# Chapter 28

# Quantitative Metabolomics in Alzheimer's Disease: Technical Considerations for Improved Reproducibility

**Sergio Veiga, Judith Wahrheit, Andrés Rodríguez-Martín, and Denise Sonntag**

## Abstract

Metabolomics is the comprehensive analysis of small molecules (metabolites) that are intermediates or endpoints of metabolism. Since metabolites change more rapidly to both external and internal stimuli than genes and proteins, metabolomics provides a more sensitive tool to study physiological changes to a wide range of factors such age, medication, or disease status. Therefore, metabolomics is being increasingly used for the study of several pathological states, including complex diseases like Alzheimer's disease (AD).

Both untargeted and targeted metabolomics have been applied for AD and both have provided diagnostic algorithms that accurately discriminate healthy patients from patients with AD by combining different metabolites. However, none of these algorithms have been replicated in larger, different cohorts, and a consensus in methodology has been claimed by the scientific community. The Absolute*IDQ*® p180 Kit (Biocrates, Life Science AG, Innsbruck, Austria) is to date the only commercially available, validated, and standardized assay that measures up to 188 metabolites in biological samples. This kit unifies methodology in a common user manual and provides quantitative measurements of metabolites, thus facilitating an easier comparison among studies and reducing the technical variability that might contribute to replication failures. Nevertheless, recent studies showed no replication even when using this kit, suggesting that additional measures should be taken to achieve replication of metabolite-based discriminative algorithms. The aim of this chapter is to provide technical guidance on how to apply quantitative metabolomic data to the definition of discriminative algorithms for the diagnosis of neurodegenerative diseases such as AD. This chapter will provide an overview of technical aspects on the whole process, from blood sampling to raw data handling, and will highlight several technical aspects in the process that could hamper replication attempts even when using validated and standardized assays, such as the Absolute*IDQ*® p180 Kit.

**Key words** Metabolomics, Replication failure, Absolute quantification, p180 Kit

## 1 Introduction

Metabolomics offers a very sensitive analytical tool for biomarker discovery in biological fluids. In particular, metabolomics could be extremely useful for multifactorial and complex diseases such Alzheimer's Disease (AD), where the application of one single blood biomarker does not provide a satisfactory level of diagnostic

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accuracy [1]. In the search of diagnostic biomarkers of AD, both the targeted and untargeted metabolomic approaches have provided diagnostic algorithms that accurately discriminate healthy patients from patients with AD, even in its early phase [2–6]. Targeted metabolomics offers absolute quantitative measurements, which are considered of higher rigor than relative quantification [7]. Absolute concentration data make different studies more easily comparable, which is key for the replication of diagnostic algorithms in different populations. Furthermore, if the algorithm is ultimately intended to be used as a medical device, absolute quantitative data is required to demonstrate that the diagnostic algorithm is reliable and reproducible for the intended use according to the FDA Guidance for industry Bioanalytical method validation [8]. Therefore, the use of quantitative data offers several advantages for replication and standardization of discriminative algorithms that could eventually be used as medical devices.

The Absolute***IDQ***® p180 Kit (Biocrates, Life Science AG, Innsbruck, Austria) is a commercially available validated and standardized metabolomics kit assay that allows simultaneous quantification of 188 metabolites in only 10 μL of plasma or serum samples. The quantification of metabolites is achieved by combining two different mass spectrometric methods (HPLC-MS/MS and FIA-MS/MS) with isotope labeled internal standards: 21 amino acids and 21 biogenic amines are analyzed by HPLC-MS/MS, while 40 acylcarnitines, 15 sphingolipids, 90 glycerophospholipids and the sum of hexoses are analyzed by FIA-MS/MS. The analysis of this selection of metabolites allows the study of several pathways that are associated with AD pathogenesis such as neurotransmitter dysfunction [9, 10], mitochondrial metabolism impairment [11, 12], lipid metabolism [13–15], or glucose homeostasis [16].

Several groups have used the Absolute***IDQ***® p180 Kit to obtain algorithms that accurately differentiate healthy patients from AD patients, including early stages of the disease [4, 5, 14, 17, 18]. However, metabolites included in the algorithms differ from one study to another, even when the results of one particular study [5] was attempted to be replicated in different, larger populations by other investigators [4, 17]. Differences in study design such as the use of different biological matrices [4] or differences in age, educational level, and race [17] were proposed as explanations for the replication failure. Additionally, we believe that different technical approaches, from sample collection to raw data handling might contribute to the definition of overfitted algorithms, with the consequent failure of replication.

The aim of this chapter is to provide technical guidance on how to obtain and analyze quantitative metabolomics data from human plasma for the creation of discriminative algorithms aiming at the diagnosis of neurodegenerative diseases, such as AD. This chapter

will provide an overview of technical aspects on the whole process, from blood sampling to raw data handling, and will offer additional strategies to increase the probability of replication of the discriminative algorithms.

## 2 Methods

### 2.1 Sample Collection

#### 2.1.1 Blood Extraction and Plasma Isolation

This step is critical and special attention should be paid to standardize this step as much as possible. The need for standardization of blood sample collection and processing has been already identified [14] and guidelines for standardization are available [19].

Sampling should be performed in overnight fasted individuals to avoid diet interferences. Serum metabolite measurements from fasted individuals have been described to show higher reliability than those from non-fasted individuals [20]. Seasonal and circadian variability should be also avoided at the time of sampling. Similarly, the influence of different potential confounders, such as age, gender, medications, smoking, diabetes status, should be controlled at the time of creation of the database.

1. Blood should be drawn directly into tubes covered with anticoagulant. The preferred anticoagulant for the use of AbsoluteIDQ® p180 Kit is EDTA, but heparin is also acceptable. The use of citrate is not recommended. In our study we used $K_2$-EDTA BD Vacutainers® tubes (BD, Ref 367525).
2. Ensure mixing of blood with anticoagulant by tube inversions after blood collection.
3. Do not cool blood before plasma separation has been completed in order to prevent hemolysis. Perform centrifugation of blood sample as soon as possible, but not later than 30 min after blood collection. Centrifuge the sample at 2500 × *g* at 20–24 °C for 10 min. Keep all the processing procedures and times standardized and use identical blood collection and storage tubes in a single study to ensure comparability.
4. Carefully transfer plasma into a precooled collection vial without aspirating blood cells, shake plasma thoroughly (vortex), and place on ice. Aliquot as necessary into the precooled and labeled storage vials before immediately freezing at −80 °C. Freeze/thaw cycles should be avoided. The use of refrozen samples is generally discouraged for metabolomic analysis of human blood [21]. Therefore, it is recommended to produce at least three aliquots of 50 μL of each plasma sample to be able to repeat the analysis with a non-refrozen sample in case of accident during shipments or analysis. According to the literature, one or two freeze/thaw cycles do not affect significantly most serum and plasma metabolites concentration, but it was reported that some analytes showed a significant reduced

concentration in serum samples after two freeze/thaw cycles, when measured in serum with the Absolute*IDQ*® p180 Kit [22].

#### 2.1.2 Sample Storage and Shipment (If Applicable)

Another factor that should be standardized as much as possible is the time from plasma isolation until analysis. Although it has been described that reliability of serum metabolites is good over 4 months [23] and 2 years [20], differences of storage time of samples until analysis have been suggested as one of the potential reasons for the replication failure of p180 Kit-based algorithms [24]. In particular, processing time and storage time has been shown to affect significantly phosphatidylcholines and lysophosphatidylcholines [14].

Ideally, samples should be analyzed as soon as possible after their collection. However, this cannot be achieved most of the times, especially in the context of a clinical trial where subjects are recruited in a time window of years. Another recommendation is that samples should be analyzed within the same measurement run or carefully randomized over measurement batches to reduce technical variability and batch effects.

Finally, if frozen samples should be sent to a different laboratory for analysis, make sure that they do not suffer any thawing during transport.

### 2.2 Sample Preparation for Analysis

Vortex the plasma samples after thawing and centrifuge at 4 °C for 5 min at 2750 × *g* before loading on the kit plate. Make sure that the centrifuge has reached the temperature before use. If the plasma samples are of high viscosity, a higher centrifugation speed should be applied (for example 5000 × *g*). Samples should not be analyzed if there is any evidence of contamination, e.g., by hemolysis.

### 2.3 Analysis with the AbsoluteIDQ® p180 Kit Assay

The Absolute*IDQ*® p180 Kit assay (BIOCRATES Life Sciences AG, Innsbruck, Austria) is a fully automated assay designed to be used with the most common triple quadrupole mass spectrometers from SCIEX, Waters, and Thermo. It combines a flow injection analysis (FIA) and a liquid chromatography (LC) method and the use of the proprietary Met*IDQ*™ software as an integral part of the Kit. The assay is based on PITC (phenylisothiocyanate) derivatization in the presence of internal standards followed by FIA-MS/MS (acylcarnitines, (lyso-) phosphatidylcholines, sphingomyelins, hexoses) and LC-MS/MS (amino acids, biogenic amines).

The experimental metabolomics measurement technique is described in detail by patents EP 1 897 014 B1 [25] and EP 1 875 401 B1 [26].

The Kit has been validated for analysis of human EDTA plasma samples. Both EDTA and heparin are suitable anticoagulants. Due to its biological similarity, human serum can also be used without impairing analytical performance.

Instructions of the AbsoluteIDQ® p180 Kit user manual need to be strictly followed. The following are general considerations on the use of the kit to avoid common mistakes to obtain high-quality data.

#### 2.3.1 System Suitability Test and Blank Sample

The Kit contains two Testmix solutions to perform separate system suitability tests for the LC and FIA part. A proper Kit performance can only be guaranteed when the Testmix run was performed successfully. First, inject a *Blank* sample to determine the background noise and to check the system for contaminations. All instrument parts must be clean and the blank levels low. Injecting a series of *Blank* samples usually improves the condition of the system. After the *Blank* sample inject the Testmix solution. The Testmix solutions contain a selection of metabolites without internal standards. The system suitability tests are intended to check instrument sensitivity, retentions times, peak shapes, column condition, and ion spray stability.

When preparing the Kit plate for sample measurement, the A1 well also contains a *Blank* sample. No sample or standard should be added to this well.

#### 2.3.2 Zero Samples

Internal standards are integrated in the B1, C1, D1 wells, but no analytes are added. These wells are used to calculate LOD (limit of detection) as described in the corresponding user manual. The use of PBS buffer is recommended for these wells if plasma or serum samples are to be analyzed. The salt content is similar to plasma, resulting in comparable ion suppression for most analytes.

#### 2.3.3 Calibration Standards

The Absolute***IDQ***® p180 Kit applies internal and external standards in order to obtain a robust, reliable, and reproducible quantification of analytes. Amino acids and biogenic amines are quantified in the LC-MS/MS part by stable isotope labeled internal standards and seven external standards. The seven external standards contain analytes of known concentration and are used to generate the calibration curves for the LC-MS assay and to determine analytical accuracies. For the lipids and a subset of acylcarnitines, which are analyzed in the FIA-MS/MS part, standards are not commercially available. Although measurement results are highly reproducible, accuracies could not be determined for these analytes.

#### 2.3.4 Quality Control (QC) Samples

The QC samples are human plasma samples with analytes added in defined concentrations. There are three different QC samples with different levels of the spiked analytes. These samples are used to calculate analytical variability (coefficient of variation, analytical precision). Alternatively, replicates of any other QC sample (commercially available reference sample or pool of unknown samples) can be analyzed with the same purpose. If samples are measured

over different measurement batches, it is recommended to run several (a minimum of 3 or 4) replicates of QC samples in order to enable a robust data normalization.

## 2.4 Data Analysis

### 2.4.1 Raw Data Processing

For the LC-MS assay the data analysis and calculation of the concentrations is performed using the corresponding MS software (Analyst, Xcalibur, MassLynx). The obtained results are then imported into the Met***IDQ***™ software and may be assessed together with the results from the FIA assay.

Clear and step-by-step instructions are provided in the user manual on how to process, convert, and import spectrometer data to generate concentration data, perform validation and quality assessment of data, and export the data for further analysis.

### 2.4.2 Data Use for the Creation of Discriminative Algorithms

Although the p180 Kit offers an unprecedented tool for quantitative and standardized analysis of metabolites in blood, there are still possible sources of variability that could make the subsequent statistical analysis prone to model errors such as data overfitting, jeopardizing the replication in different populations. We propose that raw data obtained from the p180 kit analysis should be subjected to data cleaning prior to the creation of the algorithm, with the objective that only metabolites that have a satisfactory reproducibility level are included in the algorithm creation process. Others and we have identified the following potential variability sources:

- Intrasample variability: It was described that a single measurement is sufficient for quantitative measurement of most metabolites using the Absolute***IDQ***® p180 Kit [20]. Nevertheless, some studies showed that several metabolites included in the p180 kit show a coefficient of variation >20% [18] when replicates of the same sample are analyzed, an observation made also by us and others [22]. Therefore, it is highly recommended to include at least one reference sample (usually a pool of several samples, e.g., the QC samples provided within the AbsoluteIDQ® p180 Kit) by triplicate in all the plates to control for metabolites with high intra-sample variability. The analysis of replicates of the reference samples could be used therefore to eliminate metabolites with a coefficient of variation above 25% from the statistical analysis used for algorithm creation [17, 18, 22]. Additionally, in our experience this reference sample provides an easy way to detect analytical issues or mistakes on data export if too high variability is observed within replicas.
- Metabolites with a high number of values below the LOD: Inclusion of values equal to 0 or <LOD in statistical analysis is problematic. Values below the limit of detection are characterized by a higher uncertainty and reduced accuracy. Inclusion of metabolites with values below the LOD in a high number of

patients could lead to weak algorithms with lower chances of replicability. Therefore, some authors have eliminated metabolites with more than a certain percentage of values of zero or below LOD from analysis [17, 18]. One exception for this strategy would be when a metabolite has more LOD values than the established cut-point, but such values are not equally distributed among experimental groups (for example, most control patients had an LOD value but not in AD patients). In this case, it is possible that this metabolite could be informative for the disease and careful evaluation should be made to decide whether including or not this metabolite in the analysis.

Another point to consider regarding values below the LOD is which arbitrary value should be assigned for those measurements, if they are to be included in the statistical analysis. This should be decided prior to analysis and applied consistently to all data. All values below LOD are equally probable, so several strategies can be found in the literature. Some authors chose to assign a value of LOD/2 value to concentration values below LOD [18], while others imputed the lowest nonzero measurement [17]. However, it is recommended to avoid constant values since this might result in statistical artifacts and rather replace values below LOD by a distribution of values, e.g., by imputing random values between LOD and 0 or between LOD and LOD/2.

– Interlaboratory variability: As far as possible, it is advisable to restrict the analysis to the same analytical platform and site of analysis. Although it has been described that interlaboratory precision is below 20% for most of metabolites included in the p180 kit [27], we detected some metabolites whose concentration differed by twofold or more from one platform to another when the same sample was analyzed by triplicate. Most likely, the source of this variability is the presence of metabolites near to LOD, differing MS background signals in the case of FIA-MS/MS-analyzed metabolites and differences in manual review and peak integration in LC-MS/MS-analyzed metabolites, as pointed out by Siskos and collaborators [27]. This is especially relevant if the two different platforms have significantly different sensitivity and consequently the LOD of metabolites vary from one platform to another.

## References


1. Galasko D, Golde TE (2013) Biomarkers for Alzheimer's disease in plasma, serum and blood - conceptual and practical problems. Alzheimers Res Ther 5:10
2. Olazaran J, Gil-de-Gomez L, Rodriguez-Martin A et al (2015) A blood-based, 7-metabolite signature for the early diagnosis of Alzheimer's disease. J Alzheimers Dis 45:1157–1173
3. Fiandaca MS, Zhong X, Cheema AK et al (2015) Plasma 24-metabolite panel predicts preclinical transition to clinical stages of Alzheimer's disease. Front Neurol 6:237
4. Casanova R, Varma S, Simpson B et al (2016) Blood metabolite markers of preclinical Alzheimer's disease in two longitudinally followed

cohorts of older individuals. Alzheimers Dement 12:815–822

5. Mapstone M, Cheema AK, Fiandaca MS et al (2014) Plasma phospholipids identify antecedent memory impairment in older adults. Nat Med 20:415–418
6. Proitsi P, Kim M, Whiley L et al (2017) Association of blood lipids with Alzheimer's disease: a comprehensive lipidomics analysis. Alzheimers Dement 13:140–151
7. Sumner LW, Amberg A, Barrett D et al (2007) Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 3:211–221
8. Thompson M (2002) Harmonized guidelines for single laboratory validation of methods of analysis (IUPAC Technical Report). Pure Appl Chem 74: n°5, pp 835–855.
9. Butterfield DA, Pocernich CB (2003) The glutamatergic system and Alzheimer's disease: therapeutic implications. CNS Drugs 17:641–652
10. Basun H, Forssel LG, Almkvist O et al (1990) Amino acid concentrations in cerebrospinal fluid and plasma in Alzheimer's disease and healthy control subjects. J Neural Transm Park Dis Dement Sect 2:295–304
11. Swerdlow RH, Burns JM, Khan SM (2014) The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. Biochim Biophys Acta 1842:1219–1231
12. Henderson ST, Vogel JL, Barr LJ et al (2009) Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer's disease: a randomized, double-blind, placebo-controlled, multicenter trial. Nutr Metab (Lond) 6:31
13. Han X, Rozen S, Boyle SH et al (2011) Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. PLoS One 6:e21643
14. Klavins K, Koal T, Dallmann G et al (2015) The ratio of phosphatidylcholines to lysophosphatidylcholines in plasma differentiates healthy controls from patients with Alzheimer's disease and mild cognitive impairment. Alzheimers Dement 1:295–302
15. Koal T, Klavins K, Seppi D et al (2015) Sphingomyelin SM(d18:1/18:0) is significantly enhanced in cerebrospinal fluid samples dichotomized by pathological amyloid-beta42, tau, and phospho-tau-181 levels. J Alzheimers Dis 44:1193–1201
16. Ferreira ST, Clarke JR, Bomfim TR et al (2014) Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease. Alzheimers Dement 10:S76–S83
17. Li D, Misialek JR, Boerwinkle E et al (2017) Prospective associations of plasma phospholipids and mild cognitive impairment/dementia among African Americans in the ARIC Neurocognitive Study. Alzheimers Dement 6:1–10
18. Toledo JB, Arnold M, Kastenmuller G et al (2017) Metabolic network failures in Alzheimer's disease-A biochemical road map. Alzheimers Dement 3:965–984
19. O'Bryant SE, Gupta V, Henriksen K et al (2015) Guidelines for the standardization of preanalytic variables for blood-based biomarker studies in Alzheimer's disease research. Alzheimers Dement 11:549–560
20. Carayol M, Licaj I, Achaintre D et al (2015) Reliability of serum metabolites over a two-year period: a targeted metabolomic approach in fasting and non-fasting samples from EPIC. PLoS One 10:e0135437
21. Yin P, Peter A, Franken H et al (2013) Preanalytical aspects and sample quality assessment in metabolomics studies of human blood. Clin Chem 59:833–845
22. Breier M, Wahl S, Prehn C et al (2014) Targeted metabolomics identifies reliable and stable metabolites in human serum and plasma samples. PLoS One 9:e89728
23. Floegel A, Drogan D, Wang-Sattler R et al (2011) Reliability of serum metabolite concentrations over a 4-month period using a targeted metabolomic approach. PLoS One 6:e21103
24. Mapstone M, Cheema AK, Zhong X et al (2017) Biomarker validation: methods and matrix matter. Alzheimers Dement 13:608–609
25. Ramsay SL, Stoeggl WM, Weinberger KM, Graber A, Guggenbichler W (Inventors). Biocrates Life Sciences AG (Assignee). Apparatus and method for analyzing a metabolite profile. US Patent 20070004044. Published 04 Jan 2007. 27
26. Ramsay SL, Guggenbichler W, Weinberger KM, Graber A, Stoeggl WM (Inventors). Biocrates Life Sciences AG (Assignee). Device for quantitative analysis of a drug or metabolite profile. US Patent 20070003965. Published 04 Jan 2007
27. Siskos AP, Jain P, Romisch-Margl W, Bennett M, Achaintre D, Asad Y, Marney L, Richardson L, Koulman A, Griffin JL, Raynaud F, Scalbert A, Adamski J, Prehn C, Keun HC (2017) Interlaboratory reproducibility of a targeted metabolomics platform for analysis of human serum and plasma. Anal Chem 89:656–665