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(54) Title: AUTOMATED SAMPLE QUALITY ASSESSMENT

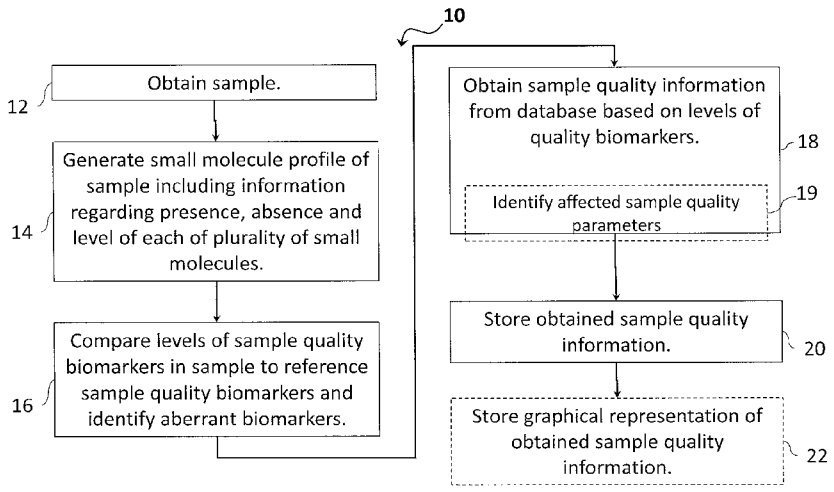


Figure 1

(57) Abstract: A method of assessing the quality of a biological sample while maintaining the viability of the biological sample for intended use analysis is described herein. The method includes analyzing a biological sample obtained from a subject for an intended use, assessing the quality of the biological sample using one or more biomarkers of sample quality, and simultaneously performing intended use analysis on the same sample. Assessing the quality of the sample can include assessing compliance with sample handling protocols and assessing subject compliance.

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AUTOMATED SAMPLE QUALITY ASSESSMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/250,627, filed November 4, 2015, the entire contents of which are hereby incorporated herein by reference.

FIELD

[0002] The invention generally relates to a system, method and apparatus to assess the quality of a sample using small molecule biomarkers indicative of sample quality parameters and methods based on the same.

BACKGROUND

[0003] Metabolomics has been widely and successfully used to identify biomarkers for various indications. Obtaining interpretable, high quality data for any application, including metabolomics analysis, depends on starting with a good study design and high quality samples, free of artifacts due to mishandling the samples or lack of subject compliance. Currently, samples may be visually inspected prior to analysis to remove samples with obvious quality issues such as temperature or sample volume. In some cases, samples can be visually identified as sub-optimal due to a physical change in the appearance of the sample. For example visual inspection of plasma or serum samples may reveal macro-hematuria, a red color from hemolysis, (high amount of red blood cell lysis) or turbidity from lipemia (high lipid content) in a sample. However, small amounts of hemolysis (e.g., micro-hematuria) or other perturbations to the sample may not be recognizable by visual inspection.

[0004] In sample types other than blood-based samples such as urine or tissue, visual inspection reveals little regarding sample quality. Furthermore, levels of metabolites in a sample may be affected by sample handling including sample collection, processing and storage conditions such as collection tubes and tube additives, processing time from collection, processing incubation temperature, time in storage, storage temperature, freeze-thaw cycles, and subject compliance such as fasting status, medication and supplement status, smoking status, and diet and exercise compliance. Protocol violations in sample handling and/or subject

compliance, in turn, may impact the utility or suitability of the sample for subsequent analysis. The ability to monitor sample acquisition and assess samples for protocol compliance regarding handling and subject compliance could ensure that samples meet quality criteria or, those samples that fail one or more criteria are removed from analysis or the analysis is modified appropriately. A method to measure and report parameters affecting sample quality is beneficial.

[0005] While sample quality parameters exist for evaluating some effects of pre-analytical processing variations in metabolomics-based research (Kamlage, *et al. Clinical Chemistry*.60:2, 399-412 (2014)), these parameters do not take into account subject compliance. Having quality parameters to evaluate subject compliance is important for studies such as clinical trials where failing to comply with a treatment regimen could affect the results of the trial or could pose a threat to subject safety. For example, non-compliance with a fasting protocol may affect results of many types of studies, including biomarker discovery and development. In addition, compliance for diagnostic tests may be important to obtaining valid results. A method to assess the quality of a sample that includes quality parameters addressing sample handling and subject compliance, and the identification of metabolite biomarkers useful in making the assessment, is needed.

[0006] The method described herein to assess the quality of a sample for studies involving the measurement of multiple small molecule analytes (e.g., metabolomics studies) represents an improvement over current methods for assessing sample quality. Current methods require a separate quality analysis on a subset of samples intended for a larger metabolomics study. The quality assessment is performed on only a few samples, and then the quality of all samples is extrapolated from that assessment. The method described herein performs quality assessment on all samples in the study simultaneously with the intended use analysis and does not require a separate sample or sample run to address sample quality. In this way, the amount of sample required is minimized, confidence that all samples meet quality criteria is assured, and the resulting data is of acceptable quality. Further, since the quality of each sample is assessed, variations observed in individual samples, such as variations due to compliance with a fasting or medication protocol, can be assessed and the need for additional or modified analysis can be determined and appropriately addressed. Current methods discourage the further use of samples for which insufficient quality

has been determined. The method described herein assesses the quality of each sample, and individual samples that do not meet specific sample quality parameters can be flagged for further analysis. Thus, the entire sample set does not need to be discarded or removed from the analysis. Included in the method, the individual sample quality parameters identified as affected are reported. This assessment provides the investigator with information relating to which aspect(s) of the handling and/or compliance protocols may have been violated.

SUMMARY

[0007] Described herein are biomarkers of sample quality and systems, methods, and apparatuses of using the biomarkers to assess sample quality. Sample quality parameters are evaluated to assess if a sample is in compliance with a protocol. The methods and markers may be used to assess the quality of archival samples which may have been collected and stored for extended periods of time and for which handling protocols and subject compliance and requirement protocols are uncertain or unavailable. Protocol compliance may include sample handling from collection to processing to storage, as well as subject compliance with protocols prior to sample collection. The metabolic profiling of samples and sample quality biomarkers can be used to assess protocol compliance associated with sample handling. Examples of criteria associated with sample handling include sample collection, collection tubes, collection tube additives, sample processing time, processing incubation temperature, time in storage, storage temperature, and freeze-thaw cycles. The metabolic profiling of samples and sample quality biomarkers can also be used to assess subject compliance with a protocol. Examples of criteria associated with subject protocol compliance include fasting status, medication and supplement status, smoking status, diet, and exercise compliance.

[0008] Some embodiments described herein include systems, methods and apparatuses that employ metabolic profiling to assess sample quality simultaneously with performing biochemical analysis or metabolic profiling analysis of the same sample according to the intended use for the sample (e.g., clinical use or experimental use). The simultaneous survey of the biological sample may include automatically generating a metabolic profile of the biological sample and automatically statistically analyzing the resulting metabolic profile data to identify small molecules that are

statistical outliers (i.e., aberrant) and can serve as sample quality biomarkers. As will be shown in the below examples, the generated metabolic profile of a sample and automated statistical analysis of the generated metabolic profile can uncover aberrant levels of small molecule sample quality biomarkers that aid in assessing the quality of the sample to determine if the sample was handled in compliance with a protocol and/or if the subject from whom the sample was collected was in compliance with a protocol (e.g., fasted). A visualization of the results of the automated statistical analysis of the generated metabolic profile may be provided to aid in assessing aberrant levels of small molecule sample quality biomarkers and/or perturbed or aberrant biochemical pathways that result from protocol non-compliance. In some embodiments, a list of one or more possible affected sample quality parameters associated with the identified aberrant sample quality biomarkers may be displayed. A list of recommended approaches for evaluating the sample may also be provided.

[0009] Automatic sample quality assessment is enabled by interrogation of sample quality biomarker levels in the sample. The sample quality assessment results can be reported as a Quality Assessment (QA) status. The sample quality biomarkers enable a user to distinguish biochemical changes due to a disease or disorder (e.g., clinical analyses) or due to experimental design (e.g., biomarker discovery analyses) from artifacts due to unacceptable sample quality. Thus, sample quality biomarkers allow a user to determine (or aid a user in determining) whether biochemical changes identified in a sample are indicative of a clinical or experimental result or of an issue with sample quality. The QA may include identifying and providing a list of affected sample quality parameters, identifying at least one intended use associated with one or more of the aberrant sample quality biomarkers, and/or providing a list of recommended approaches for evaluating the sample. The assessment allows for more than a simple inclusion/exclusion decision by providing information for further analysis of the sample based on the affected biomarkers and sample quality parameters. For example, in one aspect, the quality assessment may provide a recommendation for the application of a correction factor to one or more biomarkers before using the one or more biomarkers in an assessment. In another aspect, the quality assessment may provide a recommendation to eliminate certain biomarkers or biochemical pathways from the analysis. In another aspect, the quality assessment may provide a recommendation to substitute one biomarker with a different

biomarker for a certain biochemical pathway.

[0010] The QA may include using the measured levels of one or more biomarkers in a composite score. The quality criteria can be adjusted by the experimentalist or clinician based upon the intended use of the sample, and the criteria may be selected from one or more quality parameters (e.g., sample processing time, sample storage temperature, sample freeze/thaw, time for plasma separation from whole blood, fasting status, medication status, smoking status, exercise compliance).

[0011] The quality assessment does not require a separate analysis or a separate sample; instead, it is performed simultaneously with the experimental or informational analysis. The simultaneous assessment of sample quality with the experimental or informational analysis has a number of advantages, including but not limited to, the following: reduced workload since it is not necessary to produce, process and analyze a separate "sample quality aliquot" from the analytical samples for QA purposes; reduced time for analysis; reduced costs associated with running and maintaining the instruments; reduced costs of goods (e.g., reagents, disposables, etc.); more data (and more useful data) may be obtained from the analyzed samples; and fewer samples may be discarded. The methods assess the quality of all samples rather than a selected subset of samples so each sample is associated with a QA. The QA would provide much needed clinical utility in identifying outliers (potential protocol violations) in a study based on the physical collection and handling of samples as well as subject compliance. These assessments would be more accurate and more sensitive than qualitative visual assessments. Further, simultaneous assessment of sample quality enables improvements in efficiency.

[0012] In some aspects, the quality assessment is partially automated. In some aspects, the quality assessment is fully automated.

[0013] In one embodiment a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, and providing a sample quality assessment. In some embodiments the assessment is comprised of a composite score.

[0014] In one embodiment a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample,

listing aberrant sample quality biomarkers, associating aberrant sample quality biomarkers with sample quality parameters, and providing a list of affected sample quality parameters.

[0015] In one embodiment, a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, and identifying at least one intended use associated with one or more of the aberrant sample quality biomarkers.

[0016] In one embodiment, a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, associating aberrant sample quality biomarkers with sample quality parameters, and identifying at least one intended use associated with one or more of the aberrant sample quality biomarkers.

[0017] In one embodiment, a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, identifying at least one intended use associated with one or more of the aberrant sample quality biomarkers and providing a list of recommended approaches for evaluating the sample.

[0018] In one embodiment, a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, associating aberrant sample quality biomarkers with sample quality parameters, and providing a list of recommended approaches for evaluating the sample.

[0019] In one embodiment a method is provided for assessing a sample for sample quality by surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, associating aberrant sample quality biomarkers with sample quality parameters, providing a list of affected sample quality parameters, identifying at least one intended use associated with one or more of the aberrant sample quality biomarkers and, further, providing a list of recommended

approaches for evaluating the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1 is a block diagram of a method for assessing sample quality, in accordance with an embodiment.

[0021] Figure 2 is a block diagram of a method for screening a sample for a plurality of sample quality parameters, in accordance with an embodiment.

[0022] Figure 3 is a block diagram of a computing device for use in implementing some steps of some exemplary methods, in accordance with some embodiments.

[0023] Figure 4 is a graphical representation of levels of four exemplary biomarkers (adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine) for assessing the sample quality parameter of sample processing time. Sample processing time is indicated on the x-axis, and metabolite level is indicated on the y-axis. Results are shown for four samples, A-D.

[0024] Figure 5 is a graphical representation of sample processing time composite scores calculated using four exemplary biomarkers. Sample processing time is shown on the x-axis and sample composite score is indicated on the y-axis.

[0025] Figure 6 is a graphical representation of storage temperature scores calculated using seven exemplary biomarkers. Composite scores for samples in the reference population are indicated by open triangles, and composite score for the pseudo-observation is indicated by a closed triangle.

[0026] Figure 7 is a graphical representation of levels of 8 exemplary biomarkers for assessing the sample quality parameter of fasting status. The candidate biomarkers are indicated on the x-axis, and the biomarker level is indicated on the y-axis. Fed samples are indicated by closed triangles, and fasted samples are indicated by open diamonds.

[0027] Figure 8 is a box plot display of fasting status composite scores calculated using eight exemplary biomarkers.

[0028] Figure 9 is a graphical illustration of the composite scores obtained for the sample quality parameters of fasting status, sample storage and sample processing. The value obtained for the composite score for the indicated quality parameter for the sample is indicated by a diamond. "Score" means the composite score for that

parameter for the sample, "Reference" means the Reference Score cut-off value, "Percentile" means the percentile of the composite score for the indicated quality parameter.

[0029] Figure 10 is a graphical illustration of the data for the biomarkers for fasting status. The unacceptable range is indicated by the dashed boxes. The region outside the dashed box indicates the acceptable range. The value obtained for the biomarker in the sample is indicated by a diamond. "Z-Score" means the statistical score calculated for that biomarker for the sample, "Reference" means the Reference cut-off value, and "Percentile" means the percentile of the indicated quality biomarker.

[0030] Figure 11 is a graphical illustration of the data for the biomarkers for sample storage. The unacceptable range is indicated by the dashed boxes. The region outside the dashed box indicates the acceptable range. The value obtained for the biomarker in the sample is indicated by a diamond. "Z-Score" means the statistical score obtained for that biomarker for the sample, "Reference" means the Reference cut-off value, and "Percentile" means the percentile of the indicated quality biomarker.

[0031] Figure 12 is a graphical illustration of the data for biomarkers of sample processing. The unacceptable range is indicated by the dashed boxes. The region outside the dashed box indicates the acceptable range. The value obtained for the biomarker in the sample is indicated by a diamond. "Z-Score" means the statistical score for that biomarker for the sample, "Reference" means the Reference cut-off value, and "Percentile" means the percentile of the indicated quality biomarker.

DETAILED DESCRIPTION

[0032] A single test that can report on multiple sample quality parameters using the same sample and within the same dataset as the intended use analysis is described.

[0033] Prior to describing this invention in further detail, however, the following terms will be defined.

Definitions:

[0034] "Sample" can be any type of sample including a complex mixture or a biological sample such as a plant sample or an animal sample. The animal sample

may be from a mammal such as, for example, a human, a mouse, a non-human primate, a rabbit or other mammal, or a non-mammal sample such as, for example, a drosophila or zebrafish sample. The biological sample of interest can include blood, plasma, serum, isolated lipoprotein fraction, saliva, urine, lymph fluid, and cerebrospinal fluid, a tissue sample, a cellular sample, or a skin sample. The biological sample may contain any biological material suitable for detecting the desired biomarkers, and may comprise cellular and/or non-cellular material from the subject. The sample can be isolated from any suitable biological tissue or fluid such as, for example, blood, blood plasma, serum, skin, epidermal tissue, adipose tissue, aortic tissue, liver tissue, urine, cerebral spinal fluid, crevicular fluid, amniotic fluid, or cell samples. The sample can be, for example, a dried blood spot where blood samples are blotted and dried on filter paper. In another the example, the sample can be isolated from a skin tape such as Sebutape®. In some embodiments, the sample may be an archival sample or a large collection of archival samples which have been collected and stored for long periods of time and for which handling protocols are uncertain or unavailable.

[0035] "Test sample" means the sample to be analyzed.

[0036] "Reference sample" means a sample used for determining a standard range for a level of small molecules. "Reference sample" may refer to an individual sample from an individual reference subject (e.g., a normal (healthy) reference subject or a disease reference subject), who may be selected to closely resemble the test subject by age and gender. "Reference sample" may also refer to a sample including pooled aliquots from reference samples. "Reference sample" may also refer to a sample (or a pool of samples) that has been processed according to a protocol specific for an intended use of the test sample.

[0037] "Subject" means any animal, but is preferably a mammal, such as, for example, a human, monkey, non-human primate, rat, mouse, dog or rabbit. In some aspects the subject is a patient. In some aspects the patient is a human.

[0038] The "level" of one or more biomarkers means the absolute or relative amount or concentration of the biomarker in the sample.

[0039] "Small molecule", "metabolite", "biochemical" means organic and inorganic molecules which are present in a cell. The term does not include large macromolecules, such as large proteins (e.g., proteins with molecular weights over

2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000), large nucleic acids (e.g., nucleic acids with molecular weights of over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000), or large polysaccharides (e.g., polysaccharides with a molecular weights of over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000). The small molecules of the cell are generally found free in solution in the cytoplasm or in other organelles, such as the mitochondria, where they form a pool of intermediates, which can be metabolized further or used to generate large molecules, called macromolecules. The term "small molecules" includes signaling molecules and intermediates in the chemical reactions that transform energy derived from food into usable forms. Non-limiting examples of small molecules include sugars, fatty acids, amino acids, nucleotides, intermediates formed during cellular processes, and other small molecules found within the cell.

[0040] "Biomarker" means a compound, preferably a metabolite, which is differentially present (i.e., increased or decreased) in a test sample as compared to one or more pre-determined reference ranges or to one or more reference samples, or in a sample treated according to one condition (e.g., fasted, stored at -80°C) compared to a sample treated according to a second condition (e.g., fed, stored at -20°C). A biomarker may be differentially present at any level, but is generally present at a level that is increased by at least 5%, by at least 10%, by at least 15%, by at least 20%, by at least 25%, by at least 30%, by at least 35%, by at least 40%, by at least 45%, by at least 50%, by at least 55%, by at least 60%, by at least 65%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 100%, by at least 110%, by at least 120%, by at least 130%, by at least 140%, by at least 150%, or more; or is generally present at a level that is decreased by at least 5%, by at least 10%, by at least 15%, by at least 20%, by at least 25%, by at least 30%, by at least 35%, by at least 40%, by at least 45%, by at least 50%, by at least 55%, by at least 60%, by at least 65%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, or by 100% (i.e., absent). A biomarker may be differentially present at a level that is statistically significant (i.e., a p-value less than 0.05 and/or a q-value of less than 0.10 as determined using either Welch's T-test or Wilcoxon's rank-sum Test).

[0041] A "reference level" of a biomarker means a level of the biomarker that is indicative of sample quality or of one or more particular sample quality parameters.

A "positive" reference level of a biomarker means a level that is indicative of an acceptable sample or sample quality parameter(s). A "negative" reference level of a biomarker means a level that is indicative of an unacceptable sample or sample quality parameter(s). A "reference level" of a biomarker may be an absolute or relative amount or concentration of the biomarker, a presence or absence of the biomarker, a range of amount or concentration of the biomarker, a minimum and/or maximum amount or concentration of the biomarker, a mean amount or concentration of the biomarker, and/or a median amount or concentration of the biomarker; and, in addition, "reference levels" of combinations of biomarkers may also be ratios of absolute or relative amounts or concentrations of two or more biomarkers with respect to each other. Appropriate positive and negative reference levels of biomarkers for sample quality or for a particular sample quality parameter may be determined by measuring levels of desired biomarkers in one or more appropriate samples, referred to herein as a "reference sample", and such reference levels may be tailored to specific sample types or populations of subjects (e.g., a reference level may be age-matched so that comparisons may be made between biomarker levels in samples from subjects of a certain age and reference levels for a particular sample quality parameter in a certain age group). Such reference levels may also be tailored to specific techniques that are used to measure levels of biomarkers in samples (e.g., LC-MS, GC-MS, etc.).

[0042] "Aberrant" or "aberrant metabolite" or "aberrant level" refers to a metabolite or level of said metabolite that is either above or below a defined reference standard or range. In some embodiments the metabolite level is log-transformed prior to statistical analysis. In some embodiments the level is not log-transformed. Any statistical method may be used to determine aberrant metabolites. For example, a p-value, an IQR value or a Z-score. In one embodiment a p-value is determined and a metabolite that differs from the reference with, for example, a p-value less than 0.1 is considered aberrant. In another example embodiment a metabolite that differs from the reference with a p-value less than 0.05 is considered aberrant. In one embodiment the statistic is a Z-score. For example, for some metabolites, a metabolite having a log transformed level with a Z-score of >1 or <-1 is deemed aberrant. In some embodiments, for some metabolites, a metabolite having a log transformed level with a Z-score of >1.5 or <-1.5 is aberrant. In some embodiments, for some metabolites, a

metabolite having a log transformed level with a Z-score of >2.0 or <-2.0 is aberrant. In some embodiments, different ranges of Z-scores are used for different metabolites. In some embodiments, the defined standard range may be based on an IQR of a level, instead of an IQR of a log transformed level. For example, for some metabolites, a log transformed level falling outside of at least 1.5*IQR (Inter Quartile Range) is aberrant. In another example, for some metabolites a log transformed level falling outside of at least 3.0*IQR is identified as aberrant.

[0043] "Non-biomarker compound" means a compound that is not differentially present in a biological sample from a subject or a group of subjects having a first phenotype (e.g., having a first disease) as compared to a biological sample from a subject or group of subjects having a second phenotype (e.g., not having the first disease). Such non-biomarker compounds may, however, be biomarkers in a biological sample from a subject or a group of subjects having a third phenotype (e.g., having a second disease) as compared to the first phenotype (e.g., having the first disease) or the second phenotype (e.g., not having the first disease).

[0044] "Metabolic profile", or "small molecule profile", means a complete or partial inventory of small molecules within a targeted cell, tissue, organ, organism, or fraction thereof (e.g., cellular compartment). The inventory may include the quantity and/or type of small molecules present. The "small molecule profile" may be determined using a single technique or multiple different techniques. A small molecule profile of the sample may include information regarding presence of, absence of, and a level of each of a plurality of small molecules in the sample. In some embodiments, the plurality of small molecules may include more than 25 small molecules, more than 50 small molecules, more than 100 small molecules, more than 200 small molecules, more than 300 small molecules, more than 400 small molecules, more than 500 small molecules, more than 600 small molecules, more than 700 small molecules, more than 800 small molecules, more than 900 small molecules, more than 1000 small molecules, more than 1100 small molecules, more than 1200 small molecules more than 1300 small molecules, more than 1400 small molecules, more than 1500 small molecules, more than 2000 small molecules, more than 3000 small molecules, more than 4000 small molecules more than 5000 small molecules, more than 6000 small molecules, or more than 7000 small molecules. In other embodiments, the plurality of small molecules may include 25-25,000 small

molecules, 50-25,000 small molecules, 100-25,000 small molecules, 200-25,000 small molecules, 300-25,000 small molecules, 400-25,000 small molecules, 500-25,000 small molecules, 600-25,000 small molecules, 700-25,000 small molecules, 800-25,000 small molecules, 900-25,000 small molecules, 1000-25,000 small molecules, 1100-25,000 small molecules, 1200-25,000 small molecules, or 1300-25,000 small molecules.

[0045] "Metabolome" means all of the small molecules present in a given organism.

[0046] "Sample quality" refers to one or more defined requirements for a sample based on an intended use of the sample. The requirements may be defined by a protocol such as a sample handling or a subject compliance protocol.

[0047] A "sample quality parameter" or "quality parameter" refers to one criterion that can be measured to determine if a sample is of suitable quality for an intended use. A sample quality parameter may be associated with protocol compliance and may indicate whether the sample has been handled according to a defined protocol (i.e., compliant with a sample handling protocol) or not and/or whether a subject from whom the sample was obtained was in compliance with a defined protocol (i.e., compliance with a protocol for the subject) or not. Example sample quality parameters related to sample handling include sample collection, collection tubes, collection tube additives, sample processing time, processing incubation temperature, time in storage, storage temperature, and freeze-thaw cycles. Example sample quality parameters related to subject protocol compliance include fasting status, medication and supplement status, smoking status, diet, and exercise compliance.

[0048] Sample "intended use" refers to the purpose for which the sample was obtained. The intended use of a sample may be experimental (e.g., global metabolomic discovery study, validation study (global or targeted), hypothesis testing, hypothesis generating, associating with a gene for determining significance of variants with unknown significance) or informational (e.g., diagnostic, health assessment, precision medicine). Informational use may refer to test panels that include one or more diagnostic tests or one or more health assessments. The intended use may refer to analysis of an individual sample or to analysis of each sample in studies of large cohorts. In some embodiments, the intended use may pertain to use of archival samples which have been collected and stored for long periods of time and

for which handling protocols are uncertain or unavailable. In some embodiments the intended use may comprise performing metabolic profiling of the sample. In some embodiments the intended use may comprise performing biochemical analysis of the sample.

[0049] "Biochemical analysis" refers to the measurement of at least 1 and no more than 24 biochemicals in a sample.

[0050] "Acceptance criteria" refers to a standard used to determine if a sample is in sufficient compliance with a protocol to be suitable for its intended use. Acceptance criteria may be based on one or more sample quality biomarkers or one or more sample quality parameters. The biomarkers or parameters may be selected automatically (e.g., pre-selected default biomarkers, pre-selected default parameters) or may be modified manually by the analyst according to the intended use of the sample. The biomarkers or parameters selected may be based on the intended use of the sample. The biomarker or parameter may be associated with a threshold value that may be selected automatically (i.e., default threshold value) or may be set by the experimentalist or clinician. The acceptance criteria may use a composite score for one or more quality biomarkers or may use a composite score (e.g., a QPS) for one or more quality parameters to assess the sample quality or may use a composite score calculated from one or more selected QPS.

[0051] An "acceptable" sample or sample quality parameter refers to a sample or sample quality parameter that meets acceptance criteria for an intended use. An acceptable sample or sample parameter indicates compliance with a protocol for the sample or sample parameter.

[0052] An "unacceptable" sample or sample quality parameter refers to a sample or sample quality parameter that does not meet acceptance criteria for an intended use. An unacceptable sample or sample parameter indicates non-compliance with a protocol for the sample or sample parameter.

[0053] A "marginal" quality sample refers to a sample that meets some, but not all of the acceptance criteria for the intended use; marginal samples may be subject to additional / corrective analysis or may be removed from the analysis.

[0054] A "sample quality composite score" or "composite score" refers to a value calculated using the levels of one or more sample quality biomarkers. The composite score may be calculated for the sample as a whole (e.g., overall composite score) or

may be calculated for one or more sample quality parameters. When the score is calculated for a particular sample quality parameter, the term "quality parameter score" or "QPS" is used. One or more QPS may be combined to produce an overall quality score for the sample. When using QPS to calculate an overall composite score, the one or more QPS used for calculating the overall score may be selected by an analyst and the quality parameters selected may be based upon the intended use of the sample.

I. Biomarkers Indicative of Sample Quality

[0055] Novel biomarkers characteristic of particular sample quality parameters were identified using metabolomic profiling. Generally, metabolomic profiles were determined for biological samples obtained from samples handled according to (e.g., in compliance with) a sample collection protocol for a particular sample quality parameter, as well as from other samples not handled according to or in compliance with the sample collection protocol. Exemplary sampling considerations may include sample processing considerations such as sample storage, sample incubation, and freeze/thaw cycles, and subject non-compliance considerations such as fasting status, medication and supplement status, smoking status, and exercise compliance. The metabolomic profile for samples handled according to protocol was compared to the metabolomic profile for samples not handled according to protocol. Those molecules differentially present (*e.g.*, those molecules differentially present at a level that is statistically significant) in the metabolomic profile of samples handled according to or in compliance with the protocol as compared to samples not handled according to or in compliance with the protocol were identified as biomarkers to distinguish those groups. Similarly, biomarkers of subject compliance were identified by comparing the metabolic profile of subjects in compliance with the protocol with the metabolic profile of non-compliant subjects. Detection of a level of one or more biomarkers identified in this manner can be used to assess the quality of a sample to determine if the sample is acceptable for its intended use. Said biomarkers can be detected in a sample individually, in subsets or in combinations thereof (*e.g.*, as part of a profile).

[0056] The biomarkers are discussed in more detail herein. The biomarkers listed in Tables 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and/or 26 and the biomarkers cysteinylglycine, sphingosine 1-phosphate,

sphinganine-1-phosphate, ergothioneine and combinations thereof may be used to assess the quality of a sample. Although the markers are listed separately in Tables 1-26, one of ordinary skill in the art will understand that assessments of sample quality may be performed by combining markers found in different combinations. It will be understood that the biochemical may be ordered in any way in the Tables, for example, by significance, alphabetically, by quality parameter, by biochemical superpathway and/or subpathway, etc.

A. Sample Processing Biomarkers

[0057] The level of a metabolite in a sample may be affected by the way the sample is processed after it has been collected from a subject. Biomarkers of sample processing were identified by comparing several sample processing conditions.

Sample Incubation and Sample Separation Biomarkers

[0058] One aspect of sample processing is related to how long the sample is held (incubated) at room temperature (RT) or at 4°C (e.g., on ice or refrigerated) prior to freezing. Biomarkers of processing related to incubation time were discovered by comparing the levels of metabolites in samples that were immediately frozen after collection with samples that were held for various times before freezing. Another aspect of sample processing is the time interval between collection and serum or plasma separation from whole blood. Biomarkers of processing related to separation time of blood were discovered by comparing samples that were separated immediately after collection with samples incubated as whole blood prior to separation. Biomarkers for use in the methods disclosed herein for assessing the sample quality parameter related to sample processing include those listed in Tables 1, 2, 3, 4, 5, and/or 6. As seen in the tables, the biomarkers may include adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine, arginine, ornithine, spermine, spermidine, hypoxanthine, xanthine, oxidized glutathione, cysteine-glutathione disulfide, 5-oxoproline, thymine, 2'-deoxyuridine, 2'-deoxyinosine, 3-hydroxypropanoate, 4-phenylbutyrate, caproate (6:0), benzoate, stearoylcarnitine (C18), succinate, pyruvate, lactate, fumarate, malate, glutamate, α-ketoglutarate, linoleoylcarnitine, oleoylcarnitine, palmitoylcarnitine, myristoylcarnitine, adenosine, inosine, sphingosine, sphinganine, oleic ethanolamide (oleoyl ethanolamide),

palmitoyl ethanolamide, and combinations and subsets thereof. In one embodiment, the one or more biomarkers may be selected from the group consisting of adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine, and combinations and subsets thereof.

Sample Storage Biomarkers

[0059] The level of a metabolite in a sample may be affected by sample storage conditions. Biomarkers of sample storage were identified by comparing several sample storage conditions. One aspect of sample quality related to sample storage is the storage temperature. Another aspect is the time in storage. Biomarkers for use in the methods disclosed herein for assessing sample storage include those listed in Tables 7, 8, 9, and/or 18. As seen in the tables, the biomarkers may include α-ketoglutarate, acetylcarnitine, propionylcarnitine, caproate (6:0), 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, 3-methyl-2-oxobutyrate, gamma-glutamyl-leucine, gamma-glutamyl-valine, glutamine, methionine and combinations and subsets thereof. In one embodiment, the one or more biomarkers may be selected from the group consisting of 4-methyl-2-oxopentanoate, acetylcarnitine, α-ketoglutarate, caproate (6:0), gamma-glutamyl-leucine, glutamine, methionine, and combinations and subsets thereof.

Freeze/thaw cycles Biomarkers

[0060] The metabolite levels in a sample may be affected by the number a freeze-thaw cycles that a sample undergoes. Biomarkers for use in the methods disclosed herein for assessing sample freeze-thaw cycles include those listed in Tables 10, 11, and/or 12. As seen in the tables, the biomarkers may include adenosine, succinate, 13-HODE + 9-HODE, 3-hydroxy-2-ethylpropionate, caproate (6:0), docosahexaenoate (DHA; 22:6n3), eicosapentaenoate (EPA; 20:5n3), 2'-deoxyuridine, phenyllactate (PLA), alpha-hydroxyisovalerate, arachidonate (20:4n6), gamma-glutamylmethionine, inosine, isoleucine, myristoleate (14:1n5), phenylacetate, stearoylcarnitine (C18), 2-methylcitrate, decanoylcarnitine (C10), thymine, acetylcarnitine (C2), hexanoylcarnitine (C6), palmitoylcarnitine (C16), myristoylcarnitine, phenylpyruvate, argininosuccinate, and combinations and subsets thereof.

B. Subject Compliance Biomarkers

[0061] The metabolite levels in a sample may be affected by the compliance of a subject with a given protocol. Exemplary aspects of sample quality related to subject compliance include: fasting status, medication and supplement status, smoking status, diet, and exercise compliance.

Fasting Status Biomarkers

[0062] Biomarkers of sample quality related to fasting status were discovered by comparing the levels of metabolites in samples from subjects that were fasted with samples from subjects that were fed. Biomarkers for use in the methods disclosed herein for assessing the fasting status of an individual include those listed in Tables 13, 14, 15, 16, and/or 17. As seen in the tables, the biomarkers may include methionine sulfoxide, glycocholate, dopamine sulfate, azelate, uridine, xanthine, mannose, palmitoleate, and combinations and subsets thereof.

II. Methods of Assessing Sample Quality Using Biomarkers

General description of the method

[0063] An assessment of sample quality can be made or facilitated by analyzing a sample to determine the level of one or more biomarkers selected from Tables 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and 26 (including combinations and subsets thereof) and comparing the level(s) of the biomarkers in the sample to sample quality-positive and/or sample quality-negative reference levels of biomarkers. As mentioned above, the biomarkers listed in different tables may be combined in any combination to perform an assessment of sample quality. As seen in the tables, the biomarkers may include 3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, 4-phenylbutyrate, acetylcarnitine, adenosine, adenosine 5'-monophosphate (AMP), alpha-hydroxyisocaproate, alpha-ketoglutarate, azelate (nonanedioate), caproate (6:0), cys-gly oxidized, dopamine sulfate, gamma-glutamylleucine, gamma-glutamylvaline, glucose, glutamine, glycocholate, hexanoylcarnitine (C6), mannose, methionine, methionine sulfoxide, palmitoleate (16:1n7), propionylcarnitine, taurine, uridine, xanthine. For example, the level of one biomarker, two or more biomarkers, three or more biomarkers, four or more biomarkers, five or more biomarkers, six or more biomarkers, seven or more biomarkers, eight or more biomarkers, nine or more

biomarkers, ten or more biomarkers, etc., including a combination of all of the biomarkers in the group or any fraction thereof, may be determined and used in such methods. Determining the level(s) of combinations of the biomarkers may allow greater sensitivity and specificity in the assessment of sample quality, and may allow better differentiation of sample quality parameters. In one embodiment, the level(s) of one or more sample quality biomarkers may be assessed in combination with the level(s) of one or more currently used sample quality biomarkers or parameters. In an embodiment, ratios of the one or more biomarkers may be used to assess sample quality. Further, one or more ratios of biomarkers may be used in combination with one or more biomarkers to assess sample quality.

[0064] Any suitable method may be used to analyze the biological sample in order to determine the level(s) of the one or more biomarkers in the sample. Suitable methods include, but are not limited to, chromatography (*e.g.*, HPLC, gas chromatography, liquid chromatography), mass spectrometry (*e.g.*, MS, MS-MS), enzyme-linked immunosorbent assay (ELISA), antibody linkage, other immunochemical techniques, and combinations thereof. Further, the level(s) of the one or more biomarkers may be measured indirectly, for example, by using an assay that measures the level of a compound (or compounds) that correlates with the level of the biomarker(s) that are desired to be measured. The level(s) of the one or more biomarkers may also be determined as part of a biomarker profile, *e.g.*, a metabolomic profile, using, for example, the methods set forth herein.

Comparison to References

[0065] After the level(s) of the one or more sample quality biomarkers in a sample are determined, the measured level(s) are compared to reference levels characteristic of a sample in compliance with a protocol (*i.e.*, "positive" or "acceptable" sample quality), and/or reference levels characteristic of a sample not in compliance with a protocol (*i.e.*, "negative" or "not acceptable" sample quality). In some embodiments, the measured levels are compared to reference levels for one or more quality parameters. In some embodiments, the measured levels are compared to reference levels for all quality parameters. In some embodiments, a sample is of acceptable quality based on criteria set by the experimentalist or clinician. Levels of the one or more biomarkers matching the reference levels characteristic of a sample

positive for sample quality or a sample quality parameter (*e.g.*, levels that are the same as the reference levels, substantially the same as the reference levels, not statistically different from the reference levels, within an established range of the reference levels, etc.) are indicative of acceptable sample quality or quality parameter (in compliance with protocol). Levels of the one or more biomarkers matching the reference levels characteristic of unacceptable sample quality (out of compliance with protocol) (*e.g.*, levels that are the same as the reference levels, substantially the same as the reference levels, not statistically different from the reference levels, within an established range of the reference levels, etc.) are indicative of unacceptable sample quality or quality parameter (out of compliance with one or more protocol parameters). In addition, levels of the one or more biomarkers that are differentially present (*e.g.,* at a level that is statistically significant) in the sample, as compared to reference levels characteristic of a sample acceptable (positive) for sample quality are indicative of unacceptable sample quality or quality parameter. Levels of the one or more biomarkers that are differentially present (*e.g.,* at a level that is statistically significant) in the sample, as compared to reference levels characteristic of a sample negative for sample quality, are indicative of acceptable sample quality or quality parameter. In some embodiments, determination of acceptable or unacceptable sample quality may be determined based on a particular sample quality parameter or a combination of sample quality parameters. In some embodiments, the levels of biomarkers in a test sample compared to reference levels identifies one or more affected sample quality parameters in the test sample.

[0066] The level(s) of the one or more biomarkers may be compared to sample acceptable and/or unacceptable (quality-positive and/or quality-negative) reference levels using various techniques, including a simple comparison of the level(s) of the one or more biomarkers in the biological sample to sample quality-positive and/or sample quality-negative reference levels. The level(s) of the one or more biomarkers in the sample may also be compared to sample quality-positive and/or sample quality-negative reference levels using one or more statistical analyses (e.g., t-test, Welch's T-test, Wilcoxon's rank sum test, Z-score, random forest). In various embodiments, such comparisons may be made manually, by an automated system, or by an automated system with manual verification.

[0067] Ratios of biomarkers may be used to assess sample quality. For example,

ratios of glucose to pyruvate, glucose to lactate, glucose to succinate, glucose to fumarate, glucose to malate, arginine to ornithine, arginine to spermine, or arginine to spermidine may be used to assess sample quality. One or more ratios of biomarkers may be used in combination with one or more biomarkers to assess sample quality. In one aspect, two or more biomarkers or ratios of biomarkers may be used to assess sample quality.

[0068] In one embodiment, the biomarkers may be classified according to biochemical pathway, and assessment of sample quality can be made or facilitated by analyzing a sample to determine the level(s) of one or more biomarkers selected from one or more biochemical pathways and comparing the level(s) of the one or more biomarkers in the sample to sample quality reference levels of the one or more biomarkers in order to assess the quality of the sample. For example, the biochemical pathways and the one or more biomarkers associated with each pathway may be: (a) urea cycle metabolism (e.g., arginine, ornithine, spermine, spermidine); (b) purine degradation (e.g., hypoxanthine, xanthine, AMP, adenosine, inosine); (c) glutathione metabolism (e.g., oxidized glutathione, cys-gly oxidized, cysteine-glutathione disulfide, 5-oxoproline); (d) thymine metabolism (e.g., thymine, 2'-deoxyuridine); (e) energy metabolism (e.g., glucose, pyruvate, lactate, succinate, fumarate, malate, α-ketoglutarate, linoleoylcarnitine, oleoylcarnitine, palmitoylcarnitine, stearoylcarnitine, myristoylcarnitine); (f) sphingolipid metabolism (e.g., sphingosine, sphinganine, oleic ethanolamide (oleoyl ethanolamide), palmitoyl ethanolamide). In another aspect, two or more biomarkers or ratios of biomarkers are selected from the biomarkers listed in two or more of the biochemical pathways in groups a-f.

Composite Score

[0069] A sample quality composite score may be used to assess sample quality. The sample quality score may be an overall quality score (QS) generated for the sample based on one or more biomarkers. In another aspect, the sample quality score may be generated based on one or more biomarkers for one or more quality parameters to generate one or more quality parameter scores (QPS). The analyst may select an overall quality score (QS) or a quality score for one or more quality parameters (QPS). The selection may be based on the intended use of the sample. In

this regard, for some uses a single overall quality score may be sufficient while other intended uses may require the sample quality assessment to be based on one or more selected quality parameters. Furthermore, the parameters used in the sample quality assessment may vary with the intended use of the sample. For example, for some intended uses, the composite score may be calculated using levels of biomarkers for sample handling without regard for subject compliance biomarkers. For other intended uses, the composite score may be calculated using levels of a subset of sample handling biomarkers (e.g., storage temperature and incubation temperature, or freeze-thaw cycles). In still other intended uses, subject compliance biomarkers may be used to generate a composite score. In yet other intended uses, the composite score may be based on combinations of one or more subject compliance and one or more sample handling biomarkers. In one example, the levels of the biomarkers adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, and taurine are used to generate a composite score.

[0070] In some embodiments, the standard range for the reference small molecule profile may be based on a composite score. The method may include generating a sample quality composite score based on a combination of data from one or more of the biomarkers or ratios of biomarkers for sample quality. In some aspects, the biomarkers used to calculate the composite score may be weighted and the coefficients (weights) may be determined using, for example, regression analysis, PLS, LDA or other formal statistical methods.

Reporting

[0071] A report listing all sample quality biomarkers determined to be aberrant in the test sample may be generated. In some embodiments, the biomarkers are automatically sorted by sample quality parameter. In other embodiments, a visualization of results showing sample quality parameters and individual sample quality biomarkers is generated. In yet other embodiments, affected sample quality parameters are identified. Thus, in some embodiments, the method can comprise analyzing and reporting the information obtained from the entire metabolic profile and can identify aberrant sample quality biomarkers and associated sample quality parameters to determine affected sample quality parameters, without relying on specific sample quality tests comprised of a single internal standard for quality

assurance or quality control for specific sample quality parameters.

Sample Quality Parameters

[0072] The biomarkers for sample quality may indicate that a particular sample quality parameter is affected. For example, the sample quality parameter may provide an indication that the sample is not in compliance with a protocol. Protocol non-compliance may include issues with sample handling. Exemplary sample quality parameters related to sample handling may include: processing time, time in storage, storage temperature, freeze-thaw cycles, and collection tube additives. Protocol non-compliance may also include subject non-compliance. Exemplary sample quality parameters related to subject compliance include: fasting status, medication and supplement status, smoking status, and exercise compliance.

Assessing Sample Processing

[0073] An exemplary sample quality parameter is sample processing. Thus, an assessment of sample quality can be performed by analyzing a sample to determine the level of one or more biomarkers selected from Tables 1, 2, 3, 4, 5, and/or 6. The one or more biomarkers may include adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine, arginine, ornithine, spermine, spermidine, hypoxanthine, xanthine, oxidized glutathione, cysteine-glutathione disulfide, 5-oxoproline, thymine, 2'-deoxyuridine, 2'-deoxyinosine, 3-hydroxypropanoate, 4-phenylbutyrate, caproate (6:0), benzoate, stearoylcarnitine (C18), succinate, pyruvate, lactate, fumarate, malate, glutamate, α-ketoglutarate, linoleoylcarnitine, oleoylcarnitine, palmitoylcarnitine, myristoylcarnitine, adenosine, inosine, sphingosine, sphinganine, oleic ethanolamide (oleoyl ethanolamide), palmitoyl ethanolamide, and combinations and subsets thereof. The level(s) of the one or more biomarkers in the sample can be compared to sample quality-positive and/or sample quality-negative reference levels of the one or more biomarkers. In one embodiment, the one or more biomarkers may be selected from the group consisting of adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine, and combinations and subsets thereof. In an exemplary embodiment, an increase in the levels of adenosine 5'-monophosphate (AMP), cys-gly oxidized, and taurine, and a decrease in the level of glucose indicate prolonged sample processing time. In one example, the levels of the biomarkers adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, and taurine can be used to

generate a composite score. In another example, the levels of the biomarkers adenosine 5'-monophosphate (AMP), glucose, and taurine can be used to generate a different composite score. The composite score may comprise biomarkers that are weighted.

[0074] One aspect of sample processing is the time the sample is held prior to freezing. Assessment of sample quality related to the time the sample was held prior to freezing can be made or facilitated by analyzing a sample to determine the level of one or more biomarkers selected from Tables 1, 2, 3, 4, and/or 5, and comparing the level(s) of the one or more biomarkers in the sample to sample quality-positive and/or sample quality-negative reference levels of the biomarkers. The biomarkers may include adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine, arginine, ornithine, spermine, spermidine, hypoxanthine, xanthine, oxidized glutathione, cysteine-glutathione disulfide, 5-oxoproline, thymine, 2'-deoxyuridine, 2'-deoxyinosine, 3-hydroxypropanoate, 4-phenylbutyrate, caproate (6:0), benzoate, stearoylcarnitine (C18), succinate, and combinations and subsets thereof.

[0075] Another aspect of sample processing is the time interval between sample collection and serum or plasma separation from whole blood. Assessment of sample quality related to this time interval can be made or facilitated by analyzing a sample to determine the level of one or more biomarkers selected from Tables 1 and/or 2 and comparing the level(s) of the one or more biomarkers in the sample to sample quality-positive and/or sample quality-negative reference levels of biomarkers. The biomarkers may include adenosine 5'-monophosphate (AMP), cys-gly oxidized, glucose, taurine, pyruvate, lactate, succinate, fumarate, malate, glutamate, α-ketoglutarate, linoleoylcarnitine, oleoylcarnitine, palmitoylcarnitine, stearoylcarnitine, myristoylcarnitine, arginine, ornithine, spermine, spermidine, adenosine, xanthine, inosine, sphingosine, sphinganine, oleic ethanolamide (oleoyl ethanolamide), palmitoyl ethanolamide, and combinations and subsets thereof.

Assessing Sample Storage

[0076] Another sample quality parameter is sample storage. Assessment of sample quality related to sample storage can be made or facilitated by analyzing a sample to determine the level of one or more biomarkers selected from Tables 7, 8, 9, and/or 18 and comparing the level(s) of the one or more biomarkers in the sample to

sample quality-positive and/or sample quality-negative reference levels of the biomarkers. The biomarkers may include α-ketoglutarate, acetylcarnitine, propionylcarnitine, caproate (6:0), 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, 3-methyl-2-oxobutyrate, gamma-glutamyl-leucine, gamma-glutamyl-valine, glutamine, methionine and combinations and subsets thereof. In one embodiment, the one or more biomarkers may be selected from the group consisting of α-ketoglutarate, acetylcarnitine, propionylcarnitine, caproate (6:0), 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, 3-methyl-2-oxobutyrate, gamma-glutamyl-leucine, gamma-glutamyl-valine, glutamine, methionine, and combinations and subsets thereof. In another embodiment, an increase in the levels of caproate (6:0), gamma-glutamyl-leucine, gamma-glutamyl-valine, and a decrease in the levels of α-ketoglutarate, acetylcarnitine, propionylcarnitine, 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, 3-methyl-2-oxobutyrate, glutamine, methionine, indicate that the sample was stored at -20°C instead of -80°C. The biomarkers may be used to generate a composite score. For example, the levels of the biomarkers 4-methyl-2-oxopentanoate, acetylcarnitine, α-ketoglutarate, caproate (6:0), gamma-glutamyl-leucine, glutamine, and methionine, may be used to generate a composite score. In another example, the levels of the biomarkers 4-methyl-2-oxopentanoate, α-ketoglutarate, caproate (6:0), gamma-glutamyl-leucine, glutamine, and methionine, may be used to generate a composite score. The composite score may comprise biomarkers that are weighted.

[0077] The sample quality related to sample storage may be affected by the storage temperature and the time in storage.

Assessing Effect of Freeze-thaw Cycles

[0078] Another parameter that can affect the quality of a sample is the number of freeze-thaw cycles. In that regard, assessment of sample quality related to freeze-thaw cycles can be made or facilitated by analyzing a sample to determine the level of one or more biomarkers selected from Tables 10, 11, and/or 12 and comparing the level(s) of the biomarkers in the sample to sample quality-positive and/or sample quality-negative reference levels of the biomarkers. The biomarkers may include adenosine, succinate, 13-HODE + 9-HODE, 3-hydroxy-2-ethylpropionate, caproate (6:0), docosahexaenoate (DHA; 22:6n3), eicosapentaenoate (EPA; 20:5n3), 2'-

deoxyuridine, phenyllactate (PLA), alpha-hydroxyisovalerate, arachidonate (20:4n6), gamma-glutamylmethionine, inosine, isoleucine, myristoleate (14:1n5), phenylacetate, stearoylcarnitine (C18), 2-methylcitrate, decanoylcarnitine (C10), thymine, acetylcarnitine (C2), hexanoylcarnitine (C6), palmitoylcarnitine (C16), myristoylcarnitine, phenylpyruvate, argininosuccinate, and combinations and subsets thereof. In an exemplary embodiment, the sample is a blood or serum sample, and an increase in the levels of acetylcarnitine (C2), caproate (6:0), decanoylcarnitine (C10), succinate and a decrease in the levels of 13-HODE + 9-HODE, 2'-deoxyuridine, 2-methylcitrate, 3-hydroxy-2-ethylpropionate, adenosine, alpha-hydroxyisovalerate, arachidonate (20:4n6), docosahexaenoate (DHA; 22:6n3), eicosapentaenoate (EPA; 20:5n3), gamma-glutamylmethionine, hexanoylcarnitine (C6), inosine, isoleucine, myristoleate (14:1n5), myristoylcarnitine, palmitoylcarnitine (C16), phenylacetate, phenyllactate (PLA), stearoylcarnitine (C18), thymine indicate that plasma or serum quality is unacceptable for the freeze-thaw quality parameter. In another exemplary embodiment, the sample is a urine sample, and an increase in the levels of 2-methylcitrate, argininosuccinate, inosine, and a decrease in the levels of laurylcarnitine (C12), phenylpyruvate indicate that the urine quality is unacceptable for the freeze-thaw quality parameter. The biomarkers may be used to generate a composite score. The composite score may comprise biomarkers that may be weighted.

Assessing Fasting Status

[0079] Sample quality can be assessed by analyzing the fasting status of the subject from whom a sample is obtained. In this regard, sample quality can be assessed by analyzing a sample to determine the level of one or more biomarkers selected from Tables 13, 14, 15, 16, and/or 17 and comparing the level(s) of the biomarkers in the sample to sample quality-positive and/or sample quality-negative reference levels of biomarkers. The biomarkers may include methionine sulfoxide, glycocholate, dopamine sulfate, azelate, uridine, xanthine, mannose, palmitoleate, and combinations and subsets thereof. In one embodiment, an increase in the levels of methionine sulfoxide, glycocholate, dopamine sulfate, azelate, and a decrease in the levels of uridine, xanthine, mannose, palmitoleate, indicate that the subject was not fasted (i.e., was fed). The biomarkers may be used to generate a composite score.

For example, the levels of the biomarkers methionine sulfoxide, glycocholate, dopamine sulfate, azelate, uridine, xanthine, mannose, and palmitoleate can be used to generate a composite score. The composite score may comprise biomarkers that are weighted.

III. System, Method, Computer Program Product

[0080] Various systems and apparatuses are used in assessing sample quality according to the methods described herein. Figure 1 provides a flow chart showing an example of how such systems and apparatuses may be used.

[0081] Figure 1 is a block diagram of a method 10 for assessing sample quality. In some embodiments, the method 10 includes generating a sample quality composite score based on a weighted combination of data from one or more of the quality biomarkers, and obtaining sample quality information from the database based on the levels of the quality biomarkers and includes obtaining sample quality information from the database based on the generated sample quality composite score. The obtained sample quality information is stored (step 20). The stored sample quality information can include one or more of: i) an identification of at least one sample quality parameter associated with the quality biomarkers; ii) an identification of at least one aspect of the intended use associated with one or more of the quality biomarkers having an aberrant level; and iii) an identification of at least one recommended approach for evaluating the sample (e.g., accept sample, reject sample/remove from analysis, modify analysis to take affected parameter into account (e.g., apply a "correction factor" to adjust level of affected metabolites)). Thus, the stored sample quality information facilitates sample analysis. In some embodiments, the method may further include storing a graphical representation of the obtained sample quality information (step 22). The dashed lines around the boxes for steps 19 and 22 in Figure 1 indicate that these steps need not be present in all embodiments. The method 10 may be applied to a single sample or to any number of samples simultaneously.

[0082] As shown in Figure 1, a sample is obtained (step 12). The sample may be obtained from any source. A small molecule profile of the sample is generated including information regarding presence of, absence of, and, if appropriate, a level of each of a plurality of small molecules in the sample (step 14).

[0083] Generation of the small molecule profile of a sample requires analysis of its constituent biochemical small molecules. The analysis may include extracting at least some of the plurality of small molecules from the sample. Suitably, the analysis may be conducted using one or more different analytical techniques known in the art, for example, liquid chromatography (LC), high performance liquid chromatography (HPLC) (*see* Kristal, *et al. Anal. Biochem.* 263:18-25 (1998)), gas chromatography (GC), thin layer chromatography (TLC), electrochemical separation techniques (*see*, WO 99/27361, WO 92/13273, U.S. 5,290,420, U.S. 5,284,567, U.S. 5,104,639, U.S. 4,863,873, and U.S. RE32,920), refractive index spectroscopy (RI), ultra-violet spectroscopy (UV), fluorescent analysis, radiochemical analysis, near-infrared spectroscopy (Near-IR), nuclear magnetic resonance spectroscopy (NMR), light scattering analysis (LS), mass spectrometry (MS), tandem mass spectrometry (MS/MS^2), and combined methods such as gas-chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS), ultrahigh performance liquid chromatography/tandem mass spectrometry ($UHLC/MS/MS^2$) and gas-chromatography/tandem mass spectrometry ($GC/MS/MS^2$).

[0084] U.S. Patent No. 7,884,318, discloses systems, methods and computer-readable media for determining a composition of chemical constituents in a complex mixture, which may be employed for generating the small molecule profile of the sample, in accordance with some embodiments. U.S. Patent No. 7,884,318 is incorporated by reference herein in its entirety. As described in U.S. Patent No. 7,884,318, separation data and mass spectroscopy data are generated for a sample, and the generated separation and mass spectroscopy data is compared with a library of chemical information (a "chemical library") to determine small molecule chemical constituents of the sample.

[0085] U.S. Patent No. 7,561,975 and U.S. Patent No. 7,949,475, each of which is incorporated by reference herein in its entirety, provide additional disclosure regarding analysis for identification of small molecule constituents of a biological sample for multiple biological samples.

[0086] In some embodiments, multiple aliquots (e.g., two, three, four, five, six, etc.) of a single test sample may be tested. In some embodiments, aliquots from test samples from multiple different individuals are analyzed in the same run. In one

embodiment, the test samples may be run as individual samples, in duplicate, triplicate, quadruplicate, or etc.

[0087] In some embodiments, data analysis for generation of the small molecule profile is partially automated (e.g., with a person manually verifying the identifications of some or all small molecules based on the generated data). In some embodiments, data analysis for generation of the small molecule profile is fully automated.

[0088] The small molecule profile is compared to one or more reference small molecule profile(s) to identify sample quality biomarkers that are aberrant in the sample (step 16). The reference small molecule profile may include a standard range for each of a plurality of biomarkers. An aberrant biomarker is a biomarker with a level in the sample that is outside the standard range for the biomarker. In some embodiments, the reference small molecule profile is determined, at least in part, from statistical analysis of small molecules profiles generated for reference samples.

[0089] Sample quality information is obtained from a database based on the levels of the sample quality biomarkers (step 18). The database includes information associating an aberrant level of one or more biomarkers with information regarding sample quality or a sample quality parameter for each of the plurality of sample quality parameters. In methods in which a sample quality parameter-specific composite score is generated based on a weighted combination of data from one or more of the subset of quality biomarkers, the database further includes information associating a range of sample quality parameter-specific composite scores with information regarding the sample quality parameter for one or more of the sample quality parameters.

[0090] The obtained sample quality information is stored (step 20). The stored sample quality information can include one or more of: i) an identification of at least one sample quality parameter associated with the identified aberrant sample quality biomarkers; ii) an identification at least one intended use associated with the identified aberrant sample quality biomarkers; and iii) an identification of at least one approach for evaluating the sample based on the aberrant levels of the sample quality biomarkers. In some embodiments, the stored sample quality information includes an identification of at least one sample quality parameter associated with the identified aberrant sample quality biomarkers and one or more of an identification at least one

intended use associated with the identified sample quality biomarkers, and an identification of at least one approach for evaluating the sample based on the levels of the aberrant sample quality biomarkers. In some embodiments, the sample quality information may be stored in multiple databases (e.g., the sample analysis information may be stored in one database and the information with an identification of a sample quality parameter and information regarding approaches for evaluating the sample may be stored in another database).

[0091] Some embodiments include storing a graphical representation of some or all of the obtained sample quality information (step 22). In some embodiments, a graphical representation of the one or more sample quality parameters associated with the identified sample quality biomarkers is stored. As used herein, generation, storage and/or display of a graphical representation of some or all of the obtained sample quality information is also referred to as visualization of the information or visualization of the results.

[0092] The graphical representation may be stored in any known format for electronic storage of images (e.g., JPEG/JFIF, JPEG 2000, Exif, TIFF, RAW, GIF, BMP, PNG, PPM, PGM, PNM, WEBP, CGM, SVG, etc.) In some embodiments, the graphical representation(s) may be incorporated into a document and stored in a document file format (e.g., PDF, .ps, .doc, .docx, .ppt, .odt, .htm, .html, etc.).

[0093] In some embodiments, multiple types of sample quality information for the sample(s) may be compiled into a single document, which is referred to as a report herein. The report may include information regarding any or all of: a total number of different small molecule biochemicals detected; a total number of different sample quality biomarkers detected; identification of sample quality parameters associated with small molecules biomarkers; a listing of sample quality biomarkers associated with one or more sample quality parameters; identification of which sample quality biomarkers in the identified sample quality parameters were present in aberrant levels; identification of sample quality biomarkers present in aberrant levels and an indication of the levels; an indication of whether each aberrant sample quality biomarker is present at a level higher or lower than the standard range; an indication of a sample quality composite score for one or more sample quality parameters; an indication of whether the value for each sample quality parameter composite score is within the range of the reference score; a list of affected sample quality parameters; a

list of at least one recommended approach for evaluating the sample. The report may be stored in a suitable electronic file format. In some embodiments, the report of the results is provided to a researcher and/or health care provider in any suitable electronic or non-electronic (e.g., paper) form.

[0094] Figure 2 is a block diagram of a method 20 for assessing a sample for a plurality of sample quality parameters, in accordance with an embodiment. A sample is obtained (step 32). A small molecule profile of the sample is generated including information regarding presence of, absence of, and a level of each of a plurality of small molecules in the sample (step 34). The small molecule profile includes information regarding the levels of sample quality biomarkers for assessing sample quality. The levels of the sample quality biomarkers in the sample are compared to a reference small molecule profile that includes a standard range for a level of each of the sample quality biomarkers, and the aberrant small molecule biomarkers are identified (step 36).

[0095] If none of the sample quality biomarkers in the small molecule profile of the sample have an aberrant level, information is stored indicating that no aberrant levels were detected (step 37).

[0096] If any aberrant biomarker levels are detected, sample quality analysis proceeds, and levels of sample quality biomarkers, including the sample quality biomarkers having aberrant levels, are identified (step 38). Sample quality information is obtained from a database based on the levels of sample quality biomarkers (step 40). In some embodiments, obtaining sample quality information based on the levels of the sample quality biomarkers includes identifying one or more affected sample quality parameters (step 41). In some embodiments, the method includes generating a sample quality parameter-specific composite score based on a weighted combination of data from one or more of the sample quality biomarkers, and obtaining sample quality information from the database based on the levels of the sample quality biomarkers including obtaining sample quality information from the database based on the generated sample quality parameter-specific composite score. The sample quality biomarkers used to generate the composite score may or may not have aberrant levels. The database includes information associating a level of one or more of the plurality of sample quality biomarkers with information regarding a sample quality parameter for each of a plurality of sample quality parameters. The

obtained sample quality information is stored (step 42). The stored sample quality information includes one or more of: an identification of at least one sample quality parameter associated with the sample quality biomarkers, an identification at least one intended use associated with one or more of the sample quality biomarkers, and an identification of at least one recommended approach for evaluating the sample (e.g., analyze the sample with all measured small molecules, analyze the sample with selected measured small molecules, or remove the sample and all measured small molecules from analysis). In some embodiments, the method also includes storing a graphical representation of the obtained sample quality information (step 44). The dashed line around the boxes for steps 41 and 44 in Figure 2 indicates that these steps need not be present in all embodiments.

[0097] The steps of generating the small molecule profile of the sample, comparing the profile of the sample quality biomarkers in the sample to a reference small molecule profile to determine if any sample quality biomarkers in the sample have aberrant levels, identifying sample quality biomarkers having aberrant levels, obtaining sample quality information from a database based on levels of the sample quality biomarkers, storing an indication that no aberrant levels were found, storing obtained sample quality information, storing a graphical representation of obtained sample quality information may be performed in part or in whole using instructions executing on one or processors of one or more computing systems.

[0098] Some embodiments include storing computer-executable code with instructions for performing various steps of methods described herein.

[0099] Figure 3 is a block diagram of an exemplary computing device 100 that may be used to implement various steps of exemplary methods described herein. The computing device 100 includes one or more non-transitory computer-readable media for storing one or more computer-executable instructions or software for implementing exemplary embodiments. Suitably, the non-transitory computer-readable media may include, but are not limited to, one or more types of hardware memory, non-transitory tangible media (for example, one or more magnetic storage disks, one or more optical disks, one or more flash drives), and the like. For example, memory 106 included in the computing device 100 may store computer-readable and computer-executable instructions or software for implementing all or part of steps or methods described herein. The computing device 100 may also include a

configurable and/or programmable processor 102 and associated core 104, and optionally, one or more additional configurable and/or programmable processor(s) 102' and associated core(s) 104' (for example, in the case of computer systems having multiple processors/cores), for executing computer-readable and computer-executable instructions or software stored in the memory 106 and other programs for controlling system hardware. Processor 102 and processor(s) 102' may each be a single core processor or multiple core (104 and 104') processor.

[00100] Virtualization may be employed in the computing device 100 so that infrastructure and resources in the computing device may be shared dynamically. A virtual machine 114 may be provided to handle a process running on multiple processors so that the process appears to be using only one computing resource rather than multiple computing resources. Multiple virtual machines may also be used with one processor.

[00101] Memory 106 may include a computer system memory or random access memory, such as DRAM, SRAM, EDO RAM, and the like. Memory 106 may include other types of memory as well, or combinations thereof.

[00102] A user may interact with the computing device 100 through a visual display device 118, such as a computer monitor, which may display one or more graphical user interfaces that may be provided in accordance with exemplary embodiments. The computing device 100 may include other I/O devices for receiving input from a user, for example, a keyboard or any suitable multi-point touch interface 108, a pointing device 110 (e.g., a mouse), a microphone 128, and/or an image capturing device 132 (e.g., a camera or scanner). The multi-point touch interface 108 and the pointing device 110 may be coupled to the visual display device 118. The computing device 100 may include other suitable conventional I/O peripherals.

[00103] The computing device 100 may also include one or more storage devices 124, such as a hard-drive, CD-ROM, or other computer readable media, for storing data and computer-readable instructions and/or software for implementing exemplary embodiments described herein. Exemplary storage device 124 may also store one or more databases for storing any suitable information required to implement exemplary embodiments. For example, exemplary storage device 124 can store one or more databases 126 that include any or all of a chemical library, a reference small molecule profile, small molecules profiles of one or more reference samples, small molecule

profiles of one or more test samples, sample quality information associated with levels of one or more sample quality biomarkers for a plurality of sample quality parameters, information associating intended uses with one or more sample quality biomarkers, information associating a sample quality parameter with one or more sample quality biomarker levels for a plurality of sample quality parameters, and an identification of at least one recommended approach for evaluating the sample associated with one or more sample quality biomarker levels. In other embodiments, different databases for different steps may be associated with different computing devices. In some embodiments a database may be configured to automatically receive data corresponding to the level(s) of one or more sample quality biomarkers measured in a biological sample using an analytical device (e.g., a separation device coupled to a detection device, such as, for example, LC/MS, GC/MS) from the analytical device.

[00104] The one or more storage devices 124 may be used to store any or all of obtained sample quality information, graphical representations of obtained sample quality information, reports for a test sample, etc.

[00105] The one or more storage devices 124 and or the memory 106 may hold software 125 executable on the processor 102 for implementing an analysis facility that compares the small molecule biomarker profile of the sample to a reference small molecule biomarker profile that includes a standard range for a level of each of the plurality of sample quality biomarkers and identifies the sample quality biomarkers in the sample having aberrant levels.

[00106] The computing device 100 can include a network interface 112 configured to interface via one or more network devices 120 with one or more networks, for example, Local Area Network (LAN), Wide Area Network (WAN) or the Internet through a variety of connections including, but not limited to, standard telephone lines, LAN or WAN links (for example, 802.11, T1, T3, 56kb, X.25), broadband connections (for example, ISDN, Frame Relay, ATM), wireless connections, controller area network (CAN), or some combination of any or all of the above. In exemplary embodiments, the computing device 100 can include one or more antennas 130 to facilitate wireless communication (e.g., via the network interface) between the computing device 100 and a network. The network interface 112 may include a built-in network adapter, network interface card, PCMCIA network card, card bus network adapter, wireless network adapter, USB network adapter, modem or any other device

suitable for interfacing the computing device 100 to any type of network capable of communication and performing the operations described herein. Moreover, the computing device 100 may be any computer system, such as a workstation, desktop computer, server, laptop, handheld computer, tablet computer (e.g., the iPad™ tablet computer), mobile computing or communication device (e.g., the iPhone™ communication device), or other form of computing or telecommunications device that is capable of communication and that has sufficient processor power and memory capacity to perform the operations described herein. In some embodiments, the computing device 100 may communicate via a network with one or more computing devices or analytical devices used to obtain experimental data (e.g., a computing device associated with an LC/MS system, a computing device associated with a GC/MS system, an LC/MS system, a GC/MS system).

[00107] The computing device 100 may run any operating system 116, such as any of the versions of the Microsoft® Windows® operating systems, the different releases of the Unix and Linux operating systems, any version of the MacOS® for Macintosh computers, any embedded operating system, any real-time operating system, any open source operating system, any proprietary operating system, or any other operating system capable of running on the computing device and performing the operations described herein. In exemplary embodiments, the operating system 116 may be run in native mode or emulated mode. In an exemplary embodiment, the operating system 116 may be run on one or more cloud machine instances.

[00108] In describing exemplary embodiments, specific terminology is used for the sake of clarity. For purposes of description, each specific term is intended to at least include all technical and functional equivalents that operate in a similar manner to accomplish a similar purpose. Additionally, in some instances where a particular exemplary embodiment includes a plurality of system elements, device components or method steps, those elements, components or steps may be replaced with a single element, component or step. Likewise, a single element, component or step may be replaced with a plurality of elements, components or steps that serve the same purpose. Moreover, while exemplary embodiments have been shown and described with references to particular embodiments thereof, those of ordinary skill in the art shall understand that various substitutions and alterations in form and detail may be

made therein without departing from the scope of the invention. Further still, other embodiments, functions and advantages are also within the scope of the invention.

[00109] Exemplary block diagrams/flowcharts are provided herein for illustrative purposes and are non-limiting examples of methods. One of ordinary skill in the art will recognize that exemplary methods may include more or fewer steps than those illustrated in the exemplary block diagrams/flowcharts, and that the steps in the exemplary block diagrams/flowcharts may be performed in a different order than the order shown in the illustrative block diagrams/flowcharts.

IV. Kit

[00110] Any of the described methods, alone or in combination, may be performed using tools provided in the form of a kit. Kits may further comprise appropriate controls, standards and/or detection reagents. In an embodiment, the kit may include tools and reagents for the analysis of a blood-based sample. The kit may comprise a sample collection element and a vessel for storing the sample. For example, the kit may comprise a sample collection element, a retrieved sample collection receptacle, sample labels, sample barcodes, and instruction protocol. The instruction protocol may be provided as a printed form or booklet or on an electronic medium, such as, for example, a computer disk or other computer readable medium.

[00111] The kit may be used in accordance with the following exemplary method. A blood sample may be collected from the subject using a needle and syringe. The blood can then be extruded into a collection receptacle (e.g., a vial, a conical tube, etc.). The sample in the collection receptacle may then be subjected to metabolic profiling and intended use analysis. Barcodes and labels enable the sample identity and the analyses results to be tracked through the analysis.

[00112] The kit may further comprise a volumetric container. The volumetric container may be any container (i.e., a cup, vial, microfuge tube, microtiter plate etc.) suitable for holding a liquid sample. The volumetric container may optionally contain volumetric measurements which may be useful in measuring out a desirable amount of the sample or other reagents. The volumetric container may be made of any material (e.g., plastics, aluminum, stainless steel). The internal volume of the volumetric container depends on the type of sample to be collected. The volumetric

container can include a body and a cap. In some embodiments, the internal standard material may be attached to the cap. In some embodiments, the internal standard material may be coated on the internal volume of the body of the volumetric container.

[00113] In some embodiments, the volumetric container may additionally be configured for the type of sample collection contemplated and used for collection of the specimen. In other aspects, a specimen collection receptacle is separately provided in the kit, and may be in the form of a cup, vial, microfuge tube.

[00114] The kit may optionally comprise a transportation container. The transportation container may be any structure suitable for transportation of samples. The container is configured such that the sample material can be packed into the container and the container may be sealed.

[00115] The kit may optionally include an extraction solution.

EXAMPLES

[00116] The invention will be further explained by the following illustrative examples that are intended to be non-limiting.

I. General Methods

A. Sample Preparation.

[00117] Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a methanol extraction to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into five fractions: one for analysis by UPLC-MS/MS with positive ion mode electrospray ionization, one for analysis by UPLC-MS/MS with negative ion mode electrospray ionization, one for LC polar platform, one for analysis by GC-MS, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) under nitrogen to remove the organic solvent. For LC, the samples were stored under nitrogen overnight. For GC, the samples were dried under vacuum overnight. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS.

B. Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS).

[00118] LC/MS analysis used a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm). Extracts reconstituted in acidic conditions were gradient eluted from a C18 column using water and methanol containing 0.1% formic acid. The basic extracts were similarly eluted from C18 using methanol and water containing with 6.5mM Ammonium Bicarbonate. The third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion, and the scan range was from 80-1000 m/z.

C. Gas chromatography/Mass Spectrometry (GC/MS).

[00119] For GC/MS analysis, samples were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). The GC column was a 20m X 0.18 mm ID, with 5% phenyl; 95% dimethylsilicone phase. The temperature ramp was from 60° to 340° C in an 18 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization at unit mass resolution. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis.

D. Data Processing and Analysis.

[00120] For each biological matrix data set on each instrument, relative standard deviations (RSDs) of peak area were calculated for each internal standard to confirm

extraction efficiency, instrument performance, column integrity, chromatography, and mass calibration. Several of these internal standards serve as retention index (RI) markers and were checked for retention time and alignment. Modified versions of the software accompanying the UPLC-MS and GC-MS systems were used for peak detection and integration. The output from this processing generated a list of m/z ratios, retention times and area under the curve values. Software specified criteria for peak detection including thresholds for signal to noise ratio, height and width. Missing values, if any, were imputed with the observed minimum for that particular compound.

[00121] The biological data sets, including QC samples, were chromatographically aligned based on a retention index that utilizes internal standards assigned a fixed RI value. The RI of the experimental peak is determined by assuming a linear fit between flanking RI markers whose values do not change. The benefit of the RI is that it corrects for retention time drifts that are caused by systematic errors such as sample pH and column age. Each compound's RI was designated based on the elution relationship with its two lateral retention markers. Using an in-house software package, integrated, aligned peaks were matched against an in-house library (a chemical library) of authentic standards and routinely detected unknown compounds, which is specific to the positive, negative or GC-MS data collection method employed. Matches were based on retention index values, and the range of RI units varied relative to the detection platform. The experimental spectra were compared to the library spectra for the authentic standard and assigned forward and reverse scores. A perfect forward score would indicate that all ions in the experimental spectra were found in the library for the authentic standard at the correct ratios and a perfect reverse score would indicate that all authentic standard library ions were present in the experimental spectra and at correct ratios. The forward and reverse scores were compared and a MS/MS fragmentation spectral score was given for the proposed match. All matches were then manually reviewed by an analyst that approved or rejected each call based on the mass, RI, and scores to assess the call and manually approves the peaks if the call is retained the criteria above. However, manual review by an analyst is not required. In some embodiments the matching process is completely automated.

[00122] Further details regarding a chemical library, a method for matching

integrated aligned peaks for identification of named compounds and routinely detected unknown compounds, and computer-readable code for identifying small molecules in a sample may be found in U.S. Patent No. 7,561,975, which is incorporated by reference herein in its entirety.

E. Quality Control.

[00123] Methods were put in place to control the quality of sample extraction and instrument run procedures. These methods occur after sample collection and processing. From the biological samples, aliquots of each of the individual samples were combined to make technical replicates, which were extracted as described above. Extracts of this pooled sample were injected six times for each data set on each instrument to assess process variability. As an additional quality control, five water aliquots were also extracted as part of the sample set on each instrument to serve as process blanks for artifact identification. All QC samples included the instrument internal standards to assess extraction efficiency, and instrument performance and to serve as retention index markers for ion identification. The standards were isotopically labeled or otherwise exogenous molecules chosen so as not to obstruct detection of intrinsic ions.

Example 1. Biomarkers for assessing sample processing conditions

[00124] Biomarkers for assessing the effects related to commonly encountered sample processing conditions were identified in several studies. In one study, whole blood samples were incubated at room temperature for various times prior to separating the plasma and freezing. In another study, whole blood samples were separated following collection and the resulting plasma samples were incubated at 4°C for various times before freezing. In yet another study, whole blood samples were separated following collection and the resulting plasma samples were incubated at ambient temperature for various times before freezing. The results of each study are presented below.

[00125] In the first study, five whole blood samples were collected, divided into aliquots, and held at room temperature for 0h (Control), 0.5h, 1h, 2h, 4h, and 24h prior to plasma separation and freezing. The fold-change of each biomarker was calculated as the ratio of the mean level of the biomarker in the samples at each time point compared to the 0h time point control samples, and the p-value was determined

using matched pairs t-tests. The data is presented in Table 1. Table 1 includes, for each biomarker, the biochemical name of the biomarker, the fold change (FC) of the biomarkers in samples incubated at room temperature for 0.5h, 1h, 2h, 4h, and 24 hours prior to separation compared to control (0h time point) samples and the p-value. Thus, the study provides biomarkers related to the time interval between collection time and plasma separation time.

Table 1. Biomarkers of sample processing – Time incubated as whole blood prior to separation

	0.5h/0h		1h/0h		2h/0h		4h/0h		24h/0h	
Biochemical Name	**FC**	**p value**	**FC**	**p value**	**FC**	**p value**	**FC**	**p value**	**FC**	**p-value**
adenosine monophosphate (AMP)	1.68	6.55E-02	1.15	6.87E-01	1.55	7.34E-02	2.44	1.72E-03	11.96	2.79E-04
5-oxoproline	1.09	4.55E-01	1.16	6.45E-02	1.42	5.22E-04	1.67	6.86E-04	3.49	1.04E-04
spermine	4.30	4.33E-01	1.08	9.38E-01	0.78	7.87E-01	0.80	7.95E-01	7.27	2.48E-03
spermidine	2.79	4.85E-01	1.04	9.73E-01	1.08	9.37E-01	0.90	9.07E-01	3.76	3.50E-02
stearoylcarnitine									1.76	1.17E-01
inosine	1.20	6.89E-01	1.21	6.19E-01	1.11	8.08E-01	1.56	3.91E-01	2.33	1.64E-02
malate	1.04	8.82E-01	1.04	8.96E-01	1.19	3.53E-01	1.29	1.73E-01	2.61	2.07E-02
palmitoyl ethanolamide	1.33	4.58E-01	1.68	1.17E-01	2.41	3.10E-03	3.09	8.08E-04	4.06	6.69E-03
succinate	0.96	7.61E-01	0.99	9.47E-01	1.01	9.12E-01	1.03	8.07E-01	1.49	7.54E-03
oleic ethanolamide (oleoyl ethanolamide)	1.88	1.10E-01	3.14	2.50E-02	6.22	1.90E-04	8.94	8.31E-05	10.24	1.06E-02
adenosine	1.05	7.32E-01	0.93	6.66E-01	0.94	7.80E-01	1.05	8.24E-01	2.00	1.45E-02
S-adenosylhomocysteine (SAH)	1.25	6.55E-01	1.64	2.69E-01	1.93	1.22E-01	1.84	2.16E-01	4.80	2.47E-04
tartronate (hydroxymalonate)	1.07	6.04E-01	0.94	6.84E-01	0.99	9.66E-01	0.85	2.38E-01	0.22	2.62E-04
1-arachidonoylglyercophosphate	1.07	3.58E-01	1.00	7.89E-01	1.26	3.46E-01	4.86	8.32E-03	12.96	3.63E-04
1-methylguanosine	0.87	4.22E-01	0.87	4.78E-01	0.86	4.45E-01	0.57	2.51E-02	0.19	4.86E-04
oxalate (ethanedioate)	1.00	9.88E-01	1.04	5.78E-01	1.04	6.05E-01	0.90	1.92E-01	0.65	7.00E-04

adipate	0.99	9.66E-01	1.14	2.86E-01	1.09	6.99E-01	1.51	2.32E-02	1.84	9.09E-04
1-stearoylglycerophosphoserine	1.00	7.91E-01	1.00	5.18E-01	1.00	5.06E-01	1.00	7.28E-01	1.00	1.51E-03
1-palmitoylglycerophosphate	1.00	1.50E-01	1.00	2.92E-01	1.00	2.39E-01	1.36	3.16E-02	4.46	1.75E-03
leucylleucine	1.99	1.65E-01	1.09	8.07E-01	1.25	5.18E-01	1.16	7.00E-01	2.48	2.89E-03
5,6-dihydrouracil	1.00	9.16E-01	0.91	5.56E-01	1.08	6.97E-02	1.22	2.13E-03	1.47	3.79E-03
glycerophosphorylcholine (GPC)	1.23	5.55E-01	0.90	7.33E-01	1.37	4.64E-01	1.54	1.27E-01	2.39	5.49E-03
1-palmitoylglycerol (16:0)	0.69	2.79E-02	0.82	2.19E-01	0.86	3.42E-01	0.72	5.70E-02	0.64	7.47E-03
caproate (6:0)	0.89	3.58E-01	0.99	9.33E-01	0.95	5.36E-01	1.01	9.50E-01	1.25	8.76E-03
N-acetylaspartate (NAA)	0.97	6.40E-01	1.03	7.57E-01	1.06	5.38E-01	1.09	3.88E-01	1.26	1.12E-02
1-pentadecanoylglycerophosphocholine (15:0)	0.90	5.94E-01	1.06	6.05E-01	1.29	3.51E-02	1.16	2.73E-01	1.44	1.18E-02
isoleucylvaline	0.96	9.01E-01	1.11	6.27E-01	0.66	2.00E-01	1.40	1.23E-01	1.91	1.56E-02
1-oleoylglycerol (18:1)	0.66	1.24E-01	0.60	8.65E-02	0.63	5.66E-02	0.43	6.16E-03	0.41	1.63E-02
HWESASLLR	1.00	6.99E-01	1.00	2.23E-01	1.00	7.46E-01	1.00	9.96E-01	21.53	2.08E-02
choline	1.05	7.54E-01	1.10	5.00E-01	1.12	4.26E-01	1.28	1.02E-01	1.41	2.26E-02
glycerophosphoethanolamine	0.93	5.41E-01	0.93	4.89E-01	1.04	7.91E-01	1.05	7.32E-01	1.60	2.31E-02
prolylglycine	0.89	3.35E-01	0.88	3.46E-01	0.88	3.82E-01	1.00	9.89E-01	0.68	2.95E-02
gamma-glutamylalanine	0.95	6.71E-01	1.01	9.35E-01	1.05	7.32E-01	1.22	1.13E-01	1.54	3.02E-02
2-hydroxyglutarate	0.85	2.78E-01	0.93	5.93E-01	1.13	4.58E-01	1.03	8.41E-01	1.79	3.25E-02
uridine	0.98	9.16E-01	1.06	7.67E-01	1.09	6.40E-01	1.15	4.39E-01	1.46	3.44E-02
asparagylleucine	1.68	1.61E-01	1.00	7.66E-01	1.00	9.54E-01	3.82	3.46E-01	8.21	3.67E-02

1-palmitoyl-GPC (16:0)	0.93	6.66E-01	1.04	7.39E-01	1.24	5.34E-02	1.31	1.57E-02	1.43	3.74E-02
phenylalanyltryptophan	0.71	2.96E-01	0.95	8.08E-01	0.92	7.32E-01	0.87	5.15E-01	0.52	4.05E-02
glycylproline	0.48	3.05E-01	0.31	1.46E-01	0.12	6.09E-02	0.04	4.13E-02	0.04	4.13E-02
1-linoleoylglycerol (18:2)	0.80	4.88E-01	0.64	2.56E-01	0.51	1.16E-01	0.48	9.85E-02	0.35	4.34E-02
HWESASXX	0.86	4.87E-01	1.14	7.23E-01	0.96	8.57E-01	1.02	9.50E-01	7.19	4.99E-02
palmitoylcarnitine									1.76	1.72E-01
lactate	1.11	5.83E-01	1.25	2.35E-01	1.53	2.37E-02	1.79	1.27E-03	3.54	p<0.000 1
alpha-ketoglutarate									1.75	6.27E-02
arginine	0.86	4.34E-01	0.81	2.72E-01	0.71	1.07E-01	0.55	2.00E-02	0.26	8.38E-04
fumarate	1.06	7.74E-01	1.15	4.87E-01	1.31	2.01E-01	1.57	6.01E-02	3.09	2.09E-02
glucose									0.48	8.58E-02
glutamate	1.03	9.03E-01	1.06	8.33E-01	1.14	6.31E-01	1.51	1.94E-01	2.95	4.90E-03
linoleoylcarnitine	0.94	8.52E-01	1.30	5.14E-01	1.78	9.01E-02	2.27	3.42E-02	4.39	2.12E-02
myristoylcarnitine									1.64	1.98E-01
oleoylcarnitine (C18)	0.97	9.26E-01	1.24	4.86E-01	1.65	7.36E-02	2.05	6.67E-03	3.40	6.89E-03
ornithine	1.20	1.38E-01	1.38	1.61E-02	1.67	3.63E-03	2.02	2.44E-04	2.96	7.22E-05
pyruvate	1.20	5.36E-01	1.19	6.28E-01	1.43	4.32E-01	1.57	2.80E-01	7.83	2.59E-02
sphingosine	1.80	3.15E-02	1.85	2.67E-03	2.23	1.65E-02	2.76	3.45E-05	3.13	3.87E-03
sphingosine 1-phosphate	1.31	2.79E-01	1.42	8.67E-02	2.05	5.66E-03	2.55	2.41E-03	2.59	3.22E-02
maltose	0.68	5.62E-01	0.73	6.38E-01	0.77	6.90E-01	0.79	7.41E-01	3.21	2.51E-02
mannose	0.96	8.82E-01	1.03	8.88E-01	0.98	9.46E-01	0.76	3.04E-01	0.30	1.39E-02
taurine	1.08	4.28E-01	1.10	4.03E-01	1.08	5.19E-01	1.05	6.91E-01	1.93	8.73E-04

[00126] In a second study, sample quality biomarkers for assessing the effects of the time interval that plasma is stored at 4ºC prior to freezing at -80ºC were identified. Whole blood was collected and plasma was separated. Triplicate plasma samples were processed immediately by freezing at -80ºC within one hour of collection (control, timepoint 0h) or processing was delayed by storing the plasma samples at 4ºC for 2, 4, and 20 hours prior to freezing at -80ºC. After the levels of the metabolites were determined, the % difference between the delayed processing samples and the control samples was calculated to identify biomarkers for sample processing. Thus, the study provides biomarkers related to the time interval a plasma sample is held at 4ºC prior to freezing.

Table 2. Biomarkers for assessing sample processing – cold incubation prior to freezing

Red	Percent Change vs. Control		
Biochemical Name	2 hour	4 hour	20 hour
2'-deoxyinosine	32.34	119.73	839.44
3-hydroxypropanoate	44.05	34.39	36.39
4-phenylbutyrate	39.31	51.82	114.47
caproate (6:0)	20.60	22.24	56.44
12-HETE	-32.85	-20.98	-45.80
13-HODE + 9-HODE	-8.02	-23.39	-38.30
2'-deoxyguanosine	-9.05	-14.08	-18.25
adenosine	-9.00	-39.15	-29.82
eicosapentaenoate (EPA; 20:5n3)	-10.91	-20.72	-21.07
2-hydroxy-3-methylvalerate	8.29	8.80	62.50
argininosuccinate	-7.63	-3.51	-16.02
decanoylcarnitine (C10)	8.45	13.21	16.86
docosahexaenoate (DHA; 22:6n3)	-3.86	-14.67	-17.55
gamma-glutamylmethionine	-12.71	-11.48	-48.84
hexanoylcarnitine (C6)	-14.69	-14.67	-36.58
methionine	-2.04	4.28	32.03
myristoleate (14:1n5)	-8.44	-13.65	-19.84
uracil	11.06	11.63	56.58
sebacate (decanedioate)	-12.91	-8.53	-13.68

[00127] In a third study, four whole blood samples were separated immediately after collection and the resulting plasma samples were frozen at -80ºC within one hour of collection (control, 0h time point), or after incubation at room temperature (about

23°C) for 24, 48, 72, or 96 hours prior to being frozen at -80°C. The percent change was calculated by comparing the level of the biomarker in the sample at 24h, 48h, 72h and 96h to the 0h control. The data is presented in Table 2. Table 2 includes, for each biomarker, the biochemical name of the biomarker, and the percent difference or percent change in the level of the biomarker at each time point compared to the control (0h time point). Thus, the study provides biomarkers related to the time interval a plasma sample is held at room temperature prior to freezing.

Table 3. Biomarkers of sample processing- RT incubation prior to freezing

	Percent Change vs. Control (0 time point)			
Biochemical Name	**1 day**	**2 day**	**3 day**	**4 day**
adenosine monophosphate (AMP)	416.79	2124.06	3038.37	3941.25
glutathione, oxidized (GSSG)	361.91	3359.19	3657.22	2840.58
cys-gly, oxidized	143.20	495.89	781.35	1573.71
spermine	2897.39	21528.49	25072.27	87348.47
spermidine	874.25	10471.89	13300.03	19413.41
inosine	139.22	1998.44	4453.55	12151.70
cysteine-glutathione disulfide	306.07	1504.05	1711.69	1515.70
5-oxoproline	50.50	243.52	422.86	761.74
xanthine	-46.18	-14.33	165.70	699.72
thymine	-100.00	513.92	477.89	697.63
succinate	41.23	152.04	222.82	302.26
stearoylcarnitine (C18)	-9.04	56.75	55.79	287.17
malate	104.61	202.41	217.00	219.93
oleic ethanolamide (oleoyl ethanolamide)	24.20	63.43	51.13	136.16
adenosine	45.00	94.88	204.84	108.01
palmitoyl ethanolamide	44.63	69.67	21.10	63.99
2'-deoxyuridine	-50.97	-83.24	-100.00	-43.73
taurine	21.12	129.53	199.79	204.86
sphinganine	19.97	68.19	117.20	328.00
ornithine	17.38	14.51	9.39	7.69
glutamate	83.20	175.72	267.38	315.19
arginine	-65.57	-79.46	-80.59	-83.11
glucose	-50.31	-85.50	-96.30	-98.78
pyruvate	75.39	307.40	296.01	544.39
lactate	86.77	122.79	144.25	106.48
fumarate	28.44	21.94	15.31	14.65
alpha-ketoglutarate	56.48	100.77	159.29	119.94
oleoylcarnitine (C18)	42.01	163.77	96.76	275.06
palmitoylcarnitine (C16)	19.67	146.26	107.77	356.00

linoleoylcarnitine	42.59	158.11	111.65	288.18
myristoylcarnitine	17.73	134.39	75.70	185.46
sphingosine	-1.22	122.04	133.48	257.61
hypoxanthine	-100.00	6392.42	25003.89	75436.45

[00128] In one example, four exemplary biomarkers were selected based on significance and consistency (i.e., that the level of the small molecule biomarker either consistently increased or consistently decreased for all subsequent times). The four candidate biomarkers are shown in Table 4 below, and the levels of the biomarkers over time are shown in the graphs in Figure 4. For this example, the biomarkers were equally weighted; biomarkers that increase with longer sample processing time were assigned a positive weight, and biomarkers that decrease with longer sample processing time were assigned a negative weight. The weight for each biomarker is shown in column 2 of Table 3. It should be appreciated that the coefficients (weights) are exemplary and may be refined as additional data becomes available using formal statistical analysis methods as described herein.

Table 4. Exemplary biomarkers used to assess sample processing

Candidate Biomarker	**Weight**
Adenosine 5'-monophpsphate (AMP)	+0.25
cys-gly, oxidized	+0.25
glucose	-0.25
taurine	+0.25

[00129] The exemplary biomarkers were then evaluated using 30 plasma samples that were separated from whole blood immediately and frozen at -80ºC (0 time point) or incubated at room temperature for 0.5, 1, 2, 4, or 24 hours prior to separation from whole blood and freezing at -80ºC. Five samples were analyzed for each time point. For each sample, each exemplary biomarker was evaluated individually, and the metabolite level at each time point was assessed. The biomarkers were also used to generate a composite score to assess sample quality and the sample processing time parameter for the samples. The composite score for sample processing time for each of the five samples at each time point was calculated as follows: Sample Processing Composite Score = 0.25*(level AMP + level cys-gly oxidized – level glucose + level taurine).

[00130] In this example, the composite scores were as follows: less than one for all

ten samples incubated for 1h or less; less than one for 8 of the 10 samples held for 2h and 4h; greater than one but less than two for 2 of 10 samples held for 2h and 4h; and greater than two for all 5 samples held for 24h. A graph of the data is shown in Figure 5.

[00131] When evaluating biomarkers, a rule can be established to flag potential protocol violations in the samples and/or to fail samples as violating protocol by setting a cut-off value for the composite score. In this example, the analyst set the composite score value for accepting sample at 1.0 and the composite score value for rejecting samples at 2.0. Thus, a composite score of less than 1.0 indicates that the sample meets acceptance criteria, while a composite score greater than 1.0 but less than 2.0 indicates that the sample should be flagged for additional evaluation; and a sample with a composite score greater than 2.0 is rejected. Using the selected cut-off values in this example, two samples were flagged, five samples were rejected, and the remaining samples were accepted. It should be appreciated that the cut-off value may change with the intended use of the sample. Additionally, the cut-off values may be selected automatically by the computing system or may be selected by the analyst.

[00132] Sample quality biomarkers for assessing sample processing time (storage at 4°C prior to freezing at -80°C) were also identified in serum, and urine samples. Triplicate samples of serum, and urine were frozen at -80°C within one hour of collection (control, timepoint 0h) or were stored at 4°C for 2, 4, or 20 hours prior to freezing at -80°C. After the levels of metabolites were determined, the data were analyzed by calculating the % difference between the delayed processing samples and the control samples to identify biomarkers for sample processing time. The results are shown in Tables 5 (serum) and 6 (urine).

Table 5. Biomarkers for assessing sample processing time (serum)

	Percent Change vs. Control		
Biochemical Name	2 hour Serum	4 hour Serum	20 hour Serum
benzoate	-22.75	-18.49	-16.56
caproate (6:0)	58.20	52.30	117.71
N-acetylleucine	74.39	85.73	101.90
methylsuccinate	22.14	19.26	16.49
hypoxanthine	4.30	19.81	20.75
phenylpyruvate	-11.49	-11.01	-29.50
13-HODE + 9-HODE	14.00	4.58	25.84
alpha-hydroxyisocaproate	-19.55	-11.47	-29.27
hexanoylcarnitine (C6)	-12.96	-11.66	-33.36

Table 6. Biomarkers for assessing sample processing time (urine)

	Percent Change vs. Control		
Biochemical Name	2 hour Urine	4 hour Urine	20 hour Urine
3-methyladipate	28.10	24.45	24.16
succinate	34.34	36.48	19.08
adenosine	8.95	15.36	20.47
inosine	4.96	16.41	33.43
laurylcarnitine (C12)	-8.02	-17.04	-16.90
methionine	-4.52	-20.58	-29.25
uridine	8.67	17.79	77.30
myristoylcarnitine	-8.61	-12.00	-24.62
stearoylcarnitine (C18)	-56.04	19.40	166.25
3-hydroxypropanoate	10.85	65.73	14.85
4-guanidinobutanoate	11.08	15.82	11.12

Example 2. Biomarkers of sample storage temperature

[00133] Sample quality biomarkers for assessing sample storage temperature were identified using two sample sets. Sample set 1 consisted of 543 plasma samples that had been stored at -20ºC. Sample set 2 consisted of 401 plasma samples that had been stored at -80ºC. As a control for both sample sets, a pooled reference sample made up of aliquots taken from an independent set of plasma samples that had been stored at -80ºC was used. After the levels of metabolites were determined, the data were analyzed to identify biomarkers for assessing sample storage temperature. Metabolites that differentiated samples stored at -20ºC from those stored at -80ºC are

displayed in Table 7. Table 7 includes, for each biomarker, the biochemical name of the biomarker and the average level of the biomarker (in ion counts) in the samples stored at -20°C and in the samples stored at -80°C. The average metabolite levels are shown in columns 2 & 3 of Table 7 for sample set 1 and in columns 4 & 5 for sample set 2. For set 1, the levels of alpha-ketoglutarate were so low in the samples stored at -20°C, that the metabolite was detected in only 1 of the 543 samples. In contrast, in set 2 where all samples were stored at -80°C, alpha-ketoglutarate was detected in about 75% of the 401 plasma samples.

Table 7. Biomarker metabolites that differentiate sample storage at -20°C vs. -80°C

	Sample Set 1		Sample Set 2	
Biochemical name	**Metabolite Level -20°C Storage**	**Metabolite Level -80°C Storage (pooled reference)**	**Metabolite Level -80°C Storage**	**Metabolite Level -80°C Storage (pooled reference)**
alpha-ketoglutarate	Not Detected	100,917	31,426	108,146
acetylcarnitine	84,822	2,494,315	2,319,314	2,237,562
propionylcarnitine	76,039	332,821	362,872	320,365
caproate (6:0)	1,072,216	197,267	117,546	183,523
4-methyl-2-oxopentanoate	111,440	468,311	351,207	254,435
3-methyl-2-oxovalerate	68,646	309,257	198,062	171,348
3-methyl-2-oxobutyrate	14,046	125,751	119,800	74,419
gamma-glutamylleucine	1,060,875	114,832	280,939	182,965
gamma-glutamylvaline	1,046,714	138,055	221,598	150,513
glutamine	63,551	424,251	1,001,407	487,786
methionine	456,981	3,007,630	2,431,993	2,578,892

[00134] Next, the biomarkers were used to differentiate samples that had been stored at -20°C from samples that had been stored at -80°C. For this example, a subset of the biomarkers was used to predict the storage temperature for an independent sample set of 135 serum samples that were blinded for storage temperature. Using the biomarkers, 113 of the 135 samples were predicted to be stored at -20°C. The

exemplary biomarkers used for the analysis and the fold change for the biomarkers in samples stored at -20ºC compared to samples stored at -80ºC (-20°C/-80°C), which is the ratio of the mean level of the biomarkers in samples stored at -20ºC as compared to -80ºC mean level, are shown in Table 8.

Table 8. Exemplary serum metabolites used to differentiate sample storage at -20°C vs. -80°C

Biochemical Name	Fold Change (-20°C/80°C)
4-methyl-2-oxopentanoate	0.19
acetylcarnitine	0.68
alpha-ketoglutarate	0.77
caproate (6:0)	1.46
gamma-glutamylleucine	2.54
glutamine	0.78
methionine	0.39

[00135] In a further demonstration, a model was generated to produce a composite score using the seven exemplary biomarkers shown in Table 9 below. In this example, the biomarkers in the model were equally weighted; biomarkers that had higher levels in samples stored at -20ºC were assigned a positive weight and biomarkers that had higher levels in samples stored at -80ºC were assigned a negative weight. The weight for each biomarker is shown in column 2 of Table 9. It should be appreciated that the coefficients (weights) are exemplary and may be refined as additional data becomes available using formal statistical analysis methods as described herein.

Table 9. Exemplary metabolites used to assess sample storage temperature

Biochemical name	WEIGHT
4-methyl-2-oxopentanoate	-1/7
acetylcarnitine	-1/7
alpha-ketoglutarate	-1/7
caproate (6:0)	+1/7
gamma-glutamylleucine	+1/7
glutamine	-1/7
methionine	-1/7

[00136] The biomarkers and model were used to generate a composite score to assess sample quality and the storage temperature quality parameter. The composite

score for storage temperature for each sample was calculated as follows: Storage Temperature Composite Score = 1/7(caproate + gamma-glutamylleucine – 4-methyl-2-oxopentanoate – acetylcarnitine – alpha-ketoglutarate – glutamine – methionine). Using this model, composite scores were determined for a 4th set of samples consisting of 43 human plasma samples stored at -80°C. In addition, to evaluate how a sample stored at -20°C might differ from human plasma samples stored at -80°C, a pseudo-observation was created based on the ratios of -20°C samples to -80°C samples from sample set 1 in Table 7. The value for alpha-ketoglutarate was set to 0.1 since the metabolite was not detected in -20°C samples. The values used for the pseudo observation are as follows: alpha-ketoglutarate = 0.1, acetylcarnitine =0.034, caproate = 5.435, 4-methyl-2-oxopentanoate = 0.238, gamma-glutamylleucine = 9.238, glutamine = 0.15, and methionine = 0.15. The storage temperature composite score was calculated for the pseudo-observation which represents a sample stored at -20°C and for each of the 43 samples held at -80°C. The composite score for the pseudo-sample is 12, while the majority of composite scores for the -80°C samples is much lower; the highest composite score calculated for the samples stored at -80°C is only 2. The results are graphically illustrated in Figure 6.

Example 3. Biomarkers of sample freeze-thaw cycles

[00137] Sample quality biomarkers for assessing sample quality with freeze-thaw cycles were identified using plasma, serum, and urine samples. The samples were collected and frozen at -80°C according to sample collection protocol. Samples were thawed completely and thoroughly mixed. An aliquot of each sample (serum, urine and plasma) was taken and extracted in triplicate for metabolomics analysis (control, 1 freeze/thaw cycle). The original samples were returned to the freezer and allowed to freeze completely. The process was repeated until all five freeze thaw cycles were completed (2X = two freeze-thaw cycles, 3X = three freeze-thaw cycles, 4X = four freeze-thaw cycles, and 5X = five freeze-thaw cycles). After the levels of metabolites were determined, the data were analyzed by calculating the percent difference between the freeze-thaw samples and the control to identify biomarkers for sample freeze-thaw. One freeze-thaw cycle represents sample collection according to protocol. (The sample is collected, frozen at -80°C, shipped (frozen) to an analysis facility, and thawed at the analysis facility for metabolomics analysis.) The results are

shown in Tables 10, 11, & 12 for plasma, serum, and urine, respectively.

Table 10. Biomarkers for assessing sample freeze-thaw cycles (plasma)

	Percent Change vs. Control			
Biochemical Name	2X Plasma	3X Plasma	4X Plasma	5X Plasma
adenosine	-22.60	-25.09	-45.63	-36.58
succinate	28.59	30.73	49.50	83.13
13-HODE + 9-HODE	-7.02	-22.61	-35.66	-48.03
3-hydroxy-2-ethylpropionate	-9.91	-16.19	-41.69	-50.50
caproate (6:0)	12.65	16.32	23.08	23.83
docosahexaenoate (DHA; 22:6n3)	-7.89	-16.27	-26.30	-34.87
eicosapentaenoate (EPA; 20:5n3)	-6.37	-16.14	-27.00	-31.52
2'-deoxyuridine	-2.35	-9.26	-18.13	-21.27
phenyllactate (PLA)	-3.49	-15.79	-15.29	-16.77
alpha-hydroxyisovalerate	-3.56	-13.06	-22.62	-24.91
arachidonate (20:4n6)	-3.69	-8.23	-16.16	-18.69
gamma-glutamylmethionine	-2.35	-9.07	-16.22	-23.27
inosine	-3.09	-11.79	-16.00	-27.47
isoleucine	-2.04	-12.58	-22.05	-24.33
myristoleate (14:1n5)	-6.21	-14.78	-21.06	-25.51
phenylacetate	-3.31	-11.24	-18.82	-19.66
stearoylcarnitine (C18)	-1.44	-5.22	-18.75	-26.26
2-methylcitrate	-2.38	-6.16	-11.95	-19.03
decanoylcarnitine (C10)	4.69	9.23	11.70	19.89
thymine	-1.39	-7.55	-10.17	-18.57
4-phenylbutyrate	29.91	-62.17	-76.43	-53.47
12-HETE	42.47	-1.88	-28.12	-71.65
alpha-hydroxyisocaproate	5.36	-19.95	-26.44	-21.40
succinimide	9.45	-15.73	-19.85	-33.23
3-methyl-2-oxobutyrate	0.01	-10.43	-18.33	-21.64
3-methyl-2-oxovalerate	0.48	-10.56	-16.49	-15.53
4-methyl-2-oxopentanoate	1.42	-5.21	-16.25	-15.96
5-hydroxyhexanoate	4.46	-11.11	-19.58	-24.64
isovalerate (C5)	2.71	-11.33	-23.06	-26.32
urate	5.88	-12.72	-17.22	-24.31
1,5-anhydroglucitol (1,5-AG)	5.79	-6.90	-10.18	-16.92
3-(3-hydroxyphenyl)propionate	1.10	-11.63	-14.48	-20.56
3-(4-hydroxyphenyl)lactate (HPLA)	5.07	-9.95	-8.59	-17.79
4-hydroxyphenylacetate	4.50	-3.39	-9.88	-19.12
docosapentaenoate (n6 DPA; 22:5n6)	-0.70	-12.06	-14.83	-16.24
hexanoylcarnitine (C6)	4.65	-5.12	-13.51	-23.90

lactate	4.23	-5.86	-12.76	-16.90
S-adenosylhomocysteine (SAH)	0.17	-8.58	-13.92	-20.37

Table 11. Biomarkers for assessing sample freeze-thaw cycles (serum)

	Percent Change vs. Control			
Biochemical Name	2X Serum	3X Serum	4X Serum	5X Serum
caproate (6:0)	61.07	84.21	118.75	119.82
acetylcarnitine (C2)	16.53	13.65	29.90	21.88
adenosine	-17.74	-15.37	-15.58	-17.35
hexanoylcarnitine (C6)	-4.85	-14.36	-22.91	-22.87
palmitoylcarnitine (C16)	-6.59	-14.01	-15.98	-15.11
myristoylcarnitine	-11.31	-17.12	-23.66	-17.14
4-phenylbutyrate	18.99	-37.17	-45.93	-55.73
2-hydroxy-3-methylvalerate	8.69	3.44	-12.46	-15.42

Table 12. Biomarkers for assessing sample freeze-thaw cycles (urine)

	Percent Change vs. Control			
Biochemical Name	2X Urine	3X Urine	4X Urine	5X Urine
2-methylcitrate	40.83	95.67	38.74	53.70
phenylpyruvate	-20.29	-33.17	-34.57	-16.33
argininosuccinate	13.75	10.14	15.44	26.41
inosine	4.42	1.46	17.09	15.66
laurylcarnitine (C12)	-2.29	-10.00	-14.77	-19.27
methionine	2.33	-24.10	-23.81	-27.48
uridine	3.34	-3.62	24.35	67.91

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Example 4. Biomarkers for assessing fasting status of an individual

[00138] Sample quality biomarkers for assessing fasting status of an individual were identified using plasma samples, with 41 samples obtained from fasted subjects and 41 matched samples obtained from fed subjects. After the levels of metabolites
10 were determined, the data were analyzed using matched pairs t-test to identify biomarkers for assessing fasting status of an individual. The most significant metabolites ($p<0.0001$) from the analysis are displayed in Table 13. Table 13 includes, for each biomarker, the biochemical name of the biomarker, the biochemical superpathway and subpathway for the biomarker, the fold change (FC) of the

biomarker in samples from fed subjects compared to fasted subjects (fed/fasted) which is the ratio of the mean level of the biomarker in fed samples as compared to the fasted mean level, and the p-value determined in the statistical analysis of the data concerning the biomarkers.

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Table 13. Biomarkers for fasting/fed status in plasma

Biochemical Name	Biochemical Super Pathway	Biochemical Sub Pathway	FC (Fed / Fasted)	p-value
uridine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.66	1.15E-14
mannose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.75	3.24E-11
xanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.63	4.00E-10
5,6-dihydrothymine	Nucleotide	Pyrimidine Metabolism, Thymine containing	0.85	6.29E-10
orotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	1.34	9.03E-10
prolylglycine	Peptide	Dipeptide	1.92	1.35E-09
glycoursodeoxycholate	Lipid	Secondary Bile Acid Metabolism	1.92	3.29E-09
methionine-sulfoxide	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.57	5.14E-09
caffeine	Xenobiotics	Xanthine Metabolism	2.54	8.94E-09
1-stearoyl-2-linoleoyl-GPE (18:0/18:2)	Lipid	Phospholipid Metabolism	1.41	1.75E-08
glycodeoxycholate	Lipid	Secondary Bile Acid Metabolism	2.98	2.02E-08
glycochenodeoxycholate	Lipid	Primary Bile Acid Metabolism	2.27	2.30E-08
N-acetylglutamate	Amino Acid	Glutamate Metabolism	1.43	2.52E-08
glycolithocholate	Lipid	Secondary Bile Acid Metabolism	3.82	4.57E-08
N(2)-furoyl-glycine	Xenobiotics	Food Component/Plant	3.18	8.23E-08
oleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.80	8.42E-08
palmitoleate (16:1n7)	Lipid	Long Chain Fatty Acid	0.51	8.99E-08
glycohyocholate	Lipid	Secondary Bile Acid Metabolism	1.89	1.25E-07
myristoleate (14:1n5)	Lipid	Long Chain Fatty Acid	0.57	1.36E-07
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	1.58	1.57E-07

1,3-7-trimethylurate	Xenobiotics	Xanthine Metabolism	2.28	2.47E-07
taurocholate	Lipid	Primary Bile Acid Metabolism	2.21	2.56E-07
pyrraline	Xenobiotics	Food Component/Plant	1.90	2.77E-07
3-methyl-catechol-sulfate	Xenobiotics	Benzoate Metabolism	3.63	2.97E-07
glycocholate	Lipid	Primary Bile Acid Metabolism	2.06	3.16E-07
taurodeoxycholate	Lipid	Secondary Bile Acid Metabolism	2.39	4.35E-07
o-cresol-sulfate	Amino Acid	Phenylalanine and Tyrosine Metabolism	3.12	4.48E-07
1-oleoyl-2-linoleoyl-GPE (18:1/18:2)	Lipid	Phospholipid Metabolism	1.72	5.04E-07
1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	Lipid	Phospholipid Metabolism	1.33	6.44E-07
4-vinylguaiacol-sulfate	Xenobiotics	Food Component/Plant	4.16	6.58E-07
glycodeoxycholate-sulfate	Lipid	Secondary Bile Acid Metabolism	1.67	6.79E-07
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	Lipid	Phospholipid Metabolism	1.54	7.13E-07
myristoleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.65	9.32E-07
taurochenodeoxycholate	Lipid	Primary Bile Acid Metabolism	1.98	1.65E-06
maleate	Lipid	Fatty Acid, Dicarboxylate	2.09	1.69E-06
indole-3-carboxylic acid	Amino Acid	Tryptophan Metabolism	0.69	2.09E-06
N-acetyl-beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.86	2.28E-06
octanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.63	2.67E-06
dopamine-sulfate	Amino Acid	Phenylalanine and Tyrosine Metabolism	3.63	2.67E-06
N2,N2-dimethylguanosine	Nucleotide	Purine Metabolism, Guanine containing	0.91	2.68E-06
glycochenodeoxycholate-sulfate	Lipid	Primary Bile Acid Metabolism	1.71	2.75E-06
quinate	Xenobiotics	Food Component/Plant	2.02	3.62E-06
oleate-vaccenate (18:1)	Lipid	Long Chain Fatty Acid	0.68	3.95E-06
gamma-glutamylalanine	Peptide	Gamma-glutamyl Amino Acid	1.25	3.99E-06
decanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.64	4.68E-06
cis-4-decenoyl-carnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.72	4.97E-06
hydantoin-5-propionic acid	Amino Acid	Histidine Metabolism	1.44	5.83E-06

linoleoylcarnitine-	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.83	6.33E-06
10-heptaadecenoate (17:1n7)	Lipid	Long Chain Fatty Acid	0.58	7.10E-06
oleoyl-ethanolamide	Lipid	Endocannabinoid	0.67	8.72E-06
O-methylcatechol-sulfate	Xenobiotics	Benzoate Metabolism	1.87	1.01E-05
caprylate (8:0)	Lipid	Medium Chain Fatty Acid	7.70	1.29E-05
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	1.49	1.66E-05
catechol-sulfate	Xenobiotics	Benzoate Metabolism	1.75	1.66E-05
laurylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.70	1.78E-05
palmitate (16:0)	Lipid	Long Chain Fatty Acid	0.77	1.99E-05
N6-acetyllysine	Amino Acid	Lysine Metabolism	1.26	2.57E-05
palmitoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.88	2.64E-05
cortisone	Lipid	Steroid	0.81	2.93E-05
bilirubin-E-E-	Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	0.83	3.03E-05
alanine	Amino Acid	Alanine and Aspartate Metabolism	1.10	3.88E-05
citraconate/glutaconate	Energy	TCA Cycle	2.28	4.10E-05
C-glycosyltryptophan	Amino Acid	Tryptophan Metabolism	0.94	4.34E-05
3-hydroxy-3-methylglutarate	Lipid	Mevalonate Metabolism	1.29	1.00E-04
linoleate (18:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.35	1.00E-04
4-hydroxyphenylpyruvate	Amino Acid	Phenylalanine and Tyrosine Metabolism	1.31	1.00E-04
methylsuccinate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.88	1.00E-04
dihomo-linoleate (20:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.70	1.00E-04
cys-gly, oxidized	Amino Acid	Glutathione Metabolism	0.76	1.00E-04
docosadienoate (22:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.70	1.00E-04
eicosenoate (20:1)	Lipid	Long Chain Fatty Acid	1.74	1.00E-04
stearidonate (18:4n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.99	1.00E-04
linolenate [alpha or gamma] (18:3n3 or 6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.74	1.00E-04
2-linoleoyl-GPE (18:2)	Lipid	Lysolipid	0.66	1.00E-04
isovalerylglycine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.22	1.00E-04

N6-carbamoylthreonyladenosine	Nucleotide	Purine Metabolism, Adenine containing	0.90	1.00E-04
palmitoyl-ethanolamide	Lipid	Endocannabinoid	0.74	1.00E-04
stearoyl-ethanolamide	Lipid	Endocannabinoid	0.71	1.00E-04

[00139] Eight exemplary biomarkers were selected based on significance and biochemical superpathway/subpathway classification. In this example, only one biomarker was selected to be representative of a given subpathway. The eight candidate biomarkers are shown in Table 14 below. Biomarkers were equally weighted; biomarkers that had higher levels in fed samples were assigned a positive weight, and biomarkers that had higher levels in fasted samples (lower levels in fed samples) were assigned a negative weight. The weight for each biomarker is shown in column 2 of Table 14. The level of each of the eight candidate biomarkers as measured by Z-score, with fasted samples as the reference population, is shown in Figure 7. It should be appreciated that the coefficients (weights) are exemplary and may be refined as additional data becomes available using formal statistical analysis methods as described herein.

Table 14. Exemplary biomarkers used to assess fasting/fed status

METABOLITE	WEIGHT
methionine sulfoxide	+ 1/8
glycocholate	+ 1/8
dopamine sulfate	+ 1/8
azelate	+ 1/8
uridine	-1/8
xanthine	-1/8
mannose	-1/8
palmitoleate	-1/8

[00140] The biomarkers were used to generate a composite score to assess sample quality and the fasting status quality parameter for the samples. The composite score for fasting status was calculated for each sample as follows: Fasting Status Composite Score = 1/8(methionine sulfoxide + glycocholate + dopamine sulfate + azelate – uridine – xanthine – mannose – palmitoleate). The composite score was calculated for each of the 41 fasted samples and 41 fed samples, and the data are shown in boxplot format in Figure 8.

[00141] In an exemplary use of the composite score, a rule could be established to flag potential fasting violations in samples. In this example, a rule could be set to flag any samples with a composite score >2 as potential fasting violations. Using this rule, 34 of the fed samples and none of the fasted samples were flagged as potential fasting violations.

[00142] Sample quality biomarkers for assessing fasting status of an individual were identified using adipose, liver, and muscle tissue from 8 mice fasted for 24 hours (Fasted) and 8 mice fasted for 24 hours prior to 4 hours of re-feeding (Fed). After the levels of metabolites were determined, the data were analyzed using t-tests to identify biomarkers for assessing fasting status of an individual. Significant metabolites ($p<0.05$) from the analysis are displayed in Tables 15, 16, and 17 for liver, muscle, and adipose tissue, respectively. Tables 15-17 include, for each biomarker, the biochemical name of the biomarker, the biochemical superpathway and subpathway for the biomarker, the fold change (FC) of the biomarker in samples from fed subjects compared to fasted subjects (fed/fasted) which is the ratio of the mean level of the biomarker in fed samples as compared to the fasted mean level, and the p-value determined in the statistical analysis of the data concerning the biomarkers.

Table 15. Biomarkers for fasting status in tissue (liver)

			Fed / Fasted	
Biochemical Name	**Super Pathway**	**Sub Pathway**	**Fold Change**	**p-value**
mannose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	5.23	7.19E-13
maltopentaose	Carbohydrate	Glycogen Metabolism	327.89	1.18E-11
maltotriose	Carbohydrate	Glycogen Metabolism	310.05	2.87E-11
suberylglycine	Lipid	Fatty Acid Metabolism(Acyl Glycine)	0.11	5.81E-11
histidine	Amino Acid	Histidine Metabolism	0.46	2.26E-10
sorbitol	Carbohydrate	Fructose, Mannose and Galactose Metabolism	9.05	1.45E-09
glucose	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	6.49	2.56E-09
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	37.93	3.33E-09
stachydrine	Xenobiotics	Food Component/Plant	10.57	8.89E-09
pelargonate (9:0)	Lipid	Medium Chain Fatty Acid	0.53	1.54E-08
cysteine-glutathione disulfide	Amino Acid	Glutathione Metabolism	0.35	3.56E-08
hippurate	Xenobiotics	Benzoate Metabolism	3.22	4.74E-08
fructose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	5.15	5.20E-08
lactate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	3.21	1.19E-07
2-aminobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.39	1.28E-07
adipate	Lipid	Fatty Acid, Dicarboxylate	0.26	1.71E-07

laurate (12:0)	Lipid	Medium Chain Fatty Acid	0.6	2.36E-07
maltotetraose	Carbohydrate	Glycogen Metabolism	226.73	3.20E-07
glutathione, oxidized (GSSG)	Amino Acid	Glutathione Metabolism	1.66	3.84E-07
maltose	Carbohydrate	Glycogen Metabolism	182.59	4.44E-07
catechol sulfate	Xenobiotics	Benzoate Metabolism	4.8	4.96E-07
myristate (14:0)	Lipid	Long Chain Fatty Acid	0.51	7.15E-07
5-ketogluconate	Xenobiotics	Food Component/Plant	4.76	8.22E-07
succinylcarnitine	Energy	TCA Cycle	3	8.33E-07
adenosine 3'-monophosphate (3'-AMP)	Nucleotide	Purine Metabolism, Adenine containing	0.62	9.20E-07
ribose	Carbohydrate	Pentose Metabolism	1.98	1.29E-06
S-methylglutathione	Amino Acid	Glutathione Metabolism	2.18	1.37E-06
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	0.62	1.91E-06
caproate (6:0)	Lipid	Medium Chain Fatty Acid	2.53	2.00E-06
myristoleate (14:1n5)	Lipid	Long Chain Fatty Acid	0.45	2.14E-06
fumarate	Energy	TCA Cycle	1.77	3.11E-06
kynurenine	Amino Acid	Tryptophan Metabolism	0.39	4.08E-06
pseudouridine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.36	6.65E-06
3-methylglutarylcarnitine (C6)	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.34	6.91E-06
nicotinamide adenine dinucleotide (NAD+)	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.5	7.76E-06
tagatose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	3.99	8.27E-06
2-aminoadipate	Amino Acid	Lysine Metabolism	0.24	8.80E-06
3-hydroxybutyrate (BHBA)	Lipid	Ketone Bodies	0.4	1.00E-05
2-palmitoleoyl-GPC (16:1)*	Lipid	Lysolipid	1.99	1.08E-05
ornithine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.55	1.76E-05
cytidine 5'-monophosphate (5'-CMP)	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.65	1.78E-05
docosadienoate (22:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.83	2.39E-05
2-hydroxybutyrate (AHB)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.5	2.73E-05
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.65	2.91E-05
pentadecanoate (15:0)	Lipid	Long Chain Fatty Acid	0.63	3.95E-05
glucuronate	Carbohydrate	Aminosugar Metabolism	1.44	4.50E-05
nicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	1.52	6.14E-05
phenylacetylglycine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.57	6.34E-05
arabonate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	2.5	6.78E-05
phosphoethanolamine	Lipid	Phospholipid Metabolism	0.71	7.03E-05
ribulose	Carbohydrate	Pentose Metabolism	3.24	7.10E-05
pipecolate	Amino Acid	Lysine Metabolism	0.65	9.15E-05
xylonate	Carbohydrate	Pentose Metabolism	0.62	9.38E-05
riboflavin (Vitamin B2)	Cofactors and Vitamins	Riboflavin Metabolism	0.67	9.62E-05
dimethylarginine (SDMA + ADMA)	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.62	0.0001
myo-inositol	Lipid	Inositol Metabolism	0.73	0.0001
xanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.84	0.0001
gamma-glutamylthreonine*	Peptide	Gamma-glutamyl Amino Acid	1.96	0.0002
citrate	Energy	TCA Cycle	12.81	0.0002
succinate	Energy	TCA Cycle	1.75	0.0002
1,3-dihydroxyacetone	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	5.8	0.0003
trigonelline (N'-methylnicotinate)	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	1.28	0.0003
flavin mononucleotide (FMN)	Cofactors and Vitamins	Riboflavin Metabolism	0.77	0.0003

pantothenate	Cofactors and Vitamins	Pantothenate and CoA Metabolism	0.83	0.0003
gulono-1,4-lactone	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.72	0.0003
ophthalmate	Amino Acid	Glutathione Metabolism	0.58	0.0004
acetylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.51	0.0004
2-stearoyl-GPI (18:0)*	Lipid	Lysolipid	0.73	0.0004
hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.73	0.0004
gluconate	Xenobiotics	Food Component/Plant	1.55	0.0004
phenylpropionylglycine	Amino Acid	Phenylalanine and Tyrosine Metabolism	1.78	0.0005
malate	Energy	TCA Cycle	1.64	0.0005
tetradecanedioate	Lipid	Fatty Acid, Dicarboxylate	0.51	0.0005
2-oleoyl-GPC (18:1)*	Lipid	Lysolipid	2.59	0.0006
1-oleoyl-GPI (18:1)*	Lipid	Lysolipid	0.45	0.0006
3-methylcrotonylglycine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.52	0.0007
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.46	0.0007
N1-methyladenosine	Nucleotide	Purine Metabolism, Adenine containing	0.69	0.0007
cytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.85	0.0007
Isobar: betaine aldehyde, N-methyldiethanolamine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.77	0.0008
ribitol	Carbohydrate	Pentose Metabolism	1.5	0.0009
inositol 1-phosphate (I1P)	Lipid	Inositol Metabolism	0.75	0.0009
choline	Lipid	Phospholipid Metabolism	0.83	0.0009
uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.69	0.0009
5-oxoproline	Amino Acid	Glutathione Metabolism	0.84	0.001
tauroursodeoxycholate	Lipid	Secondary Bile Acid Metabolism	3.08	0.001
kynurenate	Amino Acid	Tryptophan Metabolism	0.39	0.0011
alanine	Amino Acid	Alanine and Aspartate Metabolism	1.28	0.0012
ribose 5-phosphate	Carbohydrate	Pentose Phosphate Pathway	1.84	0.0012
cinnamoylglycine	Xenobiotics	Food Component/Plant	1.58	0.0012
gamma-glutamylleucine	Peptide	Gamma-glutamyl Amino Acid	1.41	0.0013
beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.5	0.0013
sarcosine (N-Methylglycine)	Amino Acid	Glycine, Serine and Threonine Metabolism	4.25	0.0014
N-acetylalanine	Amino Acid	Alanine and Aspartate Metabolism	0.69	0.0014
3-(4-hydroxyphenyl)lactate	Amino Acid	Phenylalanine and Tyrosine Metabolism	1.52	0.0014
isovalerylglycine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.55	0.0014
homostachydrine*	Xenobiotics	Food Component/Plant	2.66	0.0015
palmitoyl sphingomyelin (d18:1/16:0)	Lipid	Sphingolipid Metabolism	0.76	0.0016
alpha-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.63	0.0017
urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.4	0.0017
allo-threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.55	0.0018
erythronate*	Carbohydrate	Aminosugar Metabolism	1.72	0.0023
2-docosahexaenoyl-GPC (22:6)*	Lipid	Lysolipid	2.8	0.0023
margarate (17:0)	Lipid	Long Chain Fatty Acid	0.76	0.0025
glycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.83	0.0027
glycylvaline	Peptide	Dipeptide	1.8	0.0027
valerylglycine	Lipid	Fatty Acid Metabolism(Acyl Glycine)	0.69	0.0027
1-dihomo-linolenoyl-GPC (20:3n3 or 6)*	Lipid	Lysolipid	2.15	0.0029
palmitoleate (16:1n7)	Lipid	Long Chain Fatty Acid	0.68	0.003
2-arachidonoyl-GPC (20:4)*	Lipid	Lysolipid	2.39	0.0037

hexadecanedioate	Lipid	Fatty Acid, Dicarboxylate	0.77	0.0038
undecanedioate	Lipid	Fatty Acid, Dicarboxylate	0.75	0.0039
propionylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.61	0.0039
equol sulfate	Xenobiotics	Food Component/Plant	3.56	0.0039
10-heptadecenoate (17:1n7)	Lipid	Long Chain Fatty Acid	0.74	0.0043
ergothioneine	Xenobiotics	Food Component/Plant	1.46	0.0043
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.69	0.0047
docosatrienoate (22:3n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	1.75	0.0047
9,10-DiHOME	Lipid	Fatty Acid, Dihydroxy	0.71	0.0049
daidzein	Xenobiotics	Food Component/Plant	2.2	0.0051
homoserine	Amino Acid	Glycine, Serine and Threonine Metabolism	1.88	0.0053
creatine	Amino Acid	Creatine Metabolism	0.73	0.0053
10-nonadecenoate (19:1n9)	Lipid	Long Chain Fatty Acid	0.76	0.0053
C-glycosyltryptophan	Amino Acid	Tryptophan Metabolism	0.84	0.0057
glycylproline	Peptide	Dipeptide	1.38	0.0063
hexanoylglycine	Lipid	Fatty Acid Metabolism(Acyl Glycine)	0.61	0.0066
1-palmitoleoyl-GPC (16:1)*	Lipid	Lysolipid	1.63	0.0067
flavin adenine dinucleotide (FAD)	Cofactors and Vitamins	Riboflavin Metabolism	0.85	0.0067
6-phosphogluconate	Carbohydrate	Pentose Phosphate Pathway	1.35	0.0068
1-palmitoyl-GPC (16:0)	Lipid	Lysolipid	1.9	0.0071
tauro-beta-muricholate	Lipid	Primary Bile Acid Metabolism	2.26	0.0071
2-linoleoyl-GPC (18:2)*	Lipid	Lysolipid	2.14	0.0075
kojibiose	Xenobiotics	Food Component/Plant	1.38	0.0075
beta-muricholate	Lipid	Primary Bile Acid Metabolism	2.05	0.0076
1,5-anhydroglucitol (1,5-AG)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.31	0.0082
1-oleoyl-GPC (18:1)	Lipid	Lysolipid	2.02	0.0087
6-beta-hydroxylithocholate	Lipid	Secondary Bile Acid Metabolism	2.19	0.0088
17,18-DiHETE	Lipid	Eicosanoid	0.76	0.0102
1-margaroyl-GPC (17:0)	Lipid	Lysolipid	2.2	0.0109
3-indoxyl sulfate	Amino Acid	Tryptophan Metabolism	2.02	0.0124
palmitate (16:0)	Lipid	Long Chain Fatty Acid	0.85	0.0125
chenodeoxycholate	Lipid	Primary Bile Acid Metabolism	1.56	0.0134
deoxycarnitine	Lipid	Carnitine Metabolism	1.24	0.0144
oleate (18:1n9)	Lipid	Long Chain Fatty Acid	0.72	0.0146
5-methyluridine (ribothymidine)	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.77	0.0158
ascorbate (Vitamin C)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	8.4	0.0158
glutathione, reduced (GSH)	Amino Acid	Glutathione Metabolism	1.57	0.0161
5-aminovalerate	Amino Acid	Lysine Metabolism	0.79	0.0167
15-methylpalmitate	Lipid	Fatty Acid, Branched	0.78	0.0178
1-linoleoyl-GPC (18:2)	Lipid	Lysolipid	1.73	0.0202
stearidonate (18:4n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.69	0.0204
1-stearoyl-GPI (18:0)	Lipid	Lysolipid	0.82	0.0215
2-docosapentaenoyl-GPE (22:5)*	Lipid	Lysolipid	1.56	0.0221
beta-hydroxypyruvate	Amino Acid	Glycine, Serine and Threonine Metabolism	1.28	0.0248
17-methylstearate	Lipid	Fatty Acid, Branched	0.8	0.0248
2-docosahexaenoyl-GPE (22:6)*	Lipid	Lysolipid	1.92	0.0253
glycylleucine	Peptide	Dipeptide	1.99	0.0258
pyridoxate	Cofactors and Vitamins	Vitamin B6 Metabolism	0.77	0.0271
tryptophan	Amino Acid	Tryptophan Metabolism	1.17	0.0279
biopterin	Cofactors and Vitamins	Tetrahydrobiopterin Metabolism	0.86	0.0281

2-arachidonoyl-GPE (20:4)*	Lipid	Lysolipid	1.97	0.0282
isoleucylglycine	Peptide	Dipeptide	1.91	0.0309
glutamine	Amino Acid	Glutamate Metabolism	1.17	0.0341
1-octadecanol	Lipid	Fatty Alcohol, Long Chain	0.86	0.0357
2'-deoxycytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.74	0.0363
3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.64	0.0364
2-hydroxyglutarate	Lipid	Fatty Acid, Dicarboxylate	1.46	0.0395
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.29	0.0425
1-docosahexaenoyl-GPC (22:6)*	Lipid	Lysolipid	1.77	0.046
gamma-glutamylglutamate	Peptide	Gamma-glutamyl Amino Acid	1.13	0.0489
5-dodecenoate (12:1n7)	Lipid	Medium Chain Fatty Acid	1.57	0.0491

Table 16. Biomarkers for fasting status in tissue (muscle)

			Fed / Fasted	
Biochemical Name	**Super Pathway**	**Sub Pathway**	**Fold Change**	**p-value**
stachydrine	Xenobiotics	Food Component/Plant	4.84	6.51E-11
2-hydroxybutyrate (AHB)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.15	9.65E-10
3-hydroxybutyrate (BHBA)	Lipid	Ketone Bodies	0.16	9.58E-09
guanosine	Nucleotide	Purine Metabolism, Guanine containing	0.65	1.87E-06
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.68	2.26E-06
2-aminobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.28	2.42E-06
glycolate (hydroxyacetate)	Xenobiotics	Chemical	1.83	2.43E-06
glucose	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.67	3.29E-06
glutamine	Amino Acid	Glutamate Metabolism	0.69	1.36E-05
choline	Lipid	Phospholipid Metabolism	0.46	1.43E-05
aspartate	Amino Acid	Alanine and Aspartate Metabolism	0.55	1.57E-05
leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.78	2.24E-05
uridine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.7	2.56E-05
ophthalmate	Amino Acid	Glutathione Metabolism	0.51	3.13E-05
cytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.52	8.46E-05
N-acetylglycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.4	8.48E-05
13-HODE + 9-HODE	Lipid	Fatty Acid, Monohydroxy	0.61	9.34E-05
ethanolamine	Lipid	Phospholipid Metabolism	0.49	9.39E-05
eicosenoate (20:1)	Lipid	Long Chain Fatty Acid	0.55	9.64E-05
succinylcarnitine	Energy	TCA Cycle	1.79	9.65E-05
valine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.81	0.0001
phosphoenolpyruvate (PEP)	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	3.06	0.0001
hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.63	0.0001
tiglylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.78	0.0002
2-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	3.35	0.0002
10-nonadecenoate (19:1n9)	Lipid	Long Chain Fatty Acid	0.56	0.0002
palmitoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.16	0.0002

uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.57	0.0002
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	1.44	0.0003
beta-hydroxyisovaleroylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.23	0.0003
ornithine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	2.07	0.0003
alanyltyrosine	Peptide	Dipeptide	2.51	0.0003
glutamate	Amino Acid	Glutamate Metabolism	0.67	0.0004
glycylvaline	Peptide	Dipeptide	1.55	0.0004
serylleucine	Peptide	Dipeptide	3.14	0.0004
3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	4.09	0.0004
17-methylstearate	Lipid	Fatty Acid, Branched	0.64	0.0004
oleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.15	0.0004
homostachydrine	Xenobiotics	Food Component/Plant	1.94	0.0004
lactate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.26	0.0005
maltotriose	Carbohydrate	Glycogen Metabolism	4.67	0.0005
aspartylleucine	Peptide	Dipeptide	2.9	0.0007
malate	Energy	TCA Cycle	0.78	0.0008
nonadecanoate (19:0)	Lipid	Long Chain Fatty Acid	0.67	0.001
gamma-aminobutyrate (GABA)	Amino Acid	Glutamate Metabolism	0.46	0.0012
glycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	2.09	0.0012
propionylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.8	0.0018
cytidine 5'-monophosphate (5'-CMP)	Nucleotide	Pyrimidine Metabolism, Cytidine containing	1.39	0.002
fumarate	Energy	TCA Cycle	0.69	0.0026
linoleate (18:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.7	0.0027
oleate (18:1n9)	Lipid	Long Chain Fatty Acid	0.67	0.0029
1-docosahexaenoyl-GPC (22:6)	Lipid	Lysolipid	0.32	0.0031
tryptophan	Amino Acid	Tryptophan Metabolism	0.85	0.0036
maltose	Carbohydrate	Glycogen Metabolism	1.97	0.0039
octanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.44	0.0045
dihomo-linoleate (20:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.64	0.0046
acetylphosphate	Energy	Oxidative Phosphorylation	0.78	0.0052
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	1.81	0.0055
1-margaroyl-GPC (17:0)	Lipid	Lysolipid	0.62	0.0057
alpha-hydroxyisovalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.65	0.0064
fructose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	1.58	0.0065
alanylleucine	Peptide	Dipeptide	2.4	0.007
urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.4	0.0074
alanine	Amino Acid	Alanine and Aspartate Metabolism	1.2	0.0076
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.86	0.0077
xanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.83	0.0078
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.86	0.0079
glycylleucine	Peptide	Dipeptide	1.32	0.0085
methionine sulfoxide	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	1.22	0.0086
1-arachidonoyl-GPC (20:4n6)	Lipid	Lysolipid	0.42	0.0089
2-palmitoyl-GPC (16:0)	Lipid	Lysolipid	0.39	0.0109
arabitol	Carbohydrate	Pentose Metabolism	1.39	0.0112

butyrylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.58	0.012
serylphenyalanine	Peptide	Dipeptide	1.93	0.0121
sorbitol	Carbohydrate	Fructose, Mannose and Galactose Metabolism	2.57	0.0128
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.73	0.0129
1-palmitoleoyl-GPC (16:1)	Lipid	Lysolipid	0.39	0.0141
2-arachidonoyl-GPE (20:4)	Lipid	Lysolipid	0.43	0.015
2-docosapentaenoyl-GPE (22:5)	Lipid	Lysolipid	0.41	0.0153
1-oleoyl-GPC (18:1)	Lipid	Lysolipid	0.39	0.016
margarate (17:0)	Lipid	Long Chain Fatty Acid	0.77	0.0172
mannose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	1.44	0.018
docosahexaenoate (DHA; 22:6n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.8	0.0185
1-linoleoyl-GPC (18:2)	Lipid	Lysolipid	0.39	0.0185
stearate (18:0)	Lipid	Long Chain Fatty Acid	0.83	0.0195
2-docosahexaenoyl-GPE (22:6)	Lipid	Lysolipid	0.41	0.0195
myo-inositol	Lipid	Inositol Metabolism	0.85	0.0198
hexanoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.56	0.0206
2-arachidonoyl-GPC (20:4)	Lipid	Lysolipid	0.43	0.0212
1-palmitoyl-GPC (16:0)	Lipid	Lysolipid	0.49	0.0216
2-docosahexaenoyl-GPC (22:6)	Lipid	Lysolipid	0.39	0.0225
N-glycolylneuraminate	Xenobiotics	Food Component/Plant	0.65	0.0258
2-oleoyl-GPC (18:1)	Lipid	Lysolipid	0.43	0.0265
prolylleucine	Peptide	Dipeptide	1.21	0.0277
1-(1-enyl-palmitoyl)-GPE (P-16:0)	Lipid	Lysoplasmalogen	0.75	0.0284
dehydroascorbate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	1.82	0.0284
1-myristoyl-GPC (14:0)	Lipid	Lysolipid	0.5	0.0285
pipecolate	Amino Acid	Lysine Metabolism	1.35	0.0287
3-(4-hydroxyphenyl)lactate	Amino Acid	Phenylalanine and Tyrosine Metabolism	1.2	0.029
2-linoleoyl-GPC (18:2)	Lipid	Lysolipid	0.45	0.0317
2-stearoyl-GPC (18:0)	Lipid	Lysolipid	0.53	0.0364
betaine	Amino Acid	Glycine, Serine and Threonine Metabolism	1.15	0.0384
glucose 6-phosphate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	2.41	0.0416
Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate, myo-inositol 1,4 or 1,3-diphosphate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.74	0.0484

Table 17. Biomarkers for fasting status in tissue (adipose)

			Fed / Fasted	
Biochemical Name	**Super Pathway**	**Sub Pathway**	**Fold Change**	**p-value**
2-hydroxybutyrate (AHB)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.29	7.74E-06
stachydrine	Xenobiotics	Food Component/Plant	4.14	2.52E-05
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	6.47	3.66E-05
oleate (18:1n9)	Lipid	Long Chain Fatty Acid	0.4	0.0003
phosphoethanolamine	Lipid	Phospholipid Metabolism	0.5	0.0003
maltose	Carbohydrate	Glycogen Metabolism	5.89	0.0004
glycolate (hydroxyacetate)	Xenobiotics	Chemical	1.75	0.0009

cis-vaccenate (18:1n7)	Lipid	Long Chain Fatty Acid	0.43	0.001
12,13-DiHOME	Lipid	Fatty Acid, Dihydroxy	0.42	0.0028
glucose	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	2.13	0.0029
9,10-DiHOME	Lipid	Fatty Acid, Dihydroxy	0.35	0.0031
homostachydrine*	Xenobiotics	Food Component/Plant	2.03	0.0034
spermidine	Amino Acid	Polyamine Metabolism	0.57	0.0083
taurocholate	Lipid	Primary Bile Acid Metabolism	0.13	0.0085
nonadecanoate (19:0)	Lipid	Long Chain Fatty Acid	0.47	0.012
isovalerylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.84	0.0125
17-methylstearate	Lipid	Fatty Acid, Branched	0.48	0.014
ethanolamine	Lipid	Phospholipid Metabolism	0.51	0.0157
tauro-beta-muricholate	Lipid	Primary Bile Acid Metabolism	0.1	0.0157
isopalmitic acid	Lipid	Fatty Acid, Branched	0.58	0.0162
stearate (18:0)	Lipid	Long Chain Fatty Acid	0.67	0.0167
eicosenoate (20:1)	Lipid	Long Chain Fatty Acid	0.46	0.0186
linolenate [alpha or gamma; (18:3n3 or 6)]	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.68	0.0192
1-stearoyl-GPC (18:0)	Lipid	Lysolipid	1.95	0.0195
beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.64	0.0238
margarate (17:0)	Lipid	Long Chain Fatty Acid	0.59	0.024
isobutyrylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	1.58	0.0241
1-oleoyl-GPC (18:1)	Lipid	Lysolipid	1.61	0.0241
ergothioneine	Xenobiotics	Food Component/Plant	1.78	0.0256
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.96	0.0309
docosahexaenoate (DHA; 22:6n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.74	0.0334
palmitate (16:0)	Lipid	Long Chain Fatty Acid	0.72	0.0371
1-palmitoyl-GPC (16:0)	Lipid	Lysolipid	1.63	0.0389
succinate	Energy	TCA Cycle	1.5	0.0424
stearidonate (18:4n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.71	0.0443
nicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	1.66	0.0477

Example 5. Assessing Sample Quality in Fasted and non-Fasted Samples from an Individual.

[00143] In one example, the quality of two human plasma samples was assessed using the biomarkers and methods described herein. Both samples were collected from the same subject; one was a fasted sample that was collected following an overnight fast and one was a non-fasted sample. The samples were stored and processed according to the same protocol. The levels of the quality biomarkers in each sample were determined and a composite score for each of the quality parameters of fasting status, sample processing and sample storage was generated and compared to a reference.

[00144] In this example, the Acceptable Reference Composite Score for Fasting is <0.51, for Storage is <0.61 and for Processing is <0.93. For Sample 1 the composite

score obtained for each parameter was acceptable (-0.58 for Fasting, -0.24 for Storage, -0.25 for Processing). For Sample 2 the composite score for Fasting was unacceptable (1.98) and the composite scores for Storage and Processing were acceptable (-0.44 for Storage, -0.47 for Processing).

[00145] In this example, a composite score in the 95th percentile or greater is also considered to be unacceptable. For Sample 1 the composite score percentile for the Fasting, Storage and Processing parameters was 2, 23 and 35, respectively. For Sample 2 the composite score percentile for the Fasting, Storage and Processing parameters was 100, 12 and 23, respectively.

[00146] Based on the composite score analysis, both samples were acceptable for sample storage and sample processing quality parameters, Sample 1 was determined to be a fasted sample and Sample 2 was determined to be a non-fasted sample; Sample 2 was flagged as non-fasted. The composite scores obtained for each parameter are graphically illustrated in Figure 9. In addition, the analysis of the levels obtained for the individual biomarkers that comprise the composite score for each of the quality parameters related to sample storage, sample processing and fasting status is summarized below.

[00147] The composite score for the fasting status parameter was based on the levels of the biomarkers glycocholate, palmitoleate (16:1n7), uridine, mannose, xanthine, azelate (nonanedioate), methionine sulfoxide, and dopamine sulfate measured in the sample. In Sample 1 (fasted sample), the Z-score values for all of the biomarkers were within the given reference range. For Sample 2 (non-fasted sample), three individual fasting status biomarkers (uridine, azelate, and methionine sulfoxide) had Z-scores outside the reference range, two biomarkers (xanthine, dopamine-sulfate) had borderline Z-scores and three biomarkers (glycocholate, palmitoleate (16:1n7), mannose) had Z-scores within the reference range.

[00148] Another method of evaluating the results was based on the "Percentile", which indicates where the test sample ranks relative to the reference population for the indicated biomarker. For biomarkers that decrease when a subject was fasted (methionine sulfoxide, glycocholate, dopamine sulfate, azelate), a value above the 95th percentile indicates that the test sample was from a subject that was not fasted. For biomarkers that increase when a subject is fasted (uridine, xanthine, mannose, palmitoleate), a value below 5th percentile indicates that the test sample was from a

subject that was not fasted. In the non-fasted sample, the percentile for methionine sulfoxide and azelate is above 95 and the percentile for uridine was less than 5. The fasting status biomarker data is graphically displayed in Figure 10.

[00149] The composite score for the sample storage quality parameter was based on the levels of the biomarkers alpha-ketoglutarate, methionine, glutamine, caproate (6:0), gamma-glutamylleucine and 4-methyl-2-oxopentanoate in the sample. For the fasted sample, the levels of each biomarker for sample storage were within the reference range and for the non-fasted sample alpha-ketoglutarate, methionine, glutamine, caproate (6:0), and 4-methyl-2-oxopentanoate were within the reference range, only gamma-glutamylleucine fell outside the acceptable reference range. The storage quality biomarker data is graphically illustrated in Figure 11.

[00150] The composite score for the sample processing quality parameter was based on the levels of the biomarkers adenosine 5'-monophosphate (AMP), taurine, glucose and cys-gly, oxidized in the sample. The results obtained for each biomarker for sample processing for both samples indicated that adenosine 5'-monophosphate (AMP), taurine, and glucose were within the reference range; cys-gly oxidized fell outside the acceptable reference range for Sample 1 and was borderline for Sample 2. The sample processing biomarker data is graphically illustrated in Figure 12.

Example 6. Biomarkers of Sample Storage Temperature (New to PCT)

[00151] Sample quality biomarkers for assessing sample storage over time, at -20°C and -80°C were identified. Plasma samples were stored for 1 month, 3 months, or 6 months at -20°C or at -80°C before being processed for analysis. For each time point and storage condition, ten plasma samples were used. After the levels of metabolites were determined, the data were analyzed to identify biomarkers for assessing sample storage at different storage temperatures over time. Welch's two-sample t-tests were performed to compare the two temperature conditions at each timepoint. Metabolites that differentiated samples stored at -20°C from those stored at -80°C are displayed in Table 18. Table 18 includes, for each biomarker, the biochemical name of the biomarker and the fold change for the biomarker in samples stored at -20°C compared to samples stored at -80°C (-20°C/-80°C), which is the ratio of the mean level of the biomarkers in samples stored at -20°C as compared to the -80°C mean level. The fold change values are shown for the 1 month, 3 month, and 6 month timepoints.

Table 18. Biomarker metabolites that differentiate sample storage at -20°C vs. -80°C

	Fold Change (-20°C/80°C)		
Biochemical Name	**1 Month**	**3 Months**	**6 Months**
4-methyl-2-oxopentanoate	0.8	0.43	0.25
alpha-ketoglutarate	0.84	0.46	0.26
glutamine	0.72	0.39	0.29
methionine	0.96	0.88	0.85
gamma-glutamylleucine	1.26	1.58	1.8
caproate (6:0)	1.08	1.15	1.26
pyruvate	0.32	0.15	0.16
gamma-glutamylglycine	3.34	4.44	4.92
gamma-glutamylmethionine	7.44	9.44	9.39
gamma-glutamylhistidine	1.86	2.65	3
methionine sulfoxide	1.25	1.49	2.14
isovalerate	1.36	1.94	2.46
valylvaline	1.25	1.55	1.91
gamma-glutamylglutamine	2.14	1.63	1.29
gamma-glutamyltyrosine	1.27	1.62	1.88
succinate	1.17	1.44	1.68
3-methyl-2-oxobutyrate	0.83	0.5	0.26
methionylalanine	1.14	1.21	1.28
3-methyl-2-oxovalerate	0.89	0.64	0.43
seryltyrosine	1.21	1.18	1.22
glutamate	1.11	1.15	1.22
aspartate	0.92	0.78	0.8
4-guanidinobutanoate	1.09	1.24	1.52
inosine	1.4	1.37	1.58
5-KETE	0.7	0.57	0.74
N6-methyladenosine	0.46	0.38	0.38
phenylalanyllysine	1.08	1.24	1.3
butyrylcarnitine	0.96	0.7	0.53
gamma-glutamylphenylalanine	1.16	1.34	1.46
bilirubin (E,E)	0.83	0.52	0.45
urate	0.9	0.8	0.94
asparagine	0.98	0.81	0.78
gamma-glutamylvaline	1.11	1.16	1.25
bilirubin (Z,Z)	0.81	0.53	0.35
xanthine	0.91	0.82	0.96
leucylalanine	1.09	1.08	1.17
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)	0.9	0.86	0.8

1-palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6)	0.9	0.87	0.85
histidylalanine	1.07	1.06	1.16
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	0.91	0.85	0.82
succinylcarnitine	1.24	1.31	1.6
gamma-glutamylthreonine	1.11	1.4	1.61
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	0.94	0.88	0.86
N-formylmethionine	0.98	0.88	0.86
alpha-ketobutyrate	0.56	0.45	0.34
N-acetylmethionine	0.93	0.85	0.78
16a-hydroxy DHEA 3-sulfate	1.03	0.89	0.77
1-palmityl-2-arachidonoyl-GPC (O-16:0/20:4)	0.92	0.93	0.91
cysteine sulfinic acid	1.17	1.27	1.4
biliverdin	1.66	0.84	0.37
1-(1-enyl-palmitoyl)-2-docosahexaenoyl-GPE (P-16:0/22:6)	0.91	0.83	0.77
valylglycine	1.09	1.06	1.14

[00152] A model was generated to produce a composite score using the levels of six exemplary biomarkers, caproate, gamma-glutamylleucine, 4-methyl-2-oxopentanoate, alpha-ketoglutarate, glutamine, and methionine. In this example, the biomarkers in the model were equally weighted; biomarkers that had higher levels in samples stored at -20°C were assigned a positive weight and biomarkers that had higher levels in samples stored at -80°C were assigned a negative weight. Thus, the biomarkers caproate and gamma-glutamylleucine were assigned a positive weight; the biomarkers 4-methyl-2-oxopentanoate, alpha-ketoglutarate, glutamine, and methionine were assigned a negative weight. The weighted value can be used as a coefficient in the generated model. It should be appreciated that the coefficients (weights) are exemplary and may be refined using formal statistical analysis methods as described herein.

[00153] The biomarkers and model can be used to generate a composite score to assess sample quality and a storage temperature quality parameter. For example, the composite score for storage temperature for a sample may be calculated as follows: Storage Temperature Composite Score = 1/6(caproate + gamma-glutamylleucine – 4-methyl-2-oxopentanoate – alpha-ketoglutarate – glutamine – methionine).

Example 7. Assessing Sample Quality

[00154] Metabolic profiling was used as described herein to assess sample quality simultaneously with performing intended use analysis of 98 plasma samples that were evaluated in five analytical batches. The samples were reportedly collected according to the determined sample collection protocol (i.e., plasma separated from whole blood within one hour of collection and freezing immediately at -80ºC).

[00155] Metabolic profiling identified between 476 and 806 metabolites in the samples. After the levels of the metabolites were determined, statistical analysis was performed to determine z-score values for each of the metabolites. Sample quality was assessed using the biomarkers and methods described herein. Further, the levels of quality biomarkers were used to generate a composite score for each of the quality parameters of sample processing, sample storage, stability and fasting status. Based on this analysis, samples were flagged for having aberrant scores for biomarkers of Storage (4 samples), Stability (16 samples) or Processing (2 samples), and five (5) samples were flagged as having aberrant quality biomarker scores for Storage Temperature, Stability and Processing. The aberrant quality biomarker scores indicate that the samples were not properly handled and were not processed according to protocol. No samples were flagged as being fed samples. The scores are summarized in Table 19. Failing/flagged scores are indicated by ***bold italics***. The detailed results of the analysis for the five samples that failed all three quality parameters related to sample handling and processing are detailed below.

Table 19 Summary of Quality Scores for Flagged Samples

Sample Number	Batch Number	Storage Score	Stability Score	Processing Score	Fasting
1	85	***-2.805***	***-0.321***	-2.300	Fasted
2	24	***-1.435***	0.812	-1.423	Fasted
3	85	-0.912	***-0.422***	-1.100	Fasted
4	24	***-1.356***	0.081	-1.073	Fasted
5	24	-0.517	***1.489***	-0.987	Fasted
6	24	-1.082	***1.137***	-0.963	Fasted
7	24	-0.965	***0.822***	-0.912	Fasted
8	24	-0.630	***-0.421***	-0.287	Fasted
9	91	***-1.794***	-0.174	-0.240	Fasted
10	91	-0.597	***-0.410***	-0.053	Fasted
11	24	0.221	***0.834***	-0.034	Fasted
12	85	0.021	***-0.462***	-0.014	Fasted

13	91	0.452	***-0.525***	0.013	Fasted
14	85	0.452	***-0.556***	0.029	Fasted
15	24	0.377	***-0.746***	0.183	Fasted
16	85	0.135	***-1.170***	0.215	Fasted
17	85	0.640	***1.248***	0.395	Fasted
18	73	-0.395	***-0.412***	***Flag***	Fasted
19	73	-0.615	***-0.409***	***Flag***	Fasted
A	91	***1.671***	***-0.319***	***0.083***	Fasted
B	24	***0.131***	***-0.445***	***0.800***	Fasted
C	85	***9.377***	***0.818***	***7.335***	Fasted
D	73	***2.285***	***-0.508***	***7.34***	Fasted
E	13	***-1.293***	***0.127***	***-0.97***	Fasted

[00156] In the first sample (Sample A), metabolic profiling identified 632 metabolites in the sample and analysis showed that the sample quality biomarkers 5-oxoproline, lactate, glutamate, pyruvate, arginine, glucose, and cysteinylglycine, were aberrant based on the calculated Z-scores. The composite score for sample processing was also determined to be unacceptable (Score <5th percentile). A composite score in the 95th percentile or greater is also considered to be unacceptable. For this sample, the Z-scores calculated for the quality biomarkers and the composite Z-score calculated for fasting status were acceptable while the composite score percentiles for the Storage and Processing parameters were not within the acceptable range. The Z-scores that were calculated for the levels of the aberrant biomarkers for this sample are shown in Table 20.

Table 20. Sample A: Aberrant Sample Quality Biomarkers

Biochemical Name	**Z-score**
5-oxoproline	2.83
Lactate	2.64
Glutamate	2.31
Cysteinylglycine	2.27
Pyruvate	1.95
Arginine	-3.22
Glucose	-4.81

[00157] Based on the levels of the quality biomarkers in the sample, the sample quality parameter of sample processing was flagged (i.e., identified as aberrant), and the sample was determined to not have been handled according to the defined sample

collection protocol.

[00158] In the same sample, metabolic profiling also identified biomarkers of subject compliance. The biomarkers 2-hydroxyacetaminophen sulfate, 4-acetaminophen sulfate, and 4-acetamidophenylglucuronide were elevated in the plasma sample relative to control samples with Z-score values of 3.35, 3.10, and 2.93, respectively. Based on the presence and levels of these biomarkers, the subject was determined to be undergoing acetaminophen treatment at the time of sample collection. This result may indicate that the subject was not in compliance with protocol if acetaminophen was not allowed prior to sample collection; alternatively, if acetaminophen was allowed, then the subject may be in compliance with instructions.

[00159] In a second plasma sample (Sample B), metabolic profiling identified 806 metabolites in the sample. After the levels of the metabolites were determined, statistical analysis was performed to determine Z-score values for each of the metabolites; the sample quality biomarkers pyruvate, cys-gly (oxidized), 5-oxoproline, fumarate, inosine, ornithine, lactate, glucose, arginine, oleoyl ethanolamide, sphingosine 1-phosphate, sphinganine-1-phosphate, ergothioneine, and cysteinylglycine, were determined to be aberrant. Based on the scores calculated for the levels of the quality biomarkers in the sample, the sample quality parameter of sample processing was identified as aberrant, and the sample was determined to not have been handled according to the defined sample collection protocol. The Z-scores calculated for each of the biomarkers are shown in Table 21.

Table 21. Sample B: Aberrant Sample Quality Biomarkers

Biochemical Name	Z-score
Pyruvate	2.87
Cys-gly,oxidized	2.80
5-oxoproline	2.45
Sphingosine 1-phosphate	2.34
Oleoyl ethanolamide	2.10
Sphinganine-1-phosphate	2.08
Ergothioneine	1.99
Fumarate	1.81
Inosine	1.71
Cysteinylglycine	1.65
Ornithine	1.60
Lactate	1.54

Glucose	-1.68
Arginine	-3.07

[00160] In a third plasma sample (Sample C), metabolic profiling identified 627 metabolites in the sample. After the levels of the metabolites were determined, statistical analysis was performed to calculate z-score values for each of the metabolites. Sample quality was assessed using the biomarkers described herein. The sample quality biomarkers 5-oxoproline, cys-gly (oxidized), pyruvate, fumarate, lactate, glycerol, hypoxanthine, arginine, mannose, glucose sphinganine-1-phosphate, and sphingosine 1-phosphate, were determined to be aberrant. Based on the calculated Z-scores for the levels of the quality biomarkers measured in the sample, the sample quality parameter of sample processing was identified as failing the quality metric, and the sample was determined to not have been handled according to the defined sample collection protocol. The Z-scores calculated for levels of the biomarkers are shown in Table 22.

Table 22. Sample C: Aberrant Sample Quality Biomarkers

Biochemical Name	Z-score
5-oxoproline	5.22
Cys-gly, oxidized	4.3
Pyruvate	3.66
Sphinganine-1-phosphate	3.64
Spingosine 1-phosphate	3.42
Fumarate	3.17
Lactate	2.95
Glycerol	-2.66
Hypoxanthine	-4.54
Arginine	-6.07
Mannose	-9.51
Glucose	-32.3

[00161] In a fourth plasma sample (Sample D), metabolic profiling identified 616 metabolites in the sample. The sample quality biomarkers hypoxanthine, lactate, inosine, lysine, arginine, mannose, and glucose were determined to be aberrant, indicating problems with the sample quality parameter of sample processing. Thus, the sample was determined to not have been handled according to the defined sample collection protocol. The calculated Z-scores for the measured levels of the biomarkers are shown in Table 23.

Table 23. Sample D: Aberrant Sample Quality Biomarkers

Biochemical Name	Z-score
Hypoxanthine	2.42
Lactate	2.33
Inosine	2.28
Lysine	-2.64
Arginine	-3.84
Mannose	-9.54
Glucose	-25.3

[00162] In a fifth plasma sample (Sample E), metabolic profiling identified 476 metabolites in the sample. After the levels of the metabolites were determined, statistical analysis was performed to calculate z-score values for each of the metabolites. Sample quality was assessed using the biomarkers described herein. The sample quality biomarkers 5-oxoproline, glutamate, fumarate, palmitoylcarnitine, and linoleoylcarnitine were determined to be aberrant. Based on the levels of the quality biomarkers in the sample, the time incubated as whole blood prior to separation, a quality parameter of sample processing, was flagged. The sample was determined to have had a delayed separation of plasma from whole blood, indicating that the sample was not handled according to the defined sample collection protocol. The levels of the biomarkers (Z-score) are shown in Table 23.

Table 24. Sample E: Aberrant Sample Quality Biomarkers

Biochemical Name	Z-score
5-oxoproline	13.04
glutamate	4.18
fumarate	3.47
palmitoylcarnitine	-2.38
linoleoylcarnitine	-2.28

Example 8. Assessing Sample Handling in Clinical Samples

[00163] In another example, metabolic profiling was performed as described herein and the biomarkers and algorithms described herein were used in the process as outlined in Figures 1 and 2 to simultaneously assess sample quality while performing intended use analysis of 937 clinical plasma samples. Using these systems, methods, biomarkers and algorithms, of the 937 samples tested, 47 samples were flagged as having quality problems due to sample handling in the clinic. The types of sample

quality issues that were identified in these flagged samples include use of the wrong collection tube (protocol calls for EDTA but flagged samples do not meet that requirement), delayed processing, improper or delayed separation of whole blood, improper collection temperature, and hemolysis. The results are summarized in Table 24 and notes describing the quality biomarker signatures indicating sample should be flagged for quality issues are summarized in Table 26. The numbers in Table 26 indicate the score calculated for that biomarker in that sample. The signatures for each sample indicating that the sample should be flagged for having aberrant quality are based on combinations of biomarkers. The aberrant biomarkers are not necessarily identical for each sample affected by the same quality issue; rather it is the composite score for overlapping sets of biomarkers and the various combinations of biomarkers that indicate which quality parameter is aberrant.

Table 25. Summary of Flagged Samples Based on Aberrant Sample Quality Biomarkers and Resulting Quality Scores.

Quality Issue	Number of Samples
Improper Collection Tube (non-EDTA)	26
Delayed sample processing	3
Improper or delayed whole blood separation	6
Incorrect Temperature	1
Hemolysis	11

Table 26. Quality Marker Signatures Measured in Flagged Samples

Improper Collection Tube (non-EDTA) Notes: The following scores for the biomarkers indicate improper collection tube: iminodiacetate ranged from -12.35 to -2.83 and EDTA ranged from -11.85 to -3.3; several samples having citrate levels ranging from -7 to -4 indicate heparin collection
Delayed Separation and/or Delayed Processing Notes: The following biomarkers signatures were measured indicating samples should be flagged: Example Signature #1) Pyruvate 4.26; 5-oxoproline 3.17; Lactate 3.13; Long-chain acylcarnitines >2; Arginine -5.81; Glucose -3.6 Example Signature #2) Cys-gly, oxidized 3.30; 5-oxoproline 3.28; Lactate 3.17; Malate 2.89; Cysteinylglycine 2.63; Ornithine 2.28; Pyruvate 2.12; Hypoxanthine -3.14; Glycerol -4.16; Arginine -5.16; Glucose -7.85
Improper Handling: Notes: The following biomarker signatures were measured indicating samples were not handled according to protocol: Example Signature #1) 5-oxoproline 2.83 Lactate 2.64 Glutamate 2.31 Cysteinylglycine 2.27 Pyruvate 1.95 Arginine -3.22 Glucose -4.81 Example Signature #2) 5-oxoproline 5.22 Cys-gly, oxidized 4.30 Pyruvate 3.66 Sphinganine-1-phosphate 3.64 Spingosine 1-phosphate 3.42 Fumarate 3.17 Lactate 2.95 All BCKA's have Z>2 and several acylcarntines have Z>2. Glycerol -2.66 Hypoxanthine -4.54 Arginine -6.07 Glucose -32.3 Mannose -9.51 Example Signature #3) Cys-Gly 2.205-oxoproline 3.27 lactate 2.70 pyruvate 2.69 Cys-Gly, ox 2.33 oleoylcarnitine 2.41 ornithine 2.18 hypoxanthine -5.25 glucose -3.24 arginine -1.91 (increased lactate, pyruvate, and ornithine coupled with decreased glucose and arginine is suggestive of improper sample handling) Example Signature #4) 1-palmitoylglycerol 6.37 hypoxanthine 5.26 inosine 5.12 2-palmityolglycerol 3.82 5-oxoproline 3.03 azelate 2.96 oleoylcarnitine 2.52 malate 2.39 lactate 2.18 stearoylcarnitine 2.16 IDA -3.57 urea -3.00 1,5-AG -2.76 mannose -2.67 Arg -2.35 citrulline -2.17 glucose -1.93 Example Signature #5) pyruvate 2.66, lactate 2.27, arginine -5.05, hypoxanthine -4.34, inosine -3.10, glucose -3.02
Improper Temperature: Notes: The following signature indicated the sample was not held at the proper temperature: Example Signature: Hypoxanthine 2.42 Lactate 2.33 Inosine 2.28 Lysine -2.64 Arginine -3.84 Mannose -9.54 Glucose -25.30
Hemolysis: Notes: When one or more of the listed biomarkers have Z-scores >2 the sample may be flagged for hemolysis: heme, 3-phosphoglycerate, 2,3-diphosphoglycerate, and biliverdin

We claim:

1. A method of assessing the quality of a biological sample while maintaining the viability of the biological sample for intended use analysis, the method comprising:

– analyzing a biological sample obtained from a subject for an intended use,

– assessing the quality of the biological sample using one or more biomarkers of sample quality, and

– simultaneously performing intended use analysis on the same sample.

2. The method of claim 1, wherein for a sample that is determined to meet quality acceptance criteria, the result of the intended use analysis is used and for a sample that is determined to not meet quality acceptance criteria, either not using the result of the intended use analysis or modifying the result of the intended use analysis based on the quality assessment results.

3. The method of any preceding claim, wherein determining whether a sample meets acceptance criteria includes assessing compliance with sample handling protocols.

4. The method of any preceding claim, wherein assessing compliance with sample handling protocols comprises determining whether violations of the protocols occurred for one or more of sample collection, collection tubes, collection tube additives, processing time from collection, processing incubation temperature, time in storage, storage temperature, or freeze-thaw cycles.

5. The method of any preceding claim, wherein determining whether a sample meets acceptance criteria includes assessing subject compliance.

6. The method of any preceding claim, wherein assessing subject compliance comprises determining whether violations of the protocols occurred for one or more of fasting status, medication and supplement status, smoking status, or diet and exercise compliance.

7. A method of determining whether a biological sample is acceptable to be analyzed for an intended use, the method comprising

– analyzing a biological sample obtained from a subject for the intended use,

– assessing the quality of the biological sample using one or more biomarkers of sample quality, and

– simultaneously performing intended use analysis on the same sample.

8. A method of determining whether an intended use analysis result obtained for a biological sample is acceptable to be used, the method comprising

– analyzing a biological sample obtained from a subject for the intended use,

– analyzing the sample for the intended use,

– assessing the quality of the biological sample using one or more biomarkers of sample quality, and

– for a sample that is determined to meet acceptance criteria for the intended use, using the result of the intended use analysis and for a sample that is determined to not meet acceptance criteria for the intended use, not using the result of the intended use analysis or modifying the result of the intended use analysis based on the quality assessment results.

9. A method to assess sample quality, the method comprising:

analyzing a biological sample obtained from a subject to determine the level(s) of one or more sample quality biomarkers, wherein the one or more sample quality biomarkers are selected from Tables 1 through 26 and the biomarkers cysteinylglycine, sphinganine-1-phosphate and combinations thereof; and

comparing the level(s) of the one or more biomarkers in the sample to sample quality reference levels of the one or more sample quality biomarkers to assess sample quality using these biomarkers.

10. The method of claim 9, wherein the one or more sample quality biomarkers in Tables 1-12, 18 or 26 provide an indication of at least one sample processing condition for the sample.

11. The method of claim 10, wherein the at least one sample processing condition is selected from one or more of the group consisting of time incubated as whole blood prior to separation, time of cold incubation prior to freezing, time of room temperature incubation prior to freezing, sample storage temperature, sample storage time, and number of freeze/thaw cycles.

12. The method of claim 9, wherein the one or more biomarkers in Tables 13-17 provide an indication of the fasting status of the subject.

13. The method of any preceding claim, wherein the sample quality assessment is used to distinguish biochemical changes due to disease or disorder or experimental design from artifacts due to unacceptable sample quality.

14. The method of any preceding claim, wherein the sample quality assessment is used to identify and provide a list of affected sample quality parameters, identify at least one intended use associated with one or more aberrant sample quality biomarkers, and/or provide a list of recommended approaches for evaluating the sample.

15. The method of any preceding claim, wherein the sample quality assessment provides a recommendation for the application of a correction factor to one or more biomarkers before using the one or more biomarkers in a further assessment.

16. The method of any preceding claim, wherein the sample quality assessment provides a recommendation to eliminate certain of the one or more biomarkers or biochemical pathways from the analysis.

17. The method of any preceding claim, wherein the sample quality assessment provides a recommendation to substitute one biomarker with a different biomarker for a certain biochemical pathway.

18. The method of any preceding claim, further comprising using the determined levels of the one or more biomarkers in a composite score.

19. The method of any preceding claim, wherein analyzing and comparing comprises surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, and providing a sample quality assessment.

20. The method of claim 19, further comprising associating aberrant sample quality biomarkers with sample quality parameters and providing a list of affected sample quality parameters.

21. The method of any claims 19 or 20, further comprising identifying at least one intended use associated with the one or more of the aberrant sample quality biomarkers.

22. A method as described herein.

23. A sample assessment method as described herein.

24. The method of any preceding claim, wherein the sample quality assessment is performed concomitantly with metabolomic or clinical analysis of the same sample.

25. The method of any preceding claim, wherein at least a portion of the method is automated.

26. The method of any preceding claim, wherein the sample quality is assessed simultaneously with an intended use analysis being performed on the same sample.

27. The method of any preceding claim, wherein the one or more intended uses comprise clinical or experimental analyses.

28. The method of any preceding claim, wherein the results of sample quality assessment is provided at the same time as the results of the intended use analysis.

29. The method of any preceding claim, wherein the sample quality assessment is selected based on the intended use of the sample.

30. A computing device for implementing the method of any preceding claim.

31. A computing device as described herein.

32. A kit for use in assessing sample quality.

33. A kit as described herein.

34. A system to assess sample quality, the system comprising:

a database in communication with the analytical device, said database being configured to automatically receive from the analytical device data corresponding to the level(s) of one or more sample quality biomarkers measured in a biological sample obtained from a subject, wherein the one or more biomarkers are selected from Tables 1 through 26 and the biomarkers cyteinylglycine, sphinganine-1-phosphate, and combinations thereof; and

a processor device in communication with the database, the processor device being configured to compare the level(s) of the one or more biomarkers in the sample to sample quality reference levels of the one or more sample quality biomarkers to assess sample quality.

35. The system of claim 34, wherein the information from the comparison is stored.

36. The system of claim 35, wherein the stored information may include one or more of: biomarker level(s) in the sample, a list of aberrant biomarker(s) in the sample, a list of affected sample quality parameters, statistical information, a visual indication, a composite score.

37. The system of claim 34, wherein a user interface is in communication with the database and the processor device, the user interface being configured to display a visual indication of the comparison of the one or more sample quality biomarkers, to visually indicate the sample quality.

38. The system of claim 34, wherein the one or more biomarkers in Tables 1-12, 18, 26, and the biomarkers cyteinylglycine, sphinganine-1-phosphate and combinations thereof provide an indication of at least one sample processing condition for the sample.

39. The system of claim 38, wherein the at least one sample processing condition is selected from one or more of the group consisting of time incubated as whole blood prior to separation, time of cold incubation prior to freezing, time of room temperature incubation prior to freezing, sample storage temperature, sample storage time, number of freeze/thaw cycles.

40. The system of claim 34, wherein the one or more biomarkers in Tables 13-17 provide an indication of the fasting status of the subject.

41 The system of any preceding claim, wherein the sample quality assessment is used to distinguish biochemical changes due to disease or disorder or experimental design from artifacts due to unacceptable sample quality.

42. The system of any preceding claim, wherein the sample quality assessment is used to identify and provide a list of affected sample quality parameters, identify at least one intended use associated with one or more aberrant sample quality biomarkers, and/or provide a list of recommended approaches for evaluating the sample.

43. The system of any preceding claim, wherein the sample quality assessment provides a recommendation for the application of a correction factor to one or more biomarkers before using the one or more biomarkers in a further assessment.

44. The system of any preceding claim, wherein the sample quality assessment provides a recommendation to eliminate certain of the one or more biomarkers or biochemical pathways from the analysis.

45. The system of any preceding claim, wherein the sample quality assessment provides a recommendation to substitute one biomarker with a different biomarker for a certain biochemical pathway.

46. The system of any preceding claim, further comprising using the determined levels of the one or more biomarkers in a composite score.

47. The system of any preceding claim, wherein analyzing and comparing comprises surveying the sample for the level(s) of sample quality biomarkers by statistical analysis to identify aberrant sample quality biomarkers in said sample, listing aberrant sample quality biomarkers, and providing a sample quality assessment.

48. The system of claim 47, further comprising associating aberrant sample quality biomarkers with sample quality parameters, and providing a list of affected sample quality parameters.

49. The system of any claims 47 or 48, further comprising identifying at least one intended use associated with the one or more of the aberrant sample quality biomarkers.

50. A sample assessment system as described herein.

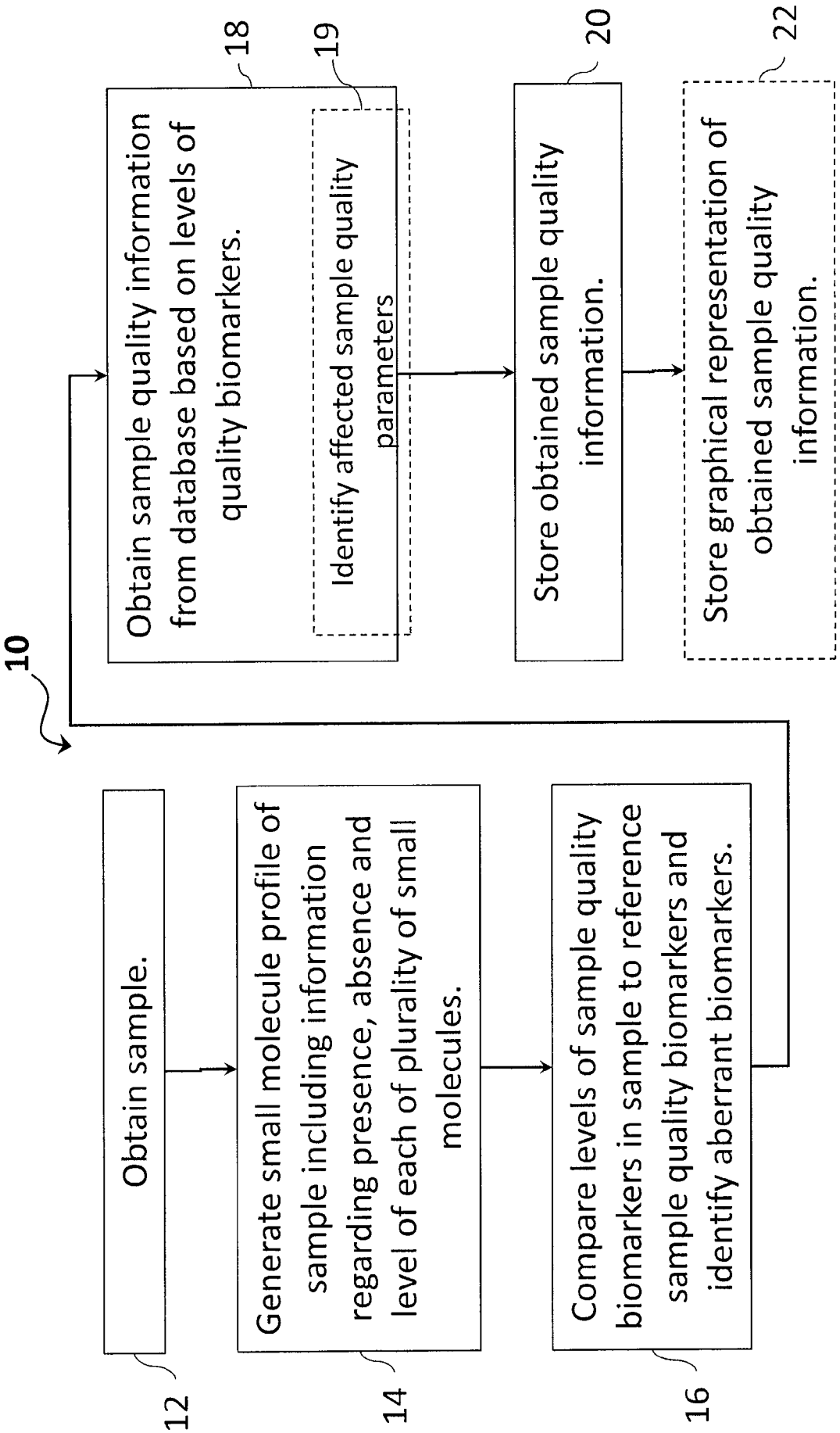

10
Obtain sample.
12
Generate small molecule profile of sample including information regarding presence, absence and level of each of plurality of small molecules.
14
Compare levels of sample quality biomarkers in sample to reference sample quality biomarkers and identify aberrant biomarkers.
16
Obtain sample quality information from database based on levels of quality biomarkers.
18
Identify affected sample quality parameters
19
Store obtained sample quality information.
20
Store graphical representation of obtained sample quality information.
22

Figure 1

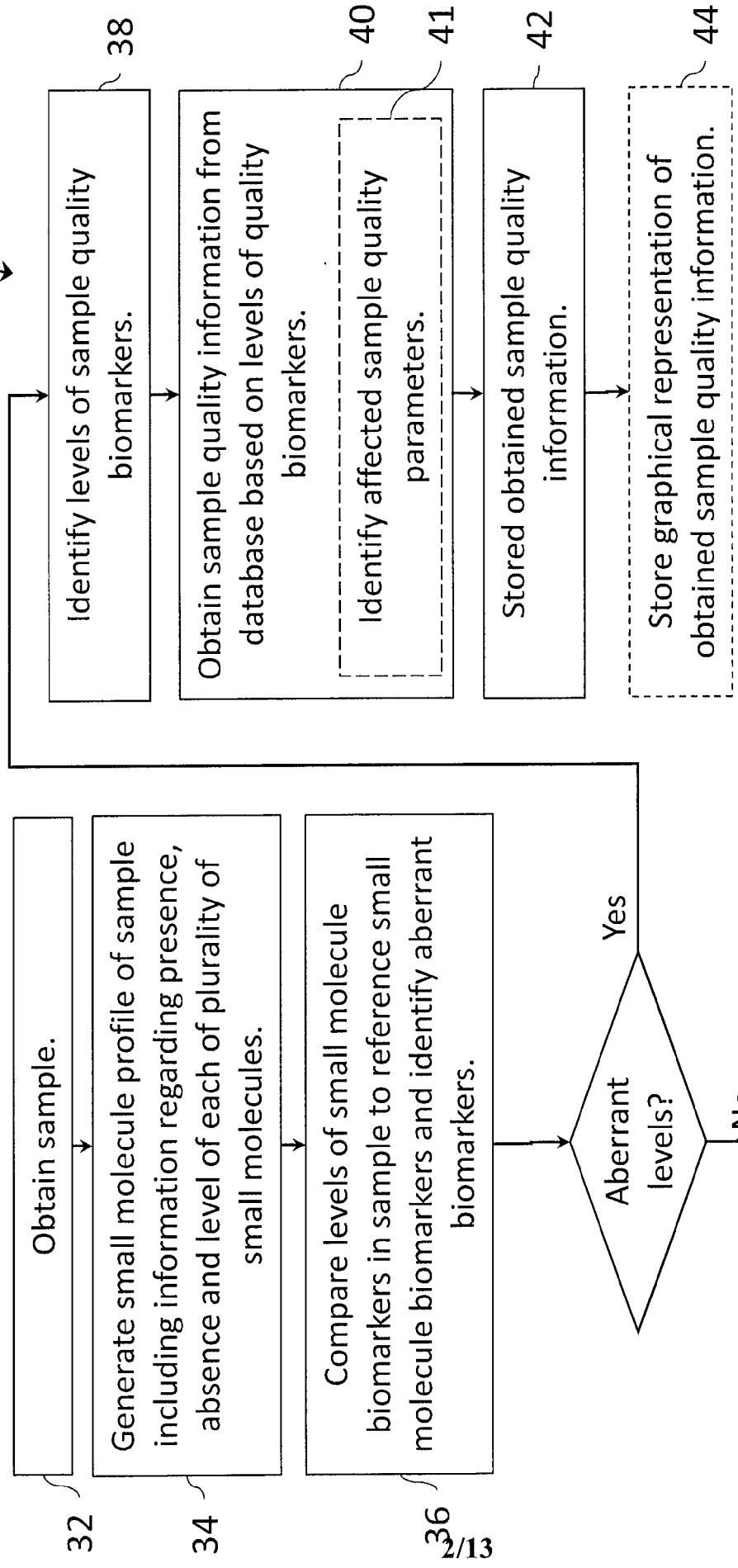

30
Obtain sample.
32
Generate small molecule profile of sample including information regarding presence, absence and level of each of plurality of small molecules.
34
Compare levels of small molecule biomarkers in sample to reference small molecule biomarkers and identify aberrant biomarkers.
36
Aberrant levels?
Yes
No
Store indication that no aberrant levels were found.
37
Identify levels of sample quality biomarkers.
38
Obtain sample quality information from database based on levels of quality biomarkers.
40
Identify affected sample quality parameters.
41
Stored obtained sample quality information.
42
Store graphical representation of obtained sample quality information.
44

Figure 2

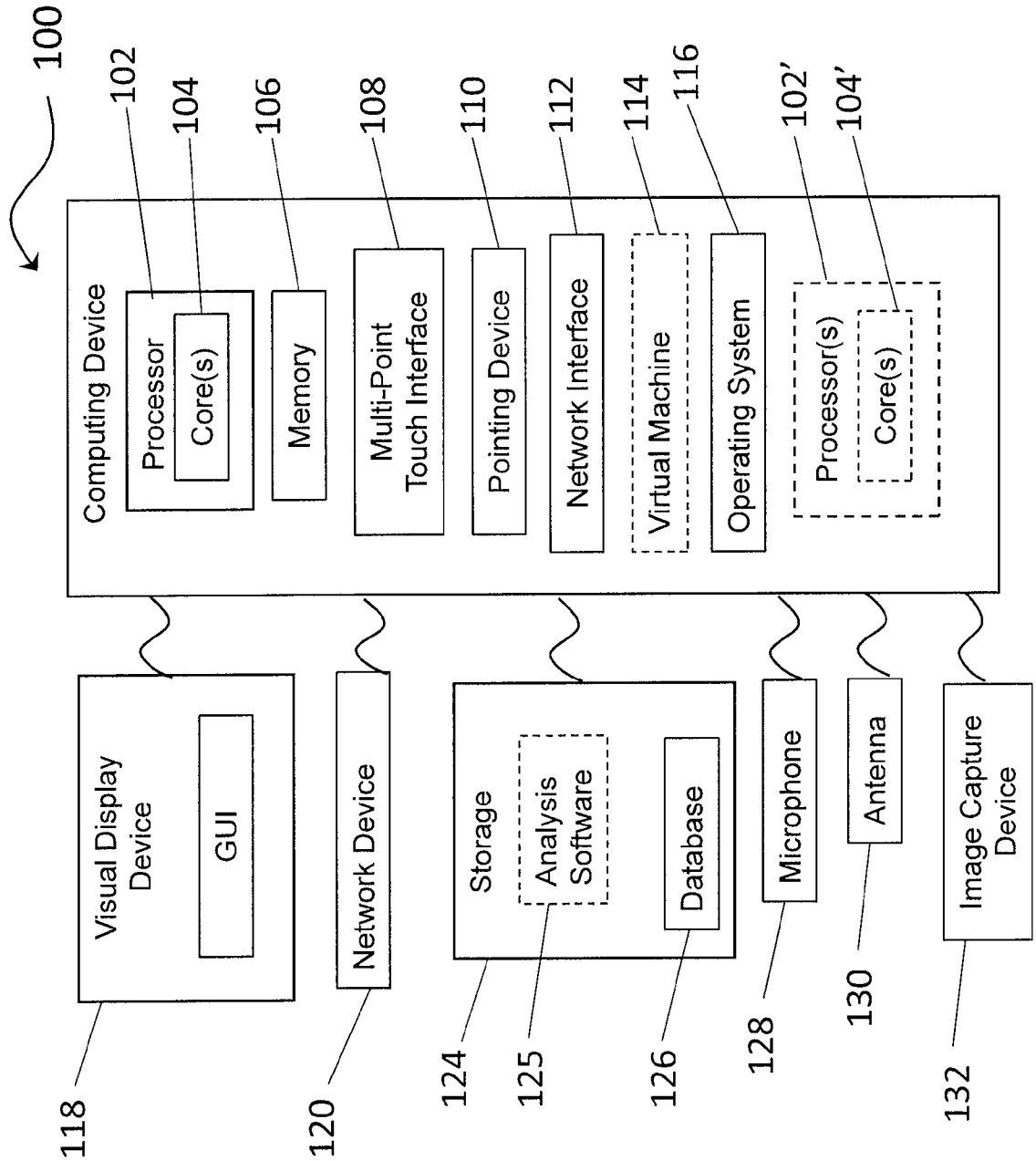

100
Computing Device
Processor
Core(s)
Memory
Multi-Point Touch Interface
Pointing Device
Network Interface
Virtual Machine
Operating System
Processor(s)
Core(s)
102
104
106
108
110
112
114
116
102'
104'
Visual Display Device
GUI
Network Device
Storage
Analysis Software
Database
Microphone
Antenna
Image Capture Device
118
120
124
125
126
128
130
132

Figure 3

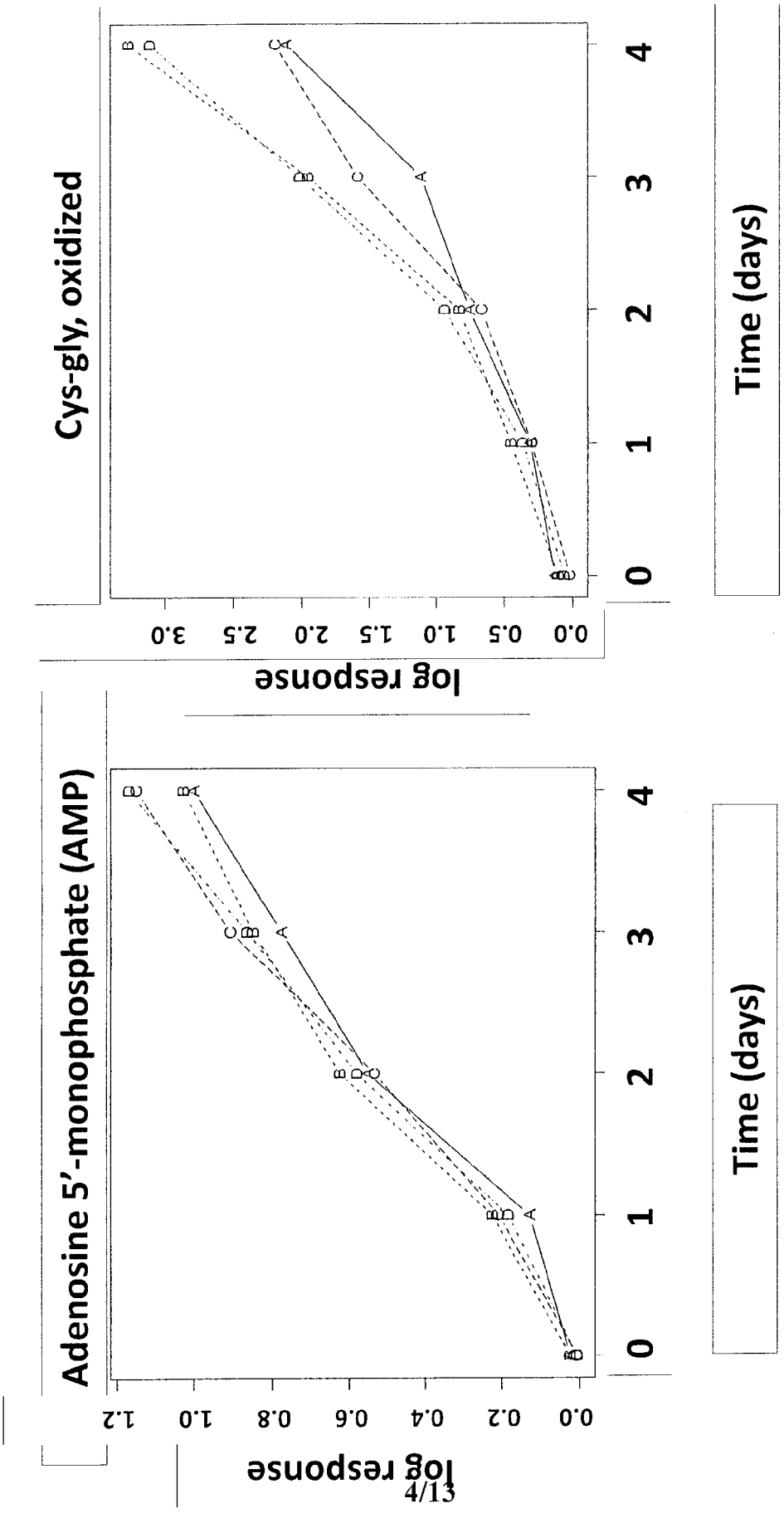

Cys-gly, oxidized
log response
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
1
2
3
4
Time (days)
Adenosine 5'-monophosphate (AMP)
log response
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
1
2
3
4
Time (days)

Figure 4

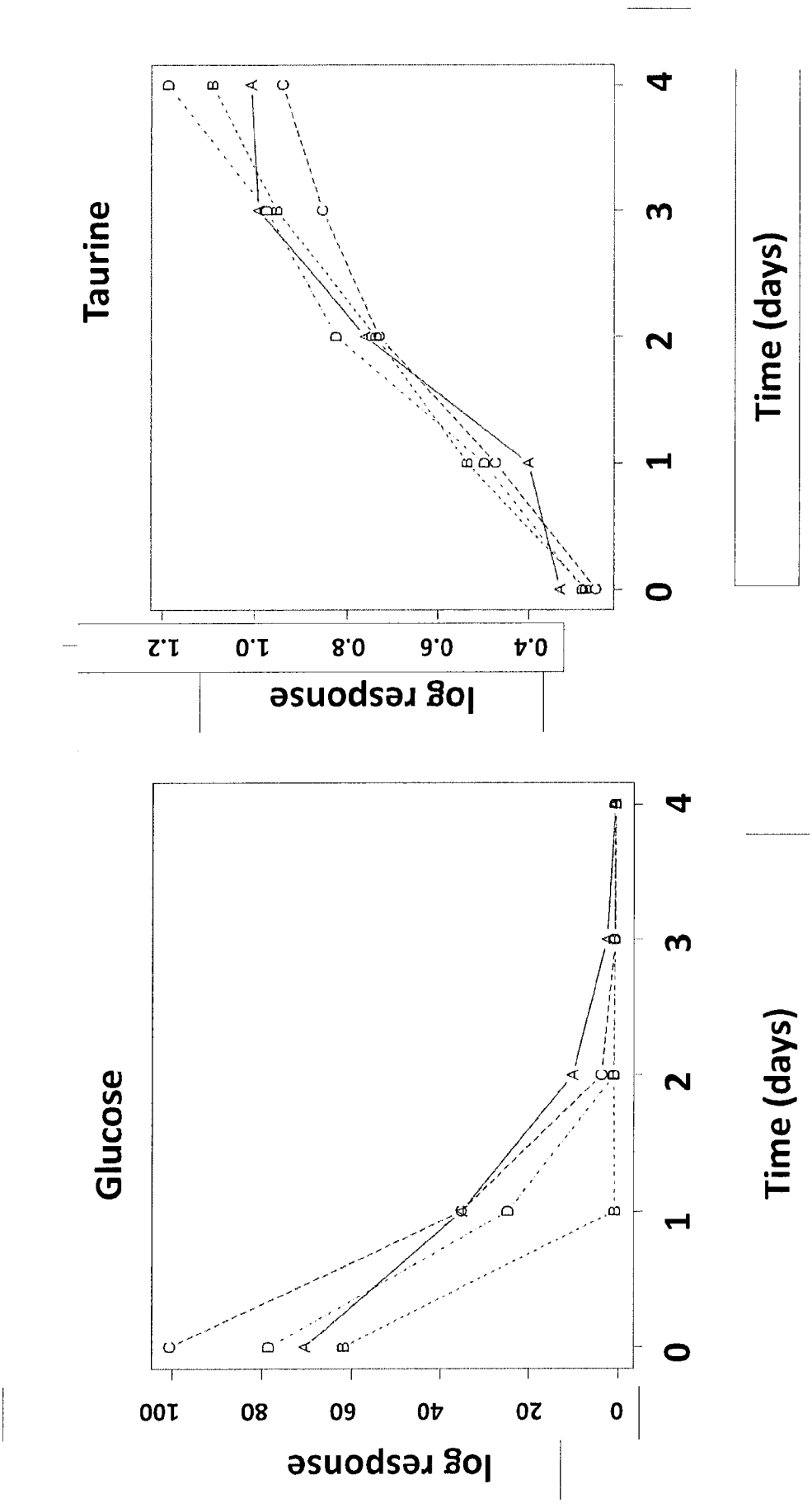

Glucose
log response
0
20
40
60
80
100
Time (days)
0
1
2
3
4
Taurine
log response
0.4
0.6
0.8
1.0
1.2
Time (days)
0
1
2
3
4

Figure 4

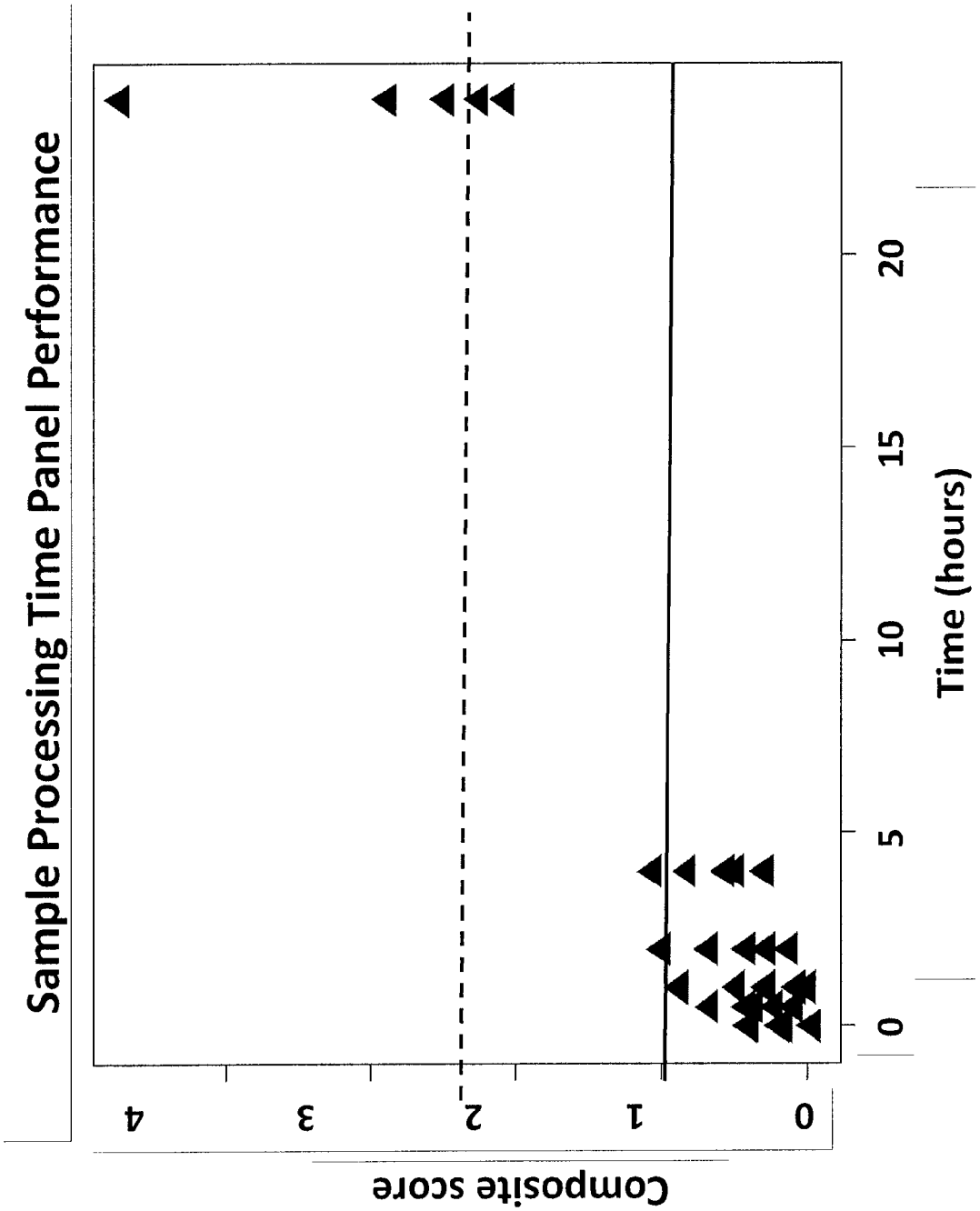

Sample Processing Time Panel Performance
Composite score
0
1
2
3
4
0
5
10
15
20
Time (hours)

Figure 5

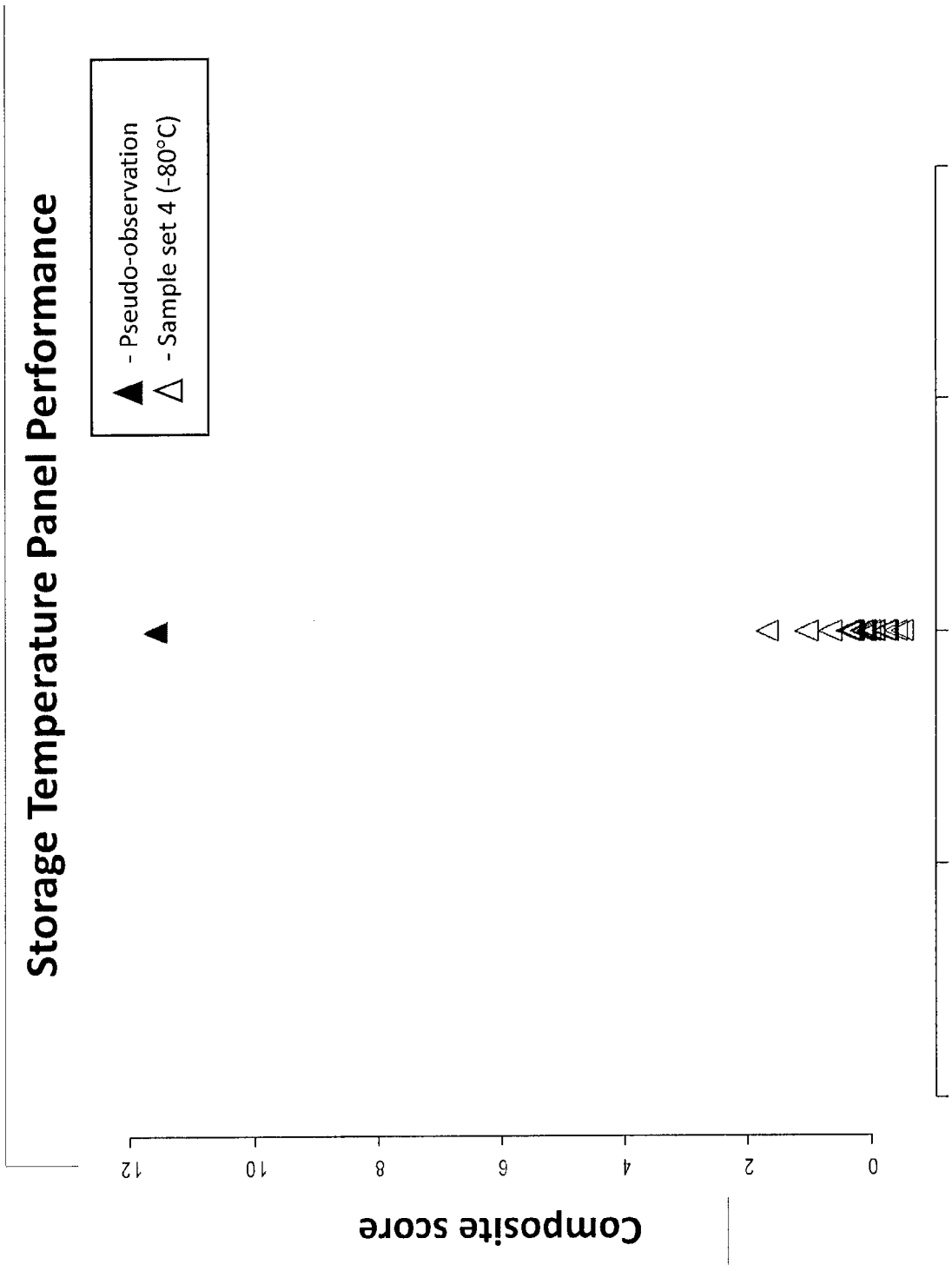

Storage Temperature Panel Performance
▲ - Pseudo-observation
△ - Sample set 4 (-80°C)
Composite score
0
2
4
6
8
10
12

Figure 6

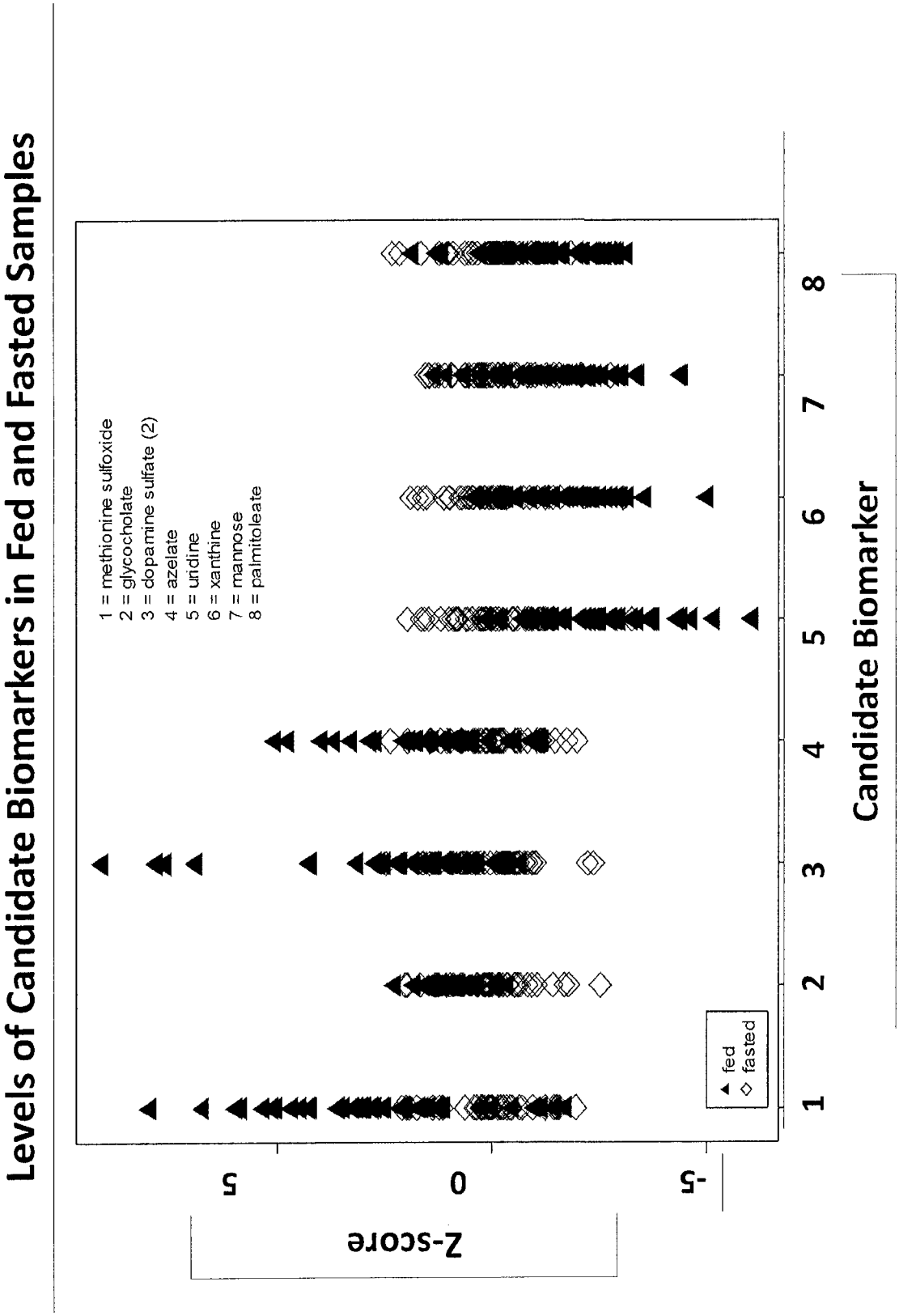

Levels of Candidate Biomarkers in Fed and Fasted Samples
1 = methionine sulfoxide
2 = glycocholate
3 = dopamine sulfate (2)
4 = azelate
5 = uridine
6 = xanthine
7 = mannose
8 = palmitoleate
fed
fasted
Z-score
5
0
-5
1
2
3
4
5
6
7
8
Candidate Biomarker

Figure 7

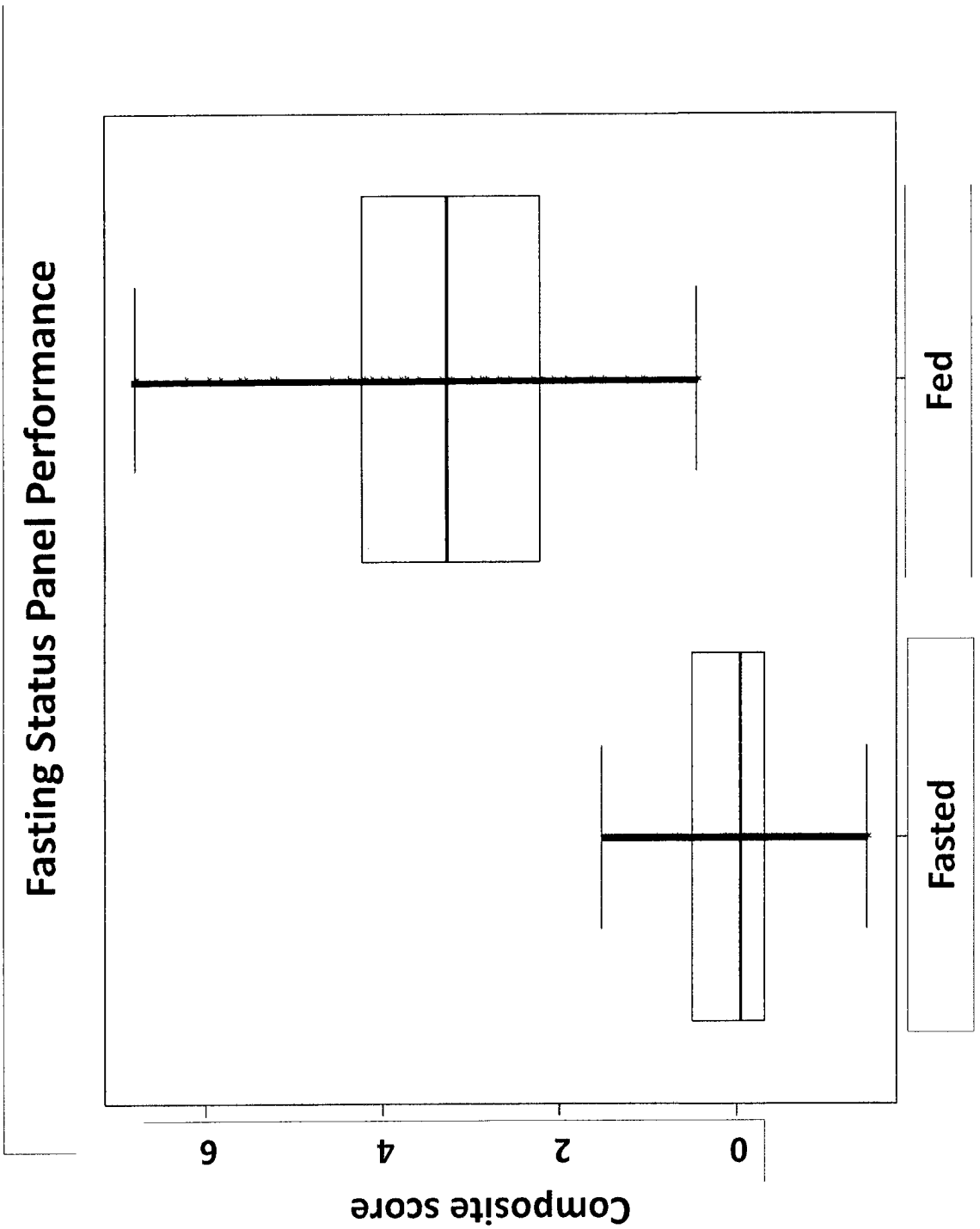
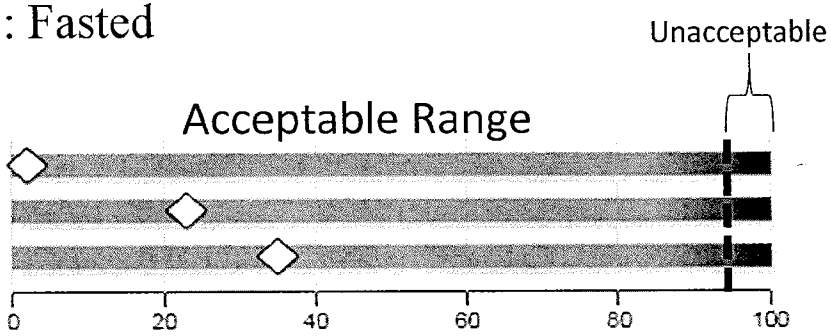

Fasting Status Panel Performance
Composite score
0
2
4
6
Fasted
Fed

Figure 8

Sample Quality Composite Scores

Sample 1: Fasted

Quality Parameter	Score	Reference	Percentile
Fasting	-0.58	< 0.51	2
Storage	-0.24	< 0.61	23
Processing	-0.25	< 0.93	35



Sample 2: Not Fasted

Quality Parameter	Score	Reference	Percentile
Fasting	1.98	< 0.51	100
Storage	-0.44	< 0.61	12
Processing	-0.47	< 0.93	23

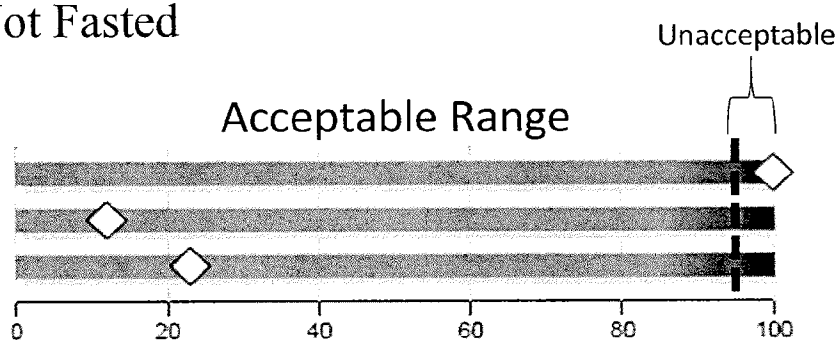
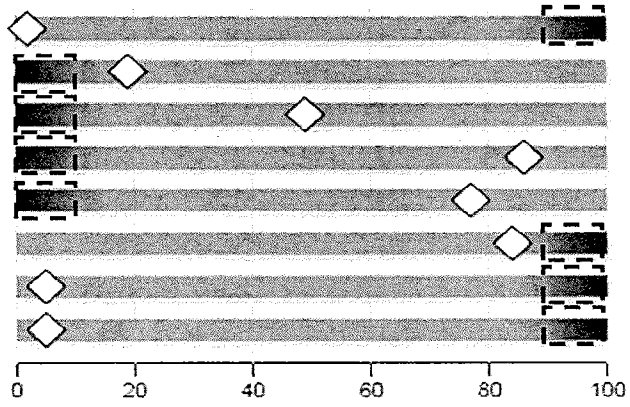


Figure 9

Quality Parameter: Fasting Status

Sample 1: Fasted

Quality Biomarker	Z-score	Reference	Percentile
glycocholate	-1.92	M < 1.66	2
palmitoleate (16:1n7)	-0.79	-1.87 < M	19
uridine	0.01	-1.47 < M	49
mannose	1.11	-2.12 < M	86
xanthine	0.57	-2.16 < M	77
azelate (nonanedioate)	1.01	M < 1.43	84
methionine-sulfoxide	-1.55	M < 1.92	5
dopamine-sulfate-2	-1.3	M < 1.61	5



Sample 2: Not Fasted

Quality Biomarker	Z-score	Reference	Percentile
glycocholate	0.65	M < 1.66	74
palmitoleate (16:1n7)	-0.57	-1.87 < M	23
uridine	-4.31	-1.47 < M	0
mannose	-0.24	-2.12 < M	42
xanthine	-1.57	-2.16 < M	7
azelate (nonanedioate)	2.97	M < 1.43	100
methionine-sulfoxide	4.34	M < 1.92	100
dopamine-sulfate-2	1.2	M < 1.61	86

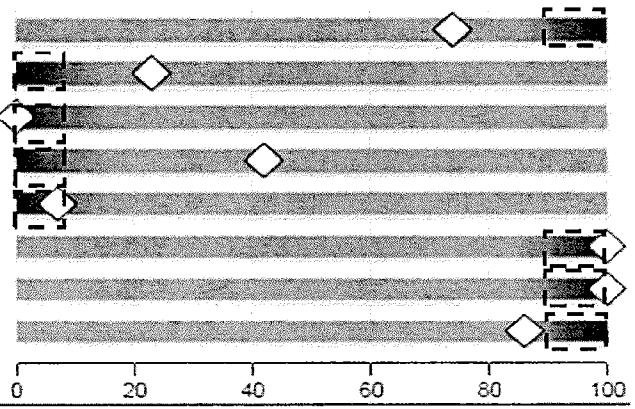
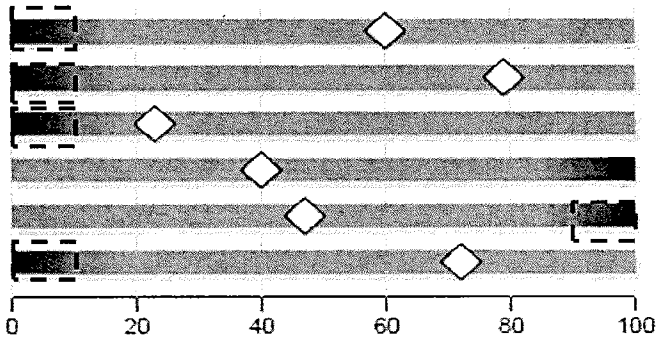


Figure 10

Parameter: Sample Storage

Sample 1: Fasted

Quality Biomarker	Z-score	Reference	Percentile
alpha-ketoglutarate	0.34	−1.68 < M	60
methionine	0.82	−1.39 < M	79
glutamine	−0.67	−1.7 < M	23
caproate (6:0)		M < 1.49	40
gamma-glutamylleucine	0.04	M < 1.29	47
4-methyl-2-oxopentanoate	0.65	−1.76 < M	72



Sample 2: Not Fasted

Quality Biomarker	Z-score	Reference	Percentile
alpha-ketoglutarate	0.05	−1.68 < M	51
methionine	3.17	−1.39 < M	100
glutamine	−0.12	−1.7 < M	42
caproate (6:0)		M < 1.49	40
gamma-glutamylleucine	2.86	M < 1.29	100
4-methyl-2-oxopentanoate	2.04	−1.76 < M	100

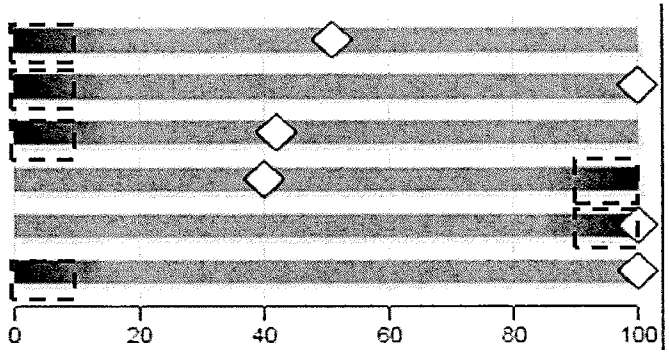
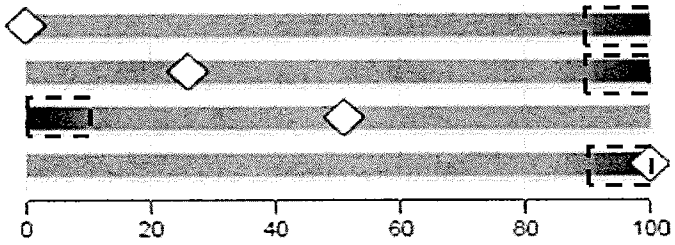


Figure 11

Parameter: Sample Processing

Sample 1: Fasted

Quality Biomarker	Z-score	Reference	Percentile
adenosine 5'-monophosphate (AMP)	-2.17	M < 1.56	0
taurine	-0.84	M < 1.37	26
glucose	-0.02	-1.7 < M	51
cys-gly, oxidized	2	M < 1.31	100



Sample 2: Not Fasted

Quality Biomarker	Z-score	Reference	Percentile
adenosine 5'-monophosphate (AMP)	-1.53	M < 1.56	5
taurine	-0.36	M < 1.37	33
glucose	1.14	-1.7 < M	84
cys-gly, oxidized	1.14	M < 1.31	88

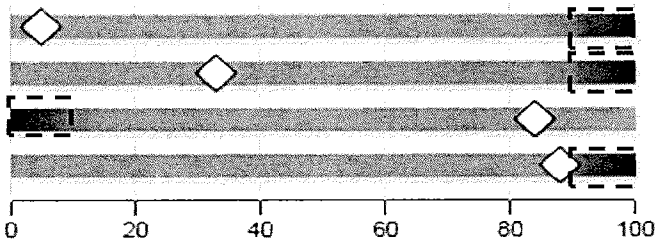


Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/60245

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 4-6, 13-21, 24-30, 41-49
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 4-6, 13-21, 24-30, 32, 41-49 are improper unsearchable omnibus type claims since they refer to a system substantially as shown and described.

3. ☒ Claims Nos.: 22-23, 31, 33 and 50
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fee must be paid.

Group I: Claims 1-3, 7, and 32 are directed towards a method of assessing the quality of a biological sample while maintaining the viability of the biological sample for intended use analysis.
Group II: Claims 8 and 32 are directed towards a method comprising acceptance criteria.
Group III: Claims 9-12, 32, and 34-40 are directed towards a method to assess sample quality.

-continued in supplemental box-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-3, 7, 32;

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/60245

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G06F 19/28, 19/10; G01N 33/50, 33/48 (2017.01)
CPC - G06F 19/28, 19/325, 19/10; G01N 33/50, 33/57484, 33/483
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): G01N 33/50, 33/48; G06F 19/00, 19/10, 19/28 (2017.01)
CPC: G01N 33/50, 33/57484, 33/48, 33/483; G06F 19/00, 19/10, 19/28, 19/30, 19/325; A61B 5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, Other Countries (INPADOC), RU, AT, CH, TH, BR, PH); EBSCO; IEEE/IEEEXplore; Google/Google Scholar; Keywords: biological, sample, biomarker, quality, standard, protocol, score, assess, analysis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2013/164278 A1 (GENERAL ELECTRIC COMPANY) 07 November 2013; page 6, lines 10-11; page 13, lines 7-18; page 67 lines 19-27.	1-2, 7, 32 --- 3
Y	US 2013/0103321 A1 (SOMALOGIC, INC.) 25 April 2013; abstract; paragraph [0097].	3
A	WO 2014/125443 A1 (METANOMICS HEALTH GMBH) 21 August 2014; entire document.	1-3, 7, 32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2017 (17.02.2017)	09 MAR 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US16/60245

-***-Continued from Box III Observations where unity of invention is lacking -***-

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I include at least simultaneously performing intended use analysis on the same sample, which are not present in Groups II-III.

The special technical features of Group II include at least for a sample that is determined to meet acceptance criteria for the intended use, using the result of the intended use analysis and for a sample that is determined to not meet acceptance criteria for the intended use, not using the result of the intended use analysis or modifying the result of the intended use analysis based on the quality assessment results, which are not present in Groups I and III.

The special technical features of Group III include at least wherein the one or more sample quality biomarkers are selected from Tables 1 through 26 and the biomarkers cysteinylglycine, sphinganine-1-phosphate and combinations thereof; and comparing the level(s) of the one or more biomarkers in the sample to sample quality reference levels of the one or more sample quality biomarkers, which are not present in Groups I-II.

The common technical features shared by Groups I-III are a method of assessing the quality of a biological sample, comprising: analyzing a biological sample obtained from a subject for the intended use; and assessing the quality of the biological sample using one or more biomarkers of sample quality.

However, these common features are previously disclosed by WO 2014/125443 A1 to METANOMICS HEALTH GMBH (hereinafter "Metanomics"). Metanomics discloses a method of assessing the quality of a biological sample, comprising: analyzing a biological sample obtained from a subject for the intended use; and assessing the quality of the biological sample using one or more biomarkers of sample quality (a method for assessing the quality of a biological sample of a subject comprising the steps of determining in a sample the amount of at least one biomarker and comparing the said amount of the at least one biomarker with a reference, whereby the quality of the sample is assessed; Abstract).

Since the common technical features are previously disclosed by the Metanomics reference, these common features are not special and so Groups I-III lack unity.