IRMS protocol

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Introduction

This protocol is designed to perform isotopic ratio analysis of biological samples for four elements — C, N, H and O. It can be applied to analyze cell cultures, bacteria, tissues, biological fluids etc. Required sample amount is normally less then 100 ug of protein (10 ug is enought for triplicate analysis).

Protein extraction

Use proper protein extraction protocol for your samples:

- Cell pellets can be lysed in 8M Urea, 1% SDS, 50mM Tris pH 8.5, Complete protease inhibitor and PhoStop from Roche). The target protein concentration in the lysate is 0.5-8 mg/mL.
- Plant samples have to be grinded in liquid nitrogen and then lyzed in appropriate buffer (Hurkman buffer, or Trizol reagent).
- Air dry the pellet (do not let the pellet completely dry otherwise it is very difficult to get back into solution.)
- Washed and dried protein pellet can be redissolved in 8M urea + 50 mM Tris pH 8.5. (~1-10 ug/ul).

First protein BCA assay (Timing 1 hr)

- Each sample should be done in triplicate, standards done in duplicate
- Dilute samples with water. Urea concentration has to be lower than 3M.
- Follow BCA assay protocol.

Notes:

(1) BCA with lysates over estimates the amount of protein. For example, if the BCA from the lysate indicates 1 mg of protein the actual amount is closer to 0.5mg.

(2) Perform BCA on lysates before the reduction/alkylation due to interference of DTT with the BCA assay.

Protein reduction and alkylation (Timing 2 hrs)

- Add DTT from a 1 M stock to a final 20 mM final concentration and incubate for 1 hr at 25 oC to reduce disulfide bonds.
- Add iodoacetamide from a 0.5 M stock to a 60 mM final concentration. Incubate for 1 hr at 25 oC in the dark to alkylate reduced cysteines.
- (optional) Quench excess iodoacetamide by adding an additional 10 mM DTT from a 1M stock. Note: 1M DTT stocks can be stored at -20C but IAA should be made fresh each day and stored in the dark

Tryptic digestion

- Dilute with 50 mM Tris pH 8.5 (final urea concentration will be 1M)
- Add Trypsin (1:60; Trypsin:Protein) and incubate at 37C overnight
- Quench the reaction by adding TFA to a final concentration of 0.5%
- Centrifuge at 4 oC, 14k for 10min, discard pellet and save supernatant

Peptide desalting (Timing 1-3 hr)

- Desalt peptides using SepPak or StageTips

LC-MS analyzis of immonium ions

- Resuspend 10 ug of peptides in 20 ul of buffer A.
- Inject 2 ul of each sample onto the LC-MS system (using Immonium method for data aquisition). Make sure instrument is in full-profile mode.

Raw to mzML data conversion (1-2h per file)

- Use MSConvert sofware to convert raw files into mzML. msLevel: 2-, activation: HCD

Data analyzis using isoms R-package (2-3h per file)

- Copy mzML files into one local folder on the computer.
- Open terminal (powershell or cmd on Windows, Terminal.app on MacOS) (e.g. by pressing Win+R, typing 'cmd' and pressing enter)
- Navigate to your directury using cd command. (e.g. `cd c:\mydata\project1`)
- Launch pick fitting procedure by executing command `Rscript --vanilla -e "isoms::mzMLtoCSV()"`.
- Prepare experimentDesign.csv file. It has to have following columns:
- **File** - csv file name in current folder, or the complete path to csv file;
- **Sample** - name of the sample;
- **Loading** - loaded amount of sample in ng;
- **Start** - start of the LC elution, in min (e.g. 10min)
- **End** - end of the LC elution, in min (e.g.60min)
- **gC, gN, gH, gO** - heavy isotope ratios in % for respective elements, has to be provided for *Control* sample. Make sure that one of the sample named *Control*.
- Run the report generation by execution command: `Rscript --vanilla -e "isoms::processExperimentDesign()" experimentDesign.csv`
- Report HTML file is generated in the working folder.