Targeted Metabolomics: Fast, Standardized Mass Spectrometric Analysis of Blood Plasma with a Kit

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Summary
There is increased interest in the discovery of metabolic biomarkers and in the routine monitoring of metabolic pathways in modern biosciences. Targeted metabolomics approaches are well suited for application in clinical, pharmaceutical and toxicological research and will complement more established genomic and proteomics technologies. Now, for the first time, the newly developed AbsoluteIDQ™ Kit enables quantification of over 160 metabolites from four different compound classes (Acylcarnitines, Amino Acids, Glycerophospho- und Sphingolipids and Hexose) in a single assay using as little as 10 µL of blood plasma.

Fig. 1: Description of the workflow for the AbsoluteIDQ™ Kit.

Targeted Metabolomics
The relatively new field of systems biology attempts to understand the complex interaction of biological systems on a molecular level. Until now systems biology was predominantly focused on the areas of genomics, transcriptomics and proteomics. However, the ongoing development of mass spectrometric methods for metabolomics now allows study of the metabolome in more detail [1-3]. This is crucial, since metabolomics represents the functional end point of physiological and pathophysiological processes. Thus, it depicts both genetic predisposition and environmental influences like nutrition, exercise or medication.

The AbsoluteIDQ Kit is based on a targeted metabolomics approach. This approach aims to simultaneously identify and quantify a high number of endogenous metabolites. One major advantage of this approach is that you generally obtain quantitative or semi-quantitative information, compared to commonly used non-targeted methods, like metabolic profiling. The observed variations in the concentration of the analytes can be more easily interpreted in a physiological context since details about the metabolomics pathways are well known and described in many cases. Furthermore, the targeted metabolomics approach is better suited for high-throughput and routine applications and will be extremely interesting for areas like clinical diagnostics in the future. It has already been shown that a multiparametric, mass-spectrometric analysis can be successfully applied in routine diagnostics for areas like neonatal screening for inborn errors of metabolism [4].

The newly developed AbsoluteIDQ Kit allows users to quantify 162 metabolites found in blood plasma. We are currently adapting the kit to other biological matrices. Over the long-term, we plan to develop more specialized versions of the kit to meet the increasing demand for standardized and validated assays for both metabolomic research and diagnostic assays. Biocrates offers a more comprehensive metabolite spectrum to customers under its proprietary TargetIDQ™ Contract Research Services.

AbsoluteIDQ™ Kit
The kit uses a standardized Flow Injection Analysis (FIA-MS-MS) method with ionization via an electrospray-ion source. Stable isotope-labelled internal standards are used for quantification. The intensity of the MS/MS signals of each analyte in relation to its isotope-labelled internal standard is in a defined range proportional to the concentration of the analyte. The MS/MS quantification employs a multiple reaction monitoring (MRM) mode to achieve high specificity and sensitivity. This detection mode measures metabolite-specific precursor/product ion transitions. The kit was developed and opti-
1. Sample registration

One component of the MetIQ Software is a flexible LIMS (Laboratory Information Management System) Module (MetLIMS). MetLIMS allows the samples and projects to be registered and can import external sample lists from other LIMS-solutions. This module also administers Biocrates’ Standards, Quality Control samples and the SOP (Standard Operating Procedure) and assigns a unique barcode to the registered samples. Detailed information can be added to the samples which might be of interest for subsequent statistical analysis. The registered samples are then distributed on the 96-well plate with quality controls, standards and blanks, and this plate report is printed for the laboratory work. The software also automatically generates a csv-file with the sample information and distribution. This csv-file is loaded into the mass spectrometric software (Analyst® Software, Applied Biosystems) and quickly generates the acquisition batch.

2. Assay preparation

Easily reproducible sample preparation and efficient extraction of the chemically different metabolites from blood plasma is a prerequisite for a robust assay. Because of this, a unique sandwich format was developed, as is shown schematically in Figure 2.

This kit design provides one of the simplest and quickest sample preparations in metabolomics today. The plasma sample (10 µL) is added to the upper filter spot in which the internal standards are already incorporated. The internal standards are essential for identifying and quantifying the metabolites. By incorporating the standards into the plate, we have eliminated many potential sources of error. The amino acids are then derivatized by addition of phenylisothiocyanate (PITC), the samples are dried under nitrogen flow and all metabolites are extracted in a single step with an organic extraction solvent. By centrifuging the plate the samples are filtered and transferred to the lower deep well capture plate. After dilution with the running solvent the samples are ready for analysis and are placed in the autosampler of the MS instrument.


The injection of the extracted samples for the FIA-ESI-MS/MS method can be performed with all autosamplers compatible with the Applied Biosystems/MDS Sciex Analyst® Software. Instrument specific Analyst® acquisition methods are provided with the kit, making the setup of the mass spectrometer easy and straightforward. The running time for the isocratic FIA method is 3 minutes with each sample analyzed twice (negative and positive mode). Thus, the total time per sample is about 7 minutes including the injection step. For 96 samples (a full plate), the data collection is usually done overnight.

4. Convert Mass Spectrometric Data

In large metabolomics projects the bottleneck is not the data collection, but rather the data analysis. In the AbsoluteIQ Kit 162 metabolites are analyzed in each of the 96 wells resulting in about 15,500 concentration values. The calculation of the concentrations is completely automated via MetIQ Software. The MetConc module interacts with Applied Biosystems/MDS Sciex Analyst® Software, converts the mass spectrometric data (wiff-files) and imports them into the MetIQ database. During this process, automated algorithms run to simultaneously calculate the final metabolite concentrations. Thus, the AbsoluteIQ Kit in combination with MetIQ software enables a high-throughput metabolomics analysis of endogenous metabolites.

5. Validate the Kit Plate

The MetVal module of the software performs an automated quality assessment of the data. It tests to determine if the obtained values for the internal standards and quality controls are within the ranges set in the SOP method. The results can be visualized in numerous graphical layouts, giving a quick, yet thorough overview of the results obtained. When values fall outside of the defined ranges, the information is shown graphically and is also transferred to the results tables.

6. Evaluate and Export Data

In the last step, the MetStat module summarizes the assay results in different tables (i.e., sorted by metabolite class, concentration values or intensities). The data validation results are also transferred to the results tables and the data can be exported in different formats (.csv or .txt). This enables further statistical or bioinformatic analysis in other programs.
Validation of method

The described kit method has been validated according to the "FDA Guidance for Industry ‘Bioanalytical Method Validation’". In addition, further tests of comparability and reproducibility have been performed in different external laboratories. In Figure 3A coefficients of variation (CVs) are shown for some metabolites, representing the different chemical classes of analytes. The CVs are generally below 15% and are fairly consistent across different labs. Some analyte-specific and class-specific differences are observed due to the significantly different endogenous concentrations. The variance of measurements on different days is also presented and yields robust, reproducible values.

The absolute values obtained in the different labs are shown in Figure 3B. The concentrations deviate only slightly, illustrates that the kit can be used for combined studies in different labs. The standardized sample preparation and the use of internal standards for the calculation of the concentrations significantly minimize the influence of many site specific factors. Due to the high number of analyzed metabolites, some compromise was accepted in absolute quantification. Semi-quantitative data was generated for many phospho- and sphingolipids because of the lack of corresponding standards. For other metabolites the concentration in normal plasma is below the quantification range. Thus, these parameters are only accurately determined in cases where the metabolite levels are increased as a result of anomalies, disease states or certain treatments.

Spectrum of Metabolites and Application Areas

Table 1 summarizes the metabolite classes measured with the kit and the number of metabolites in each class. It also gives some examples for biological relevance of the metabolites class. Naturally, the patho (physiological) function of the metabolites cannot be comprehensively described, but it should give an impression of how metabolomics data can be used to elicit detailed information on diverse metabolic pathways. The major breakthrough of this kit is the

<table>
<thead>
<tr>
<th>Metabolite Class</th>
<th>Measured Analytes</th>
<th>Biological Relevance (selected examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acylcarnitine</td>
<td>40</td>
<td>Energy metabolism, fatty acid transport and mitochondrial fatty acid oxidation (e.g., inborn disorders like MCAD), ketosis, oxidative stress, mitochondrial membrane damage (apoptosis)</td>
</tr>
<tr>
<td>Amino Acids</td>
<td>13 proteinogenic Amino Acids + Omihtine</td>
<td>Amino acid metabolism (e.g., inborn disorders like PKU, MSUD), urea-cycle, activity of gluconeogenesis and glycolysis, insulin sensitivity / - resistance, neurotransmitter metabolism, oxidative stress</td>
</tr>
<tr>
<td>Hexose</td>
<td>Sum of Hexose (90-95 % Glucose)</td>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
<td>Lyso-Phosphatidylycholine</td>
<td>15</td>
<td>Degradation of phospholipids (phospholipase activity), membrane damage, signaling cascades, fatty acid profile</td>
</tr>
<tr>
<td>Phosphatidylycholine</td>
<td>77</td>
<td>Dyslipidemia, membrane composition and damage, fatty acid profile, activity of desaturases</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>15</td>
<td>Signaling cascades, membrane damage (e.g., neurodegeneration)</td>
</tr>
</tbody>
</table>

Figure 3: Representative results of a field test in 3 independent laboratories (test site I, II, III).
Part A shows the coefficients of variation (in %) of 5 different analytes. The reproducibility is depicted on 3 different days (3 columns per Analyte) and also between the labs. Part B gives the median concentration (in µM) inclusive standard deviation for the field test data. 9 plasma samples per kit plate have been analyzed.

Table 1: Overview of Metabolite Classes
simultaneous quantification of analytes from 4 compound classes using as little as 10 µL and the high number of phospholipids and sphingolipids identified and quantified.

Because of the broad metabolite spectrum, the application areas are diverse. Table 2 lists a few of the main application areas. Besides basic research, the major areas of application are pharmaceutical and clinical research. Metabolomics methods are also well suited for translational research studies. A given metabolite is (unlike a transcript or protein) the same in every organism and the central metabolic pathways are generally conserved during evolution. Therefore studies from cell culture to pre-clinical animal models to clinical trials can be performed using the same analytical method. In this context, because the kit only needs a small amount of sample, it is ideal for investigating mouse models and even taking multiple time points without having to sacrifice the animal. To summarize, the AbsoluteIDQ Kit is the first product in a long line of metabolomics tools that will be essential for routine metabolic screening in the future. For more information on how to bring the strength of the AbsoluteIDQ™ Kit into your lab, please email sales@biocrates.com.

References