Targeted Metabolomics Analysis of Cell Culture Lysates using the Absolute*IDQ*™ p180 Kit

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Introduction

The Absolute*IDQ*™ p180 Kit is a commercially available product for targeted metabolomics which can simultaneously identify and quantify a large number of endogenous metabolites in small sample amounts. The Kit enables the quantification of 186 metabolites from 5 different compound classes (biogenic amines, amino acids, acylcarnitines, glycerophospho-/sphingolipids and hexoses). The assay is performed using a combined high performance liquid chromatography (HPLC) and mass spectrometry-based flow injection analysis (FIA) method. The Kit was developed on the AB Sciex API 4000™ and 4000 QTrap® triple quadrupole mass spectrometers and is validated for human plasma.

Cell cultures are commonly used as *in vitro* models to address a wide variety of biological questions, ranging from understanding the complexity of cancer to the discovery of therapeutic effects [1,2]. Furthermore, biotechnological processes utilize the capacity of cells to synthesize pharmaceutically or technically relevant biological compounds [3]. Cellular changes that occur during cell development as a result of cell treatment or during fermentational production can be comprehensively assessed using various genomic, transcriptomic, proteomic and metabolomic techniques. The metabolome mirrors the functional end-point of biological processes, triggered by genetic information and various external influences. Therefore, we used a targeted metabolomics approach for metabolite identification and quantification to enable the functional annotation and characterization of biological changes.

We tested and evaluated the analytical performance of the Absolute*IDQ* Kit on lysates prepared from lymphoblast cells with and without treatment by ionizing radiation. In this application note, we present a cell preparation method and report on the performance of the Kit with regards to detectability of metabolites and reproducibility.

Methods

Sample preparation:

A lymphoblast cell line (GM07057) was obtained from the Coriell Institute (Camden, NJ, USA) and grown at 37°C and 5% CO_2 in RPMI 1640 media (Gibco, Carlsbad, CA, USA) supplemented with 15% heat-inactivated Fetal Bovine Serum (Hyclone, Logan, UT, USA), 100 units/mL of penicillin, and 100 µg/mL streptomycin (Gibco). Cells were suspended at 10^6 cells/mL in T25 flasks (Nunc, Rochester, NY, USA) containing fresh growth medium and allowed to equilibrate at 37°C, 5% CO_2 for 36 hours before treatment with ionizing radiation. Treated cells were given a dose of 10 Gy (5.1 Gy per minute) in a Cesium-137 irradiator (JL Shepherd, San Fernando, CA, USA). Control cells were mock-irradiated. Cells were incubated at 37°C, 5% CO_2 for 5 hours and then harvested.

Irradiated and mock-irradiated cells were transferred to 50 mL conical tubes and counted in triplicate with a Z1 Coulter Counter (Beckman Coulter, Brea, CA, USA). Cells were centrifuged 180 x g for 8 minutes at 4°C, and then media was removed. Cells were washed two times with 50 mL ice cold phosphate buffered saline, and then lysed in either 10 mM Phosphate Buffer (hereinafter referred to as PB) or in a solution of 85% ethanol (hereinafter referred to as EtOH). For the EtOH lysis buffer, cells were first resuspended in 15% final volume of 10 mM PB and then 85% final volume of 100% ice cold ethanol was added. Cells were lysed in both lysate buffers at two different cell concentrations: $4x10^7$ cells/mL and $8x10^7$ cells/mL. Cells in both lysis buffers were

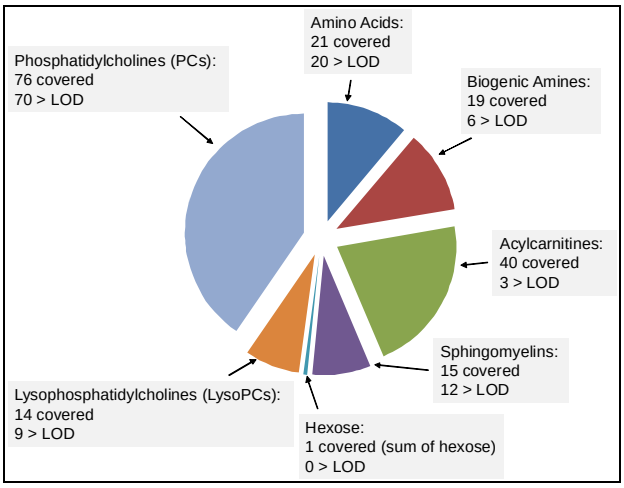

Figure 1: Detected metabolites in the analyzed cell lysates. *The pie chart illustrates the Kit metabolite panel separated into metabolite classes. In the description field of each class, the number of covered metabolites is given (Kit panel) as well as the number of metabolites above the limit of detection (> LOD).*

subjected to three rounds of sonication followed by a rapid freeze-thaw procedure using liquid nitrogen as follows: cells were sonicated for 15 seconds at a power setting of 5 using a 550 Sonic Dimembrator (Fisher Scientific, Pittsburgh, PA, USA), frozen for 30 seconds in liquid nitrogen, rapidly thawed in a 98°C heat block, and returned to ice. After the third round of freeze-thaw, cell lysates were cleared by centrifugation at 20,000 x g for 10 minutes at 4°C. The supernatants were transferred to cryogenic vials and stored in liquid nitrogen vapor phase. Lysates were thawed on ice prior to use.

Sample analysis:

The Absolute*IDQ* Kit was prepared as described in detail in the User Manual. Eight samples (two radiation treatments x two buffers x two cell concentrations) were added to the Kit's 96 well plate in five replicates at both 10 µL per well and 20 µL per well. There were two different zero samples (PB and EtOH), each run in triplicate on the Kit plate. The Biocrates' quality control samples QC1 and QC2 were also added, while QC3 was omitted due to space considerations. The QCs represent lyophilized human plasma samples containing metabolites at several concentration levels.

For sample analysis, we used the AB Sciex 4000 QTrap® in combination with an Agilent 1100 autosampler and HPLC system. The standard acquisition methods of the Absolute*IDQ* Kit were applied, comprising a LC and two subsequent FIA acquisitions (one in positive, one in negative ion mode). The LC part covers the amino acids and biogenic amines (positive mode), the FIA part measures acylcarnitines, lipids (both positive mode) and hexoses (negative mode). Metabolites were quantified using internal standards. Multiple reaction monitoring (MRM) was used for identification and quantification.

Data analysis:

The proprietary Met*IQ*™ software is an integral part of the Kit and was used for data processing. The concentrations of metabolites that were analyzed by FIA (acylcarnitines, lipids and hexoses) were automatically calculated by the software. The analyte peaks obtained by LC (amino acids and biogenic amines) were integrated by the AB Sciex Analyst® software. The concentrations were determined based on the measured standards for calibration and by applying the associated quantification method (provided with the Kit).

Results and Discussion

Metabolites:

The Absolute*IDQ* p180 Kit measures a total of 186 metabolites. In the present study on lymphoblast cell lysates, a total number of 120 metabolites were detected: amino acids (20), biogenic amines (6), acylcarnitines (3), (lyso-)glycerophospholipids (79), and sphingomyelins (12). Hexose was not detected. The pie chart (Figure 1) gives an overview of the detected metabolites of each metabolite class, referring to the total number that is covered by the Kit.

Extraction solvents:

In recent studies on brain tissue samples, we found that 85% ethanol in 10 mM PB (EtOH) is a good compromise to cover all classes of metabolites for analyses [4]. Therefore, this extraction buffer was the first choice for our studies on cell lysates. In addition, we tested pure 10 mM PB, in order to compare the extraction efficiency of a high organic solvent versus an aqueous buffer. When comparing the two buffers, we found that both allowed the detection of a similar total number of metabolites (Figure 2), with slightly more detected when EtOH buffer was used. Considering the extraction efficiency in more detail, we found that EtOH was more efficient for lipids, while PB led to higher yields of amino acids and biogenic amines (Figure 3). This confirmed the findings of the above mentioned brain studies [4]. We conclude that EtOH buffer is a good choice for the extraction of cell lysates to cover all metabolite classes of the Kit.

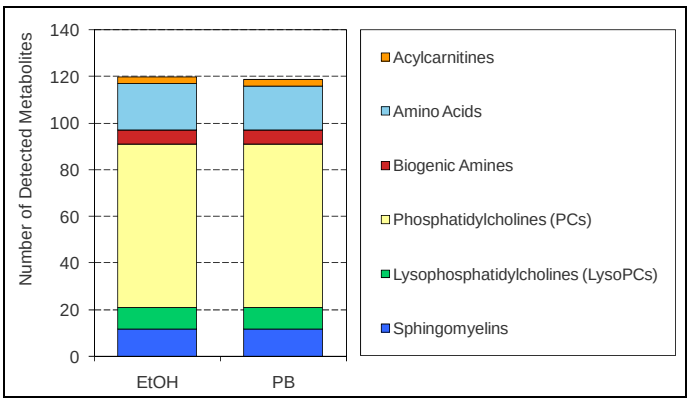

Figure 2: Number of detected metabolites in EtOH and PB
The figure shows that the use of EtOH and PB buffers resulted in a similar total number of metabolites. The figure represents the measurements with the following sample conditions: $4x10^7$ cells/mL, 10 µL sample volume per well.

Reproducibility / Coefficient of Variation:

Median and standard deviation values of the coefficient of variation (CV) were calculated for the different metabolite classes for all sample preparation conditions used (Figure 4). Only analytes with values above the limit of detection (LOD) were considered. The LOD is defined as three times the median value of the zero samples. The figure shows that cell extracts processed in both EtOH and PB solvents provided similarly good reproducibility. Regarding the different cell concentrations and sample volumes, the CV values compare well with each other too. Since we have focused on the reproducibility when analyzing cell lysates in general, irradiated and mock irradiated cells were treated identically in the figure.

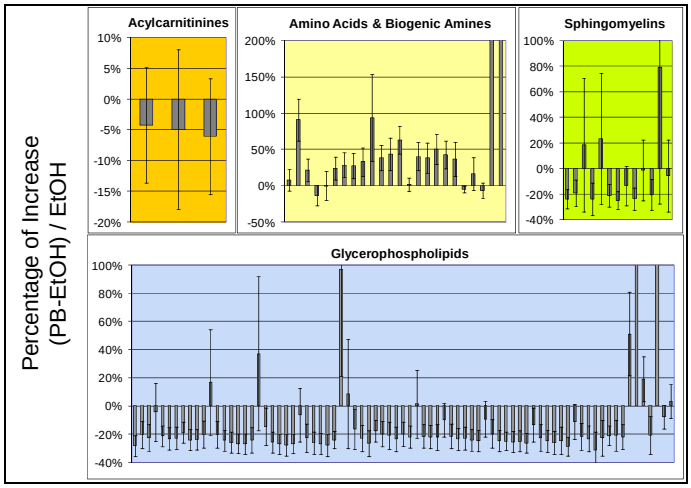

Figure 3: Comparison of extraction efficiency (EtOH vs PB). *The figure shows the average percentage ratio of detection (PB compared to EtOH) as calculated from mean concentrations for each metabolite, separated into metabolite classes. Positive percent bars indicate higher metabolite concentrations in the PB extract and vice versa. Standard deviations of mean metabolite concentrations of all sample types (n=8) are represented as error bars. PB was superior for most amino acids and biogenic amines, while EtOH buffer was superior for most lipids.*

Internal Standards / Ion Suppression:

The internal standards (IS), which are already incorporated in the Kit plate, are essential for quantification. Chemically homologous IS are used for quantification of the lipids, stable isotope-labeled IS are used to quantify the other compound classes. The amount of IS is identical in each well, and the IS intensities of zero sample and sample wells were compared to allow conclusions on ion suppression effects. When comparing the two cell concentrations for each extraction buffer (PB and EtOH), the higher cell concentration ($8x10^7$ cells/mL) was correlated with a slightly decreased IS intensity level compared to the respective lower cell concentration ($4x10^7$ cells/mL). The same trend was observed when comparing a 20 µL sample volume to a sample volume of 10 µL. This might have been due to increased suppression effects caused by an increased amount of matrix. When comparing EtOH and PB extracts within identical preparation conditions (cell concentration and sample volume), we found that PB resulted in considerably higher suppression effects, but did not negatively impact the analytical performance of the Kit.

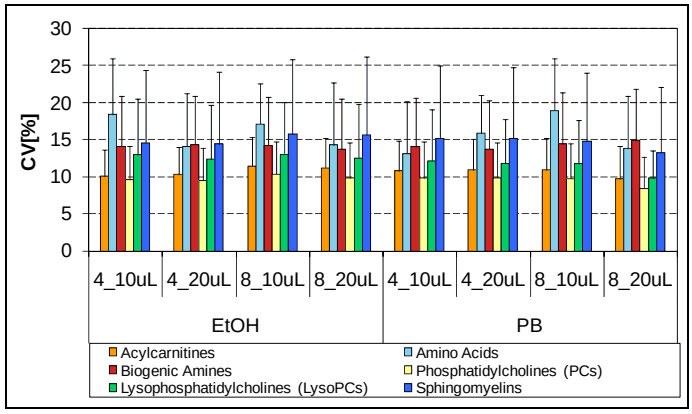

Figure 4: Comparison of CV values.
Lymphoblast cell line samples were analyzed with eight different sample preparation conditions. For example, "4_10µL" corresponds to a cell concentration of $4x10^7$ cells/mL and a sample volume of 10 µL, the other legends accordingly. Median and standard deviation values of the coefficient of variation (CV) were calculated for the different metabolites classes. Only analytes with values above LOD were used. Irradiated cells and normal cells were treated identically.

Conclusion

The results presented in this application note clearly reveal that the Absolute*IDQ*™ p180 Kit is well suited for analyzing cell lysates. 120 metabolites were detected, and good analytical performance was achieved. To analyze all classes of metabolites that are covered by the Kit, we recommend using 85% ethanol in 10 mM sodium phosphate as an extraction solvent for the cell lysates. The results also show that all tested sample preparation conditions are well suited for reproducible metabolite quantification.
The standard sample volume that is usually applied to the Kit plate for plasma or serum samples is 10 µL. Since we observed lower suppression effects with this sample volume compared to 20 µL, we recommend 10 µL for cell lysates as well. A clear statement of which cell concentration is better suited for this Kit cannot be given, suggesting that a broad range of concentrations can be used with the Kit. Therefore, the cell concentrations that were used in this study can be considered as a good starting point for cell analysis optimization.

References

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