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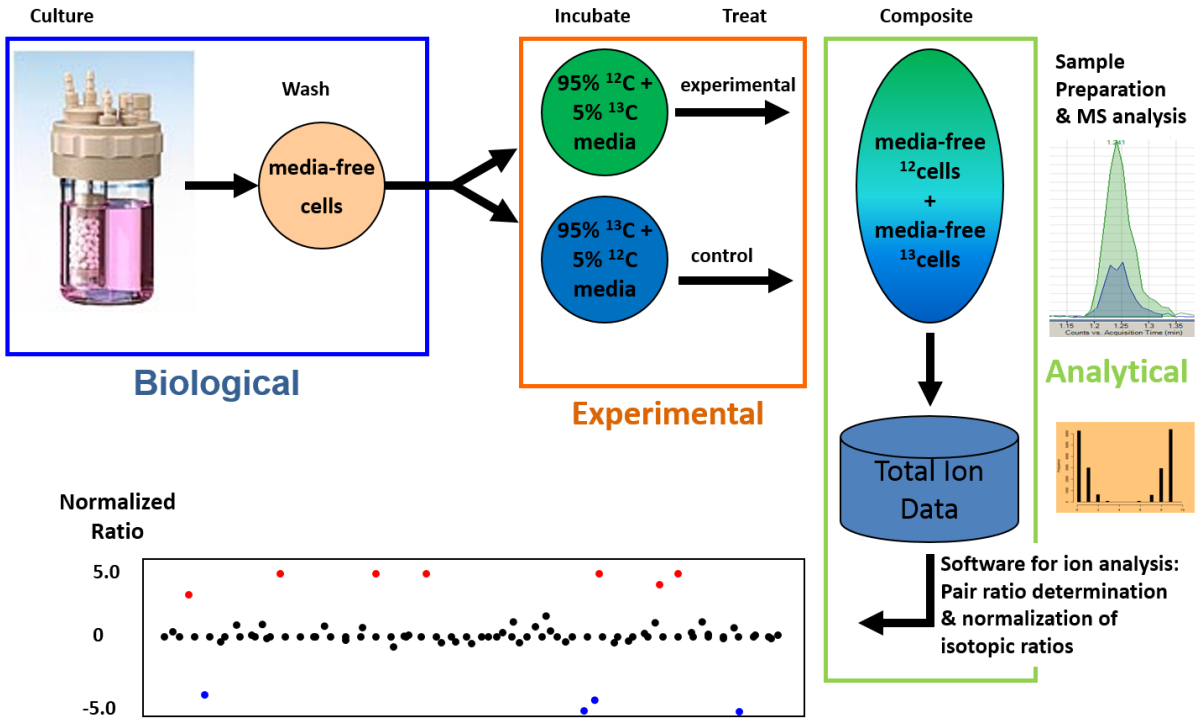
IROA Metabolic Profiling Kits FAQ

Q: What does IROA stand for?
A: *The acronym refers to* Isotope Ratio Outlier Analysis.

Q: What does the IROA protocol involve?
A: *For the Basic IROA 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- MS analysis (see workflow schematic below).*



Q: What is metabolic profiling?
A: *Quantitative analysis of metabolites, generally performed by either NMR or MS, to measure a change of the metabolome in response to a perturbation or stressor.*

Q: What is the cost to use IROA's 100 and 200 biochemical quantitation kits for yeast and bacteria metabolic profiling?

A: *The supplied media in these kits is sufficient for a minimum of 48 experimental and 48 control cell sample analyses or 96 samples of each, experimental and control, if only 0.5 mL are used per sample. Procedurally, 0.5 mL of media is used per sample during the cell growth phase, sufficient for 5 cell doublings to assure full label incorporation. As a guideline generally 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.*

Q. What does IROA's ClusterFinder™ software and portal provide?

A: *The software automates peak identification, quantitation and normalization from the raw MS data, while the portal provides assistance on data interpretation and statistical analysis. Further to the portal, it provides 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.*

Q: How is the issue of sample-to-sample variance overcome with the IROA protocol?

A: *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.

Q. How does one distinguish the amino acids that are added to the media as standards from the ones that are metabolized from glucose?

A. We have two sources of amino acids in the IROA media. Once is from the labeled AAA mix (supplied by CIL) supplied in both the bacteria and mammalian kits and the other is from the labeled yeast extract supplied in the mammalian kits. The media objective is to supply all of the necessary nutrients for growth and reproduction for multiple cell types so that following 5 cell doublings all of the metabolic pools are fully labeled (the C12 content is replaced by either %5 or 95% C13). The experiment using the IROA Basic methodology is to determine which metabolic pools are affected by perturbation.

Should the researcher wish to follow particular precursor(s) through biochemical pathways, i.e. distinguish one or more amino acids from amino acids metabolized from glucose, then the IROA Fluxomic protocol is followed, whereby cells are fully labeled with 5% C13 IROA media and 99% C13 labeled precursors (purchased through CIL) are fed to the cells. During a time-course, samples are taken and the fate of the precursor(s) determined using the ClusterFinder software.

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Q. How does the IROA Flux analysis work?

A: Our flux analysis works very simply. It is an unbiased flux analysis. Using the 5% IROA media you grow the cells to label every metabolic pool with a 5% signal. After the cells are labeled, a flux reagent is added at 99% isotopic abundance/labeling. The software can automatically find all of the metabolic pools based on their 5% C13 signal and knows from the pattern the formula and number of carbons in the molecule (and, of course, if you have run authentic compounds to build a library, the name of the metabolic pool). Since the software knows the number of carbons (n) in the molecule, it examines all positions from the M+2 to M+n for flux of carbons from the flux reagent. The software reports all metabolic pools that show incorporation and the number of carbons that were incorporated. The beauty of the technique is that it is an unbiased flux method, i.e. it examines in an unbiased manner every metabolic pool. Normal, i.e. non-IROA, flux experiments cannot identify metabolic pools unassisted so finding each one is a manual act which generally limits the number of things you examine. We think the IROA approach is much easier and has a broader perspective.

Q: How is the IROA technique analytically superior to SILAC?

A: *See table below for a side-by-side comparison.*

Key Bottlenecks	IROA	SILAC
Extent of labeling	All biomolecules are labeled	Some biomolecules are labeled
Analyte identification	Molecular formula can be calculated	Standards or library references are required for ID
Data processing/reduction	Simplifies biological data by removing artifacts and confounding information automatically	Artifact removal is not possible
Assignment of biological Significance	Media and software are used to directly compare metabolic pools between control and experimental systems; can easily determine which metabolic pools are increased or decreased following experimental protocol	Can tell what peptides are up- or down-regulated; however, since there are so many proteins and these are largely unmapped, it is very difficult to compute significance; also difficult to assign all the peptides to specific proteins

Statistical analysis	Possible since controls are embedded into every sample	No difference
Validation	Complete labeling is easily achieved and interpretation is reproducible	Complete labeling is difficult, making it difficult to obtain reproducible results

Q. When and how is the Phenotypic IROA protocol used?

A: The Phenotypic IROA® Experimental protocol provides an alternative to the Basic IROA protocol when it is not practical or possible to label experimental samples, such as tissue biopsies, field-grown plants and cells/spent media from large-scale fermentation runs. Using the Basic IROA protocol both Control and Experimental samples are fully isotopically labeled, whereas with the Phenotypic IROA protocol the sample is collected at natural abundance and mixed with a fully predefined "Internal Standard" that has been isotopically labeled using IROA 95% ^{13}C media *(see workflow schematic below)*. An ideal Standard is one that represents the entire metabolome of the fluid or cells under study, and as such, the Phenotypic experiment may be considered a complex targeted analysis. The Standard is generated by growing a comparable cell line using 95% ^{13}C media. For example, if the Experimental samples are breast tissue biopsies, an IROA-labeled breast cell line could be considered as a complex Internal Standard. All the IROA-labeled Standard peaks may be readily identified by the IROA ClusterFinder™ software according to the presence of their characteristic peaks. When applying the Basic IROA protocol, the ratio (Experimental: Control) of the carbon envelope peaks areas presented in the resultant dataset represents the relative deviation of the metabolic pool sizes brought about by the experimental condition. When applying the Phenotypic IROA experimental protocol, the overall pattern of deviation from the Standard will define phenotype by difference from the Standard.

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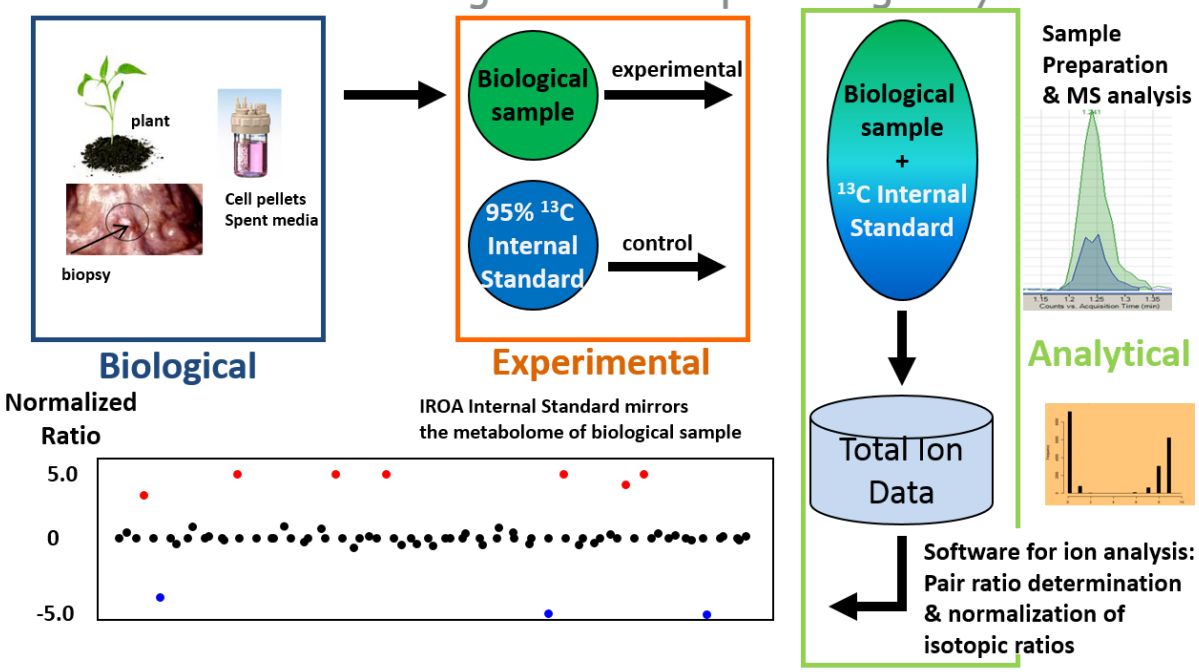

The material to be phenotyped is mixed with ^{13}C (IROA) Internal Standard which allows all peaks to be found and paired. The deviation from the standard is diagnostic of the sample's biochemical phenotype.

Q. What is the shelf life of the kits?

A: The kits have a two-year shelf life and then are retested. The bacterial and mammalian kits both have labeled amino acids and the mammalian kit also has yeast extract which are dissolved in the liquid media and filtered. Once the solutions are made up, it is recommended to make aliquots and store these at -20 degrees C in the dark. They will be stable for at least 12 months. Defrost, then add dialyzed FBS.

Q. How are the IROA kits shipped and how should they be stored?

A: The kits are shipped with ice packs and it is recommended to store the kits in the refrigerator at 4 degrees C.