In vitro Synthesis of Stable Isotopically Labeled Proteins for Use as Internal Standards for Mass Spectrometric Quantitation of Clinical Protein Biomarkers

While the use of proteins as clinical biomarkers has become more prevalent, the clinical methods employed to measure these analytes are often based on immunological affinity or enzyme activity. In order for such tests to be traceable to standards of a higher order, reference materials and methods must be established for clinically relevant proteins. Although liquid chromatography/mass spectrometry (LC/MS) methods are increasingly utilized for quantitative protein measurements, the lack of appropriate internal standards hinders the development of standardized analytical methods for clinical proteins. To address this problem, NIST is investigating potential sources of stable-isotope labeled proteins for use as internal standards in quantitative mass spectrometric methods.

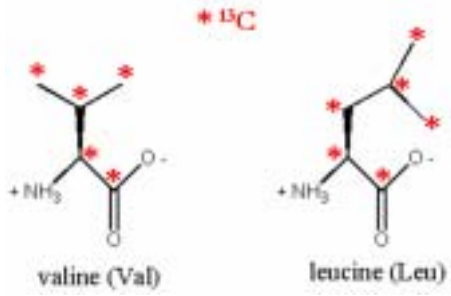
J. Camara, D.M. Bunk, N. Dodder (Div. 839), and P. Reddy (Div. 831)

In clinical proteomics, a "bottom up" approach is often used: in order to make full-length proteins more amenable to mass spectrometric detection, protein mixtures are often digested with a proteolytic enzyme, such as trypsin, prior to analysis. Known amounts of stable isotope-labeled peptides are added as internal standards to allow for quantitation by isotope dilution mass spectrometry. While peptide standards are relatively easy to synthesize, they are not ideal. Ideally, the internal standard should mimic the analyte throughout the analytical procedure. However, peptides cannot account for loss of full-length protein or incomplete enzyme digestion that may occur during sample processing. In this work, cell-free *in vitro* protein expression systems were investigated for their ability to produce customized, full-length proteins for use as internal standards. A full-length, stable isotope-labeled protein standard spiked into a sample prior to processing would account for the multiple variables that may affect the final levels of measured peptides.

NIST is establishing capabilities to produce synthetic proteins for use as standard reference materials to be used to calibrate critical proteomics tools, such as mass spectrometry.

Traditionally, stable isotope-labeled proteins are expressed in cells cultured in stable isotope-labeling growth media. However, these media are extremely expensive, and the total amount of target protein, as well as the level of stable-isotope incorporation, is inefficient. More recently, cell-free, *in vitro* protein expression systems have become available in which cell extracts contain the components necessary for coupled transcription/translation, resulting in the expression of a protein of interest from a DNA template. In many *in vitro* expression systems, individual amino acids can be completely replaced by stable isotope-labeled versions for high-efficiency incorporation into the target protein.

We have evaluated the protein production efficiency of these expression kits and have engineered a DNA expression template with the necessary elements for synthesis of C-reactive protein (CRP) in an *Escherichia coli*-based system. (CRP is used as a clinical marker of inflammation.) We are also investigating methods such as size-exclusion LC and native gel electrophoresis to characterize CRP pentamer formation, which would be required to mimic native CRP. In addition, we are exploring computer simulations of mass spectrometric ion distributions, which will allow us to characterize the extent to which stable isotopes are incorporated into various target proteins. This knowledge will be required to utilize the labeled proteins as internal standards for quantitative mass spectrometry.



Examples of stable isotope-labeled amino acids

Future plans:

Because there is an ongoing need for reference materials and methods for protein measurements, NIST will continue to evaluate cell-free *in vitro* protein expression systems as a source of stable isotope-labeled proteins for internal standards. We will proceed with the expression and characterization of labeled C-reactive protein as our first clinical candidate and plan to extend the project to include additional clinically relevant proteins.