Incorporation Rate of Lysine-$^{13}C_6$ in Non-Generational Metabolic Labeling in Mice

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1. Abstract and Introduction

Protein quantification by MS is made most precise by comparing signature peptides with identical peptides enriched in heavy isotopes. Although isotope enriched peptides are often used as internal standards, intact labeled proteins will yield more accurate results because they will undergo the same degree of enzymatic cleavage as the protein being investigated. The use of labeled cells, such as in SILAC (1), or cell line mixtures, for use as internal standards (2), has proven powerful because differences in the entire or large portions of the proteome may be studied simultaneously in a direct quantitative fashion. Labeled intact tissue, however, may be even more useful than labeled cells because of the overall chemical similarity to sample tissue. Although labels may be introduced at the peptide level by chemical tagging or introduced during protein synthesis *in vivo*, metabolic labeling is preferred over chemical tagging because every protein will be labeled and systematic errors present during sample preparation are reduced.
Mice labeled with Lysine-$^{13}C_6$ for use as an experimental control and the production of labeled tissue for use as internal standards have been described (3-5). Sequence homology between mouse and man even permits quantification of some human proteins (6).

Although the use of labeled mouse tissue represents a powerful proteomic work-flow, the current expense of heavy labeled lysine mouse feed may limit wide-spread use of this new technology.

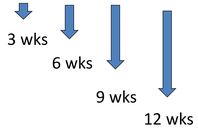
2. Objective

To broaden the potential use of this technology, we evaluate the efficiency and safety of non-generational labeling in mice by using MouseExpress® (Lysine $^{13}C_6$, 99%) mouse feed and a modified diet with a lower Lysine content.

3. Workflow

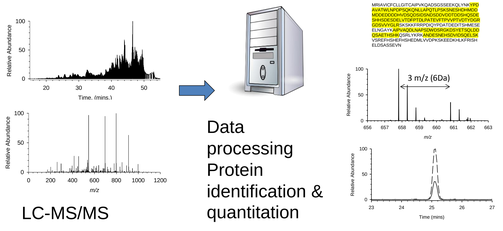
Cohort A. Diet A: 12g Lysine 13C6 per kg feed
Cohort B. Diet A: 8g Lysine 13C6 per kg feed

Plasma and ear punch collections from each animal



1. Depletion of abundant plasma proteins
2. Tissue homogenization and protein extraction

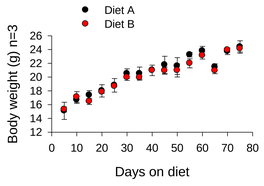
1. SDS-PAGE
2. Excision of 30 and 75 kDa bands
3. In gel digestion



LC-MS/MS

Data processing
Protein identification & quantitation

4. Health Assessment



Weight Gain

- [x] Normal
- [ ] Under-weight
- [ ] Over-weight

Clinical Observations

All animals appeared healthy through-out the study. Weight gain was in the "normal range" compared to growth curve for the C57 BL6 strain (weight chart is available at www.criver.com)

Animal A3 developed a head tilt and atrophy of the left eye on day 22 of the study but this was considered a feature of the background strain and unrelated to feed.

5. Material and Methods

Three-week old female C57 Bl/6 mice were fed exclusively 3-4 g/day of **Diet A (3 animals),** 3-4 g/day of **Diet B (3 animals)** for 12 consecutive weeks. Upon collection plasma samples were stored at -80 °C, ear punch tissue was flash frozen at -80 °C .

Diet A: MouseExpress® L-Lysine ($^{13}C_6$,99%) Mouse Feed containing **12 g** of $^{13}C_6$ Lysine:2HCl per Kg feed

Diet B: Modified MouseExpress® L-Lysine ($^{13}C_6$,99%) Mouse Feed containing **8 g** of $^{13}C_6$ Lysine:2HCl per Kg feed

Plasma samples were depleted of albumin and IgG using Proteome Purify™ Mouse Serum Protein Immunodepletion Resin (R&D Systems). Depleted fractions (flow-through) were concentrated using Thermo Scientific Protein Concentrators PES, 10K MWCO. Protein concentrations were determined using the Thermo Scientific Pierce 660nm Protein Assay Kit.

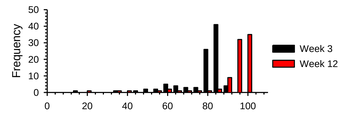
Ear punch tissue samples were homogenized in lysis buffer (100 mM Tris-HCl pH 8, 4% SDS containing Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail) using a Mini-Beadbeater-8 (Bio Spec Products Inc.) and CK14/28 ceramic beads (Precellys). Samples were incubated at 95 °C for 5 min and centrifuged for 10 min at 15,000 × g. Protein content was determined using the Thermo Scientific BCA Protein Assay Kit.

10µg of protein from plasma and ear punch samples was processed by SDS-PAGE. The 30 and 75 kDa molecular weight regions were excised and the gel bands were digested with trypsin (Promega) using a ProGest robot (DigiLab).

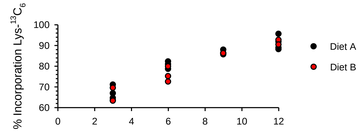
Gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher LTQ Orbitrap Velos Pro. Peptides loaded on a trapping column and eluted over a 75µm analytical column at 350nL/min; columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The top fifteen most abundant ions were selected for MS/MS. Protein/ peptide identification and quantitation were performed with MaxQuant 1.3.0.5.

6. Results

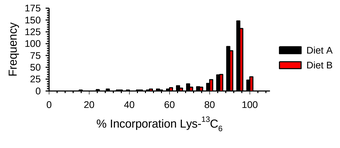
Fractional Enrichment in Plasma after 3 and 12 wks of labeling with Diet B



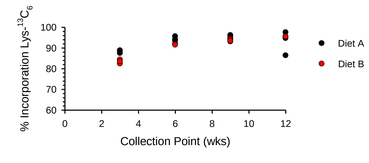
Percent incorporation $^{13}C_6$ Lysine observed in tissue samples



Fractional enrichment in Tissue after 12 weeks of Labeling with Diet A and Diet B



Percent incorporation $^{13}C_6$ Lysine observed in plasma samples



7. Conclusion

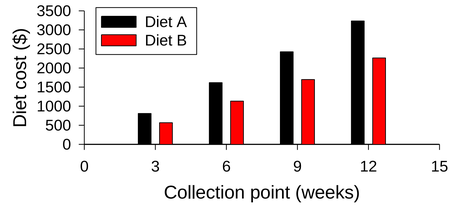
Does the reduced Lysine $^{13}C_6$ diet perform equally well to the current diet? Yes

Mice showed no adverse effects on either Diet A or B.
No statistical difference in incorporation efficiency in plasma or ear tissue at 9 and 12 weeks using these diets.

Can non-generational Lysine-$^{13}C_6$ labeled mice be used in SILAC-type experiments? Yes

Longer labeling times and perhaps generational labeling will be required for studies using organs/tissue with slower protein turn-over rates than blood or liver (7). The effects of a high sucrose, protein-free diet should be considered in the study design.

How does a reduced Lysine diet transfer to cost savings? Yes
A 30% savings may be incurred by using a reduced Lysine $^{13}C_6$ diet. In a 12 week labeling period, this transfers to savings of ~$970 per mouse.



Time (weight)	Diet A	Diet A enrichment (plasma)	Diet A enrichment (tissue)	Diet B	Diet B enrichment (plasma)	Diet B enrichment (tissue)	$ savings
3 Wk (73.5g)	$809	88	68	$566	83	65	243
6 Wk (147g)	$1,617	94	84	$1,132	92	79	485
9 Wk (220.5g)	$2,426	95	87	$1,698	94	86	728
12 Wk (294g)	$3,234	93	91	$2,264	95	92	970

8. Next steps

The data presented here support the possibility of a non-generational metabolically labeled mouse model. Further time points are necessary to validate the model.

9. References

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10. Acknowledgments

CIL contracted Charles River, provided mouse feeds and protocol
Charles River (GEMS) housed/fed/weighed/observed mice, sampled plasma/tissues, shipped samples to Thermo Scientific.
Thermo Scientific solubilized tissue, depleted plasma, resolved samples on gels, and excised bands
MSBioworks performed in-gel digestions, ran LC/MS/MS, processed data