Cambridge Isotope Laboratories, Inc.
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IROA® Biochemical Quantitation Kits

For Metabolic Profiling

An innovative technology termed isotopic ratio outlier analysis (IROA) was developed to overcome the analytical challenges associated with current, stable isotope-assisted methods used in untargeted/targeted metabolic profiling.[1] These challenges minimally pertain to analytical variance, artifactual peaks, and metabolite identification. The basic IROA method uses metabolic incorporation of heavy (95% ^{13}C) and light (5% ^{13}C) nutrients, such as D-glucose and other carbon energy sources, to give rise to unique labeling patterns that can be readily identified and distinguished by MS.[1-4] Such a method would be useful in interlaboratory quantitative evaluations, if widely available.

Cambridge Isotope Laboratories, Inc. (CIL) is proud to offer a series of IROA® Biochemical Quantitation Kits for metabolic profiling of various cell populations and biological samples. These kits contain the reagents and tools necessary for the successful labeling, identification, and quantitation of metabolites in various cell populations (see general product info below).

^{13}C biochemical quantitation kits available:

Catalog No.	Description
IROA-200-50	IROA 200 Kit for Bacterial Metabolic Profiling
IROA-200-UL	IROA Unlabeled Bacterial Media
IROA-300-250	IROA 300 Kit for Mammalian Metabolic Profiling
IROA-300-UL	IROA Unlabeled Mammalian Media
IROA-PHENO-95-300	IROA 300 Kit for Phenotypic Metabolic Profiling
IROA-FLUX-05-300	IROA 300 Kit for Fluxomic Metabolic Profiling

See isotope.com for pricing.

IROA TECHNOLOGIES
...making metabolic profiling easy®

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Kit Features and Benefits

- Eliminates technical and analytical variance → increases reliability
- Removes artifacts and noise → increases precision and determination of metabolome
- Reproducible identification of knowns/unknowns
- Accurate, relative quantitation
- Automated solution (via software) → saves time
- Easy statistical interpretation of sample results
- Broad applicability with experimental perturbations being user-defined

Applications

By using specific isotopic balances to create definable patterns in metabolites, these IROA Biochemical Quantitation Kits can be used to study flux, systems biology, and biomarkers in a wide variety of areas, as exemplified below (see references 1-7 for application examples):

- Health and diagnostics
- Toxicology
- Pharmacology
- Drug development
- Crop breeding
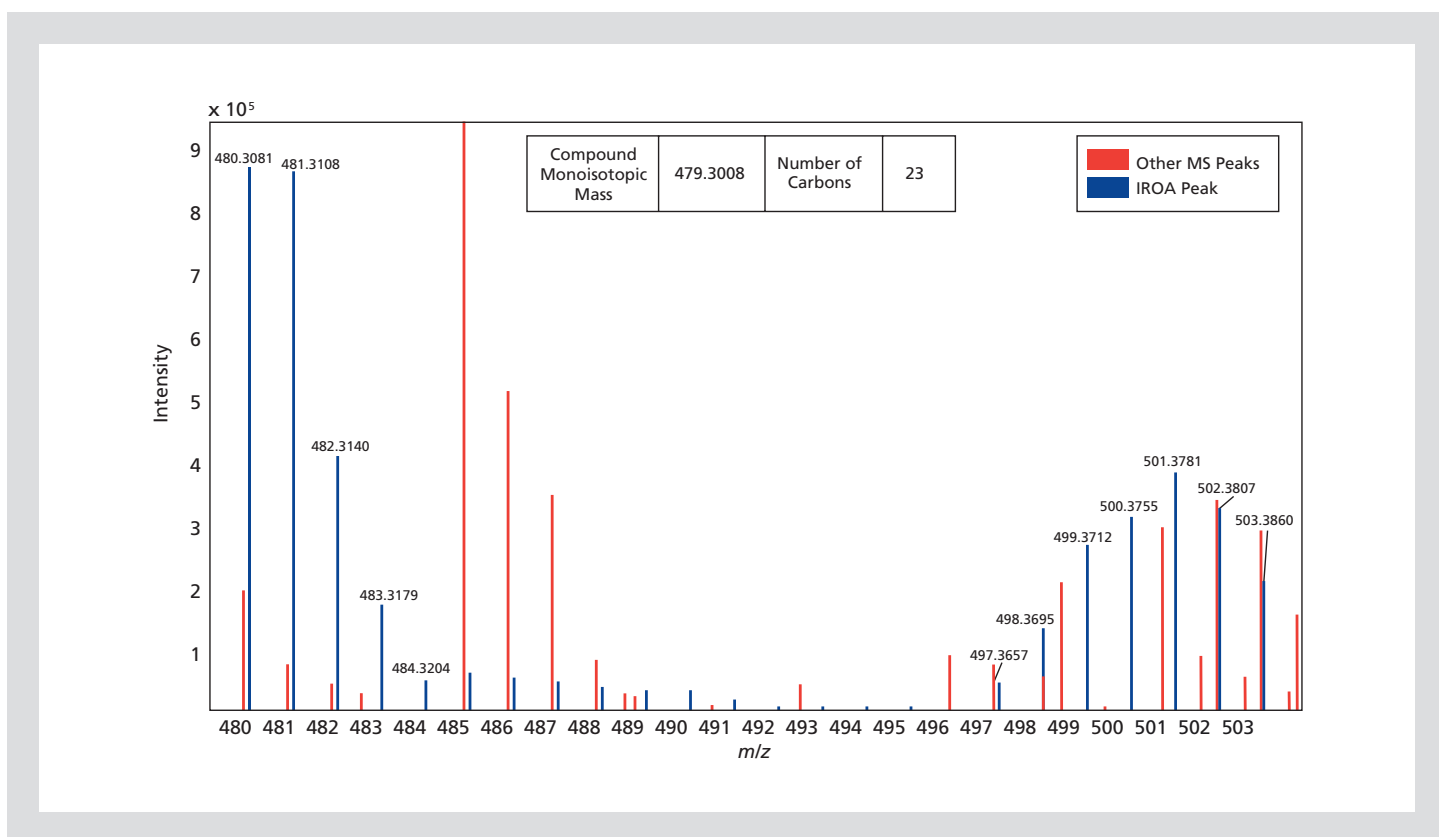- Plant biotechnology
- Biogenesis

Figure. LC-MS analysis of the endometabolome of heat-shocked *Caenorhabditis elegans* grown on agar plates with IROA ^{13}C-labeled *Escherichia coli* as its food.[7] Based on mass spectral spacing and relative intensities, biogenic peaks (in blue) could be differentiated from artifactual noise (in red), which lead to the identification of lysophosphatidylethanolamine 18:1 having a formula of $C_{23}H_{46}NO_7P$.

Performing cellular metabolic profiling? Use the IROA protocol.

In the basic IROA protocol, biomolecules in two cell populations (control and experimental) are randomly labeled with stable isotopic media (^{13}C-based). After a defined incubation period (with 5+ cell doublings), the experimental group is perturbed (through a chemical, genetic, or environmental stressor) before uniform mixing, sample preparation, and LC-MS analysis. **Note:** unlabeled bacterial and mammalian media are available for cell growth testing and adaptation.

Compared to conventional labeling approaches (e.g., SILAC), the reduced enrichment enables isotopic distributions to be readily detected by MS in a predictable and distinguishable manner. These distributions can then be used to: (i) differentiate biological signals from artifacts, (ii) calculate accurate molecular formulae, and (iii) determine relative concentrations of the metabolites of biogenic origin. This data analysis process is aided by IROA Technologies ClusterFinder™ software (see figure above). For experimental samples that cannot be labeled in culture systems (such as biopsies and large-scale fermentations), the IROA Phenotopic protocol can be applied to prepare a relevant labeled internal standard (at 95% ^{13}C) to use as a yardstick to compare differences between experimental cell populations.[1]

Biochemical quantitation kit contents:
- Detailed user manual (provided via email upon product shipment)
- Labeling media (i.e., 5% and 95% ^{13}C$_6$ D-glucose for basic protocols, with 5% ^{13}C$_6$ D-glucose only in flux and 95% ^{13}C$_6$ D-glucose only in phenotypic)
- Kit-specific, basal media components

Kit	Kit-specific, basal media components
Bacterial Kit	M9 minimal medium, amino acid mix (^{13}C, 95% and 5%)
Mammalian Kit	EBSS/RPMI 1640 vitamins, amino acid mix (^{13}C, 95% and 5%), and yeast extract
Phenotypic Kit	EBSS/RPMI 1640 vitamins, 95% ^{13}C amino acids and yeast extract
Fluxomic Kit	EBSS/RPMI 1640 vitamins, 5% ^{13}C amino acids and yeast extract

- Access to IROA Technologies ClusterFinder software (presently version 3.0; login details provided with product shipment) available at iroatech.com/portal/

ClusterFinder is a trademark of IROA Technologies.

Overall, the IROA protocol increases the information content that can be extracted from the mass spectral signals, overcoming current analytical bottlenecks and achieving accurate metabolic profiling.

FAQs

What does the IROA protocol involve? In the basic protocol, biomolecules in two cell populations – control and experimental – are randomly labeled with stable ^{13}C (95% and 5% labeled media for the control and experimental groups, respectively). After a defined incubation period, the experimental group is perturbed (through a chemical, genetic, or environmental stressor) before uniform mixing, sample preparation, and LC- or GC-MS analysis.

What does IROA's ClusterFinder software and portal provide? The software automates peak identification and quantitation from the raw MS data, while providing tools for statistical analysis and interpretation. The integrated Assay Portal enables basic statistics (e.g., regressions and variances) and analysis (e.g., principal component, random forest, and correlation), along with summary plots (e.g., volcano, hybrid, and metabolic mapping) of the distributions.

What are acceptable MS data formats for ClusterFinder? ClusterFinder directly supports the following MS data file formats: mzXML, mzML, and mzDATA; although, mzXML is preferable and the data should be centroided. **Note:** all major instrument vendors provide software for converting their proprietary data files into one of the formats supported by ClusterFinder. It must also be noted that although ClusterFinder does not currently support GC-MS data inputs, these kits have been applied to metabolomic GC-MS applications (see Qiu et al. reference as an example).

What are the requirements for running ClusterFinder software?

Hardware requirements:
1. Intel Pentium III/800 MHz or higher (or compatible); dual-core processor or higher
2. 16 GB RAM minimum
3. Computer needs to be network accessible

Software requirements:
1. Windows XP (x64 and x84)
2. Windows 7 SP1 (x86 and x84)
3. Windows Server 2008 R2 SP1 (x64)
4. Windows Server 2008 SP2 (x86 and x84)
5. Windows 8
6. Windows Server 2012
7. Java 8 must be installed and callable
8. Windows 7 or higher

How is normalization achieved in the IROA datasets? Spectral TIC normalization is achieved using the MS total useful signal (MSTUS) approach.[8] In this approach, only the components that are common to all signals are used after baseline correction and removal of the artifacts (from xenobiotics or chemical noise, for instance) and nonbiological compounds that carry no IROA signatures.

How is the issue of sample-to-sample variance overcome with the IROA protocol? Ion suppression, stemming from the variability of ionization efficiency, is one of the biggest problems facing MS data interpretation. There is no sample-to-sample variance in the IROA datasets because the experimental and control samples are prepped and analyzed together. Further, since the standards and analytes are chemically identical and measured in an identical environment, they share identical ionization efficiencies making the measurements more accurate.

According to the protocol, amino acid mixes are to be added to the bacterial and mammalian kits. How does one distinguish the amino acids that are added to the media as standards from the ones that are metabolized from glucose? The purpose of the media is to provide the necessary nutrients for growth and reproduction of multiple cell types. This will allow complete metabolic labeling (i.e., the ^{12}C content is converted to either 5% or 95% ^{13}C from D-glucose) after five cell doublings. If a researcher is interested in following particular precursor(s) through biochemical pathways to distinguish one or more amino acids from amino acids metabolized from glucose, then the IROA Fluxomic protocol should be employed. In this protocol, cells are fully labeled with 5% ^{13}C IROA media, and 99% ^{13}C-labeled precursors (tracers) are fed to the cells. During a time course, samples are taken, with the fate of the precursor(s) determined with the ClusterFinder software.

How many samples can be minimally analyzed with IROA's 200 biochemical quantitation kit? Also, what is the per-sample cost? The labeling media supplied with this kit can minimally accommodate 48 experimental and 48 control cell sample analyses. Procedurally, 0.5 mL of media/sample during the cell growth phase is sufficient for five cell doublings to assure full label incorporation. As a general guideline, LC-MS injections of 7 or 12 µL for positive or negative ESI, respectively, are recommended from a final volume of 400 µL in the 96-well plate. This equates to approximately $15-30 USD per experimental sample and includes the software for analysis.

How many samples can be minimally analyzed with IROA's 300 biochemical quantitation kit? Also, what is the per-sample cost? The labeling media supplied with these kits can minimally accommodate 46 experimental and 46 control cell sample analyses. Procedurally, using a six-well plate and 3 mL of media /sample/well/ passage is sufficient for five cell doublings to assure full label incorporation. This assumes a total volume of 278 mL media (250 mL plus 28 mL dialyzed fetal bovine serum), 2.5 generations per passage or a total 6 mL per sample. As a general guideline, LC-MS injections of 2 or 4 µL for positive or negative ESI, respectively, are recommended from a final volume of 500 µL in the six-well plate. This equates to approximately $40 USD per experimental sample and includes the software for analysis. Note that for this minimalist discussion, there is sufficient volume in each well for both positive and negative analysis, as well as HILIC or other analyses.

Please visit isotope.com for a complete list of isotope-labeled compounds.

To request a quotation or place an order, please contact our sales team

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