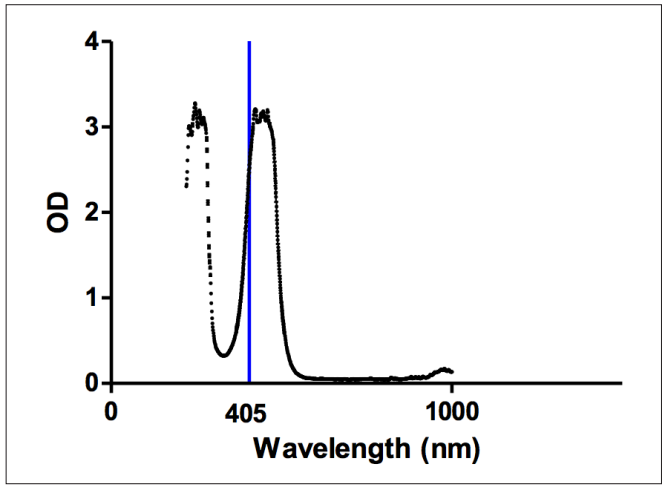
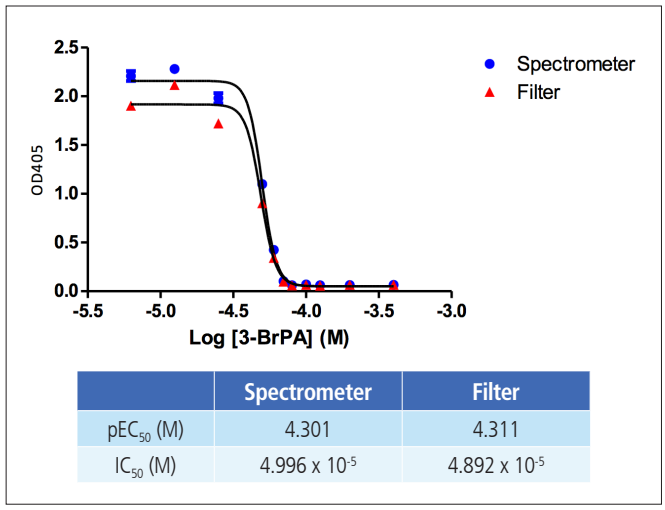


Figure 3. Full spectrum measurement of the optical density in the protein extract sample of untreated cells.

As expected, the absorbance spectrum shows a peak around the wavelength of 450 nm. According to the obtained values, a measurement at 405 nm shows 81% of the maximum signal, which provides absorbance values that are reasonable for the establishment of the sensitivity window of the assay.

The effect of the 3-BrPA on the GAPDH cellular level is shown by plotting the optical density values at 405 nm against the 3-BrPA concentrations used (Figure 4).



	Spectrometer	Filter
pEC_{50} (M)	4.301	4.311
IC_{50} (M)	4.996×10^{-5}	4.892×10^{-5}

Figure 4. 3-BP effect on GAPDH cellular level expressed in optical density measured in the filter and spectrometer modes together with the calculated IC_{50} values of 3-BrPA.

Clearly, the 3-BrPA decreases the GAPDH cellular level at high concentrations, describing an inhibition effect of 3-BrPA on the glycolysis process for which the IC_{50} of about 50 µM was calculated. The similarity between the two dose-response curves generated from the two absorbance measurement modes along with the very close IC_{50} values of the 3-BrPA show the adaptability of the assay for both VICTOR Nivo 5F and VICTOR Nivo 5S instruments.

Conclusion

This study shows the optimization of the ELISA-based assay for the evaluation of GAPDH expression level derived from the cellular activity of 3-Bromopyruvic acid in HepG2 cells. The accurate linear regression of the GAPDH standard along with the similarity between the filter-based and spectrometer-based absorbance measurements describe the high suitability of both VICTOR Nivo 5F and VICTOR Nivo 5S instruments for absorbance-based assays. Moreover, the user-friendly set-up protocols of the two micro plate readers make the use and the storage of assay-specific parameters easy and clearly structured.

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