

# The Use of Differential Quantitation in Targeted Proteomics

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## Overview

The expression level of a given protein in a “disease state” vs. the “normal state” of a cell or tissue is an important indication the mechanism of disease and may point the way to potential treatments.

An approach to measuring differential protein expression levels has been published lately by Aebersold et. al. using ICAT™ reagent with an LCQ™ ion trap mass spectrometer (Nature Biotechnology Vol 17, pp. 994, 1999).

We have demonstrated a methodology to quantitate the differences of a low level (femtomole) protein, human growth hormone (hGH), in human plasma by a new LC-MS system (ProteomeX™) with a new software program (BioWorks™ 3.0) and the ICAT reagent.

## Introduction

Targeted proteomics often focuses on the selective identification and quantitation of a protein of interest in an array of proteins. Mass spectrometers are highly selective detectors, but for complex samples multi-step sample preparation may be required prior to MS analysis. We have demonstrated here a methodology to identify and quantitate the differences in a low level (femtomole) protein, hGH in human plasma, by a new multi-dimensional LC-MS system with new protein analysis software.

## Methods

A sample of human plasma (5mg/mL) containing 15fmol hGH (HSA to hGH ratio of about 40,000:1) was divided in two. Both samples were dissolved in 8M urea, reduced and alkylated (one with D0-ICAT, the other with D8-ICAT), and buffer exchanged. The samples were then combined, digested with trypsin, desalted, and injected on a cation exchange column, and eluted onto a reversed phase column with sequential salt steps. The eluted peptides were analyzed by an LCQ™ Deca XP ion trap mass spectrometer. All of the 2D LC-MS/MS operations were performed automatically with a ProteomeX Workstation.

Protein identifications were made using TurboSEQUENT™ within BioWorks 3.0 software. Relevant hGH peptides (cys-containing peptides bound with D0 or D8 ICAT) were identified using MS/MS spectra. Differential quantitation was determined by calculation of ratios of these peptides by XPRESS™, embedded in BioWorks 3.0 software, using MS spectra or selected daughter ions from MS/MS spectra.

## Results

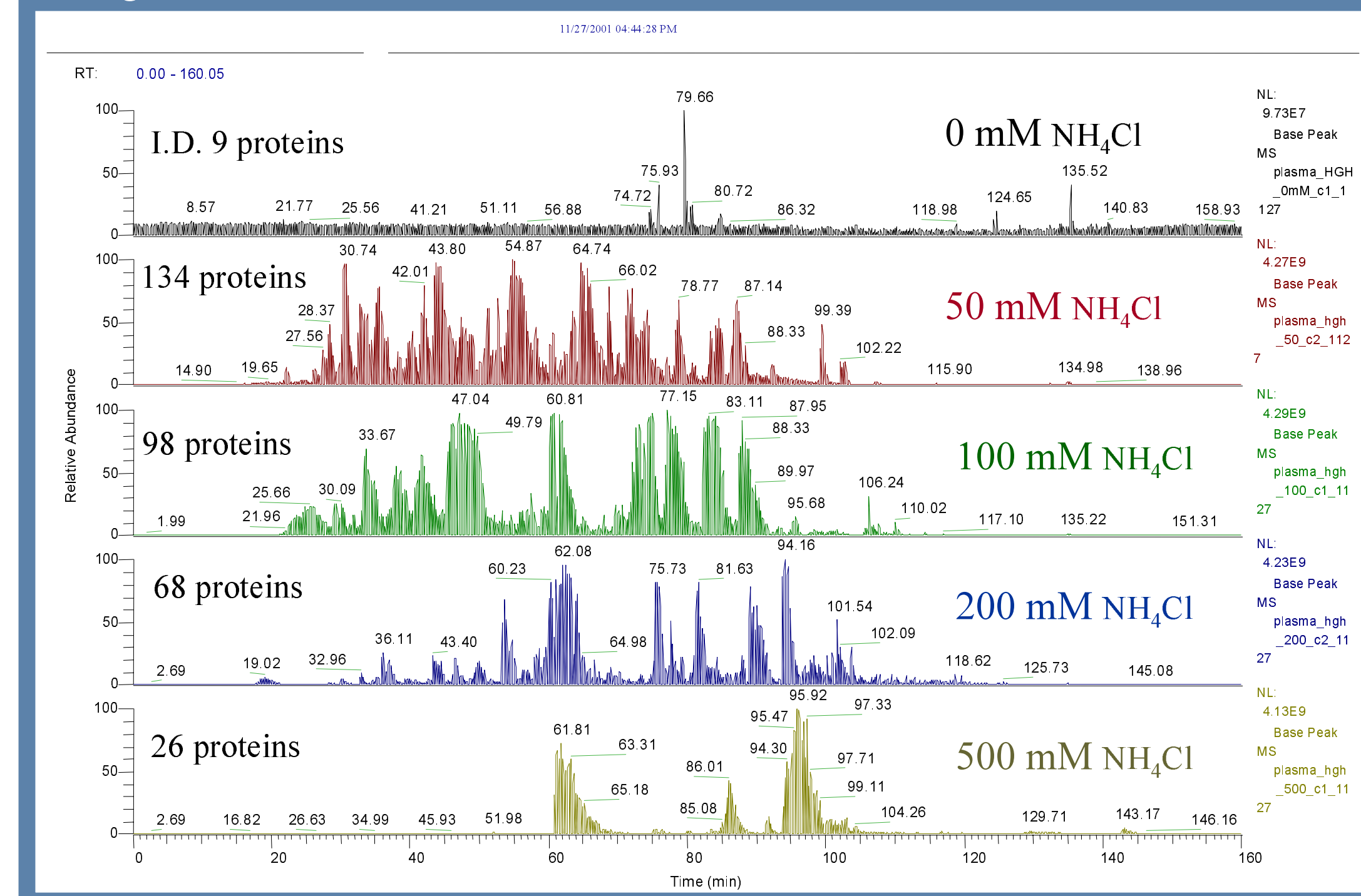
FIGURE 1. Procedure for Determination of Differential Protein Quantitation with ICAT and ProteomeX.

1. Divide sample (hGH in plasma) into two identical pools.
2. Reduce and alkylate (ICAT with D0 for one plasma pool and ICAT with D8 for the other plasma pool), separately. Mix the two pools and digest the whole mixture with trypsin.
3. Clean the digested plasma proteins by ion-exchange (remove excess ICAT reagent) and then capture the ICAT-peptides (biotinylated) by avidin affinity column.
4. Collect the ICAT-peptide fractions, and run the collected fraction by LC-MS with MS/MS.
5. Run BioWorks software, which includes TurboSEQUENT and XPRESS, for data analysis.

ProteomeX  
(2D LC-MS)

Collect the flow through Frxn

FIGURE 2. Analysis of Trypsin-Digested Human Plasma Mixture using 2D LC-MS/MS on ProteomeX Workstation



Human plasma containing femtomole-level hGH was suspended in 8M urea, reduced, and alkylated with either D0-ICAT or D8-ICAT, buffer exchanged, and then digested with trypsin. The trypsin-digested mixture was loaded on a strong cation exchange column (BioBasic SCX, Thermo Hypersil-Keystone), then step-eluted with NH<sub>4</sub>Cl steps of increasing molarity onto alternating reversed phase columns (BioBasic-18, Thermo Hypersil-Keystone), prior to be analyzed by an ion trap mass spectrometer (LCQ Deca XP). The chromatograms of the peptides captured after each salt step are overlaid and displayed here.

FIGURE 3. hGH Peptides Identified with 2D LC-MS/MS

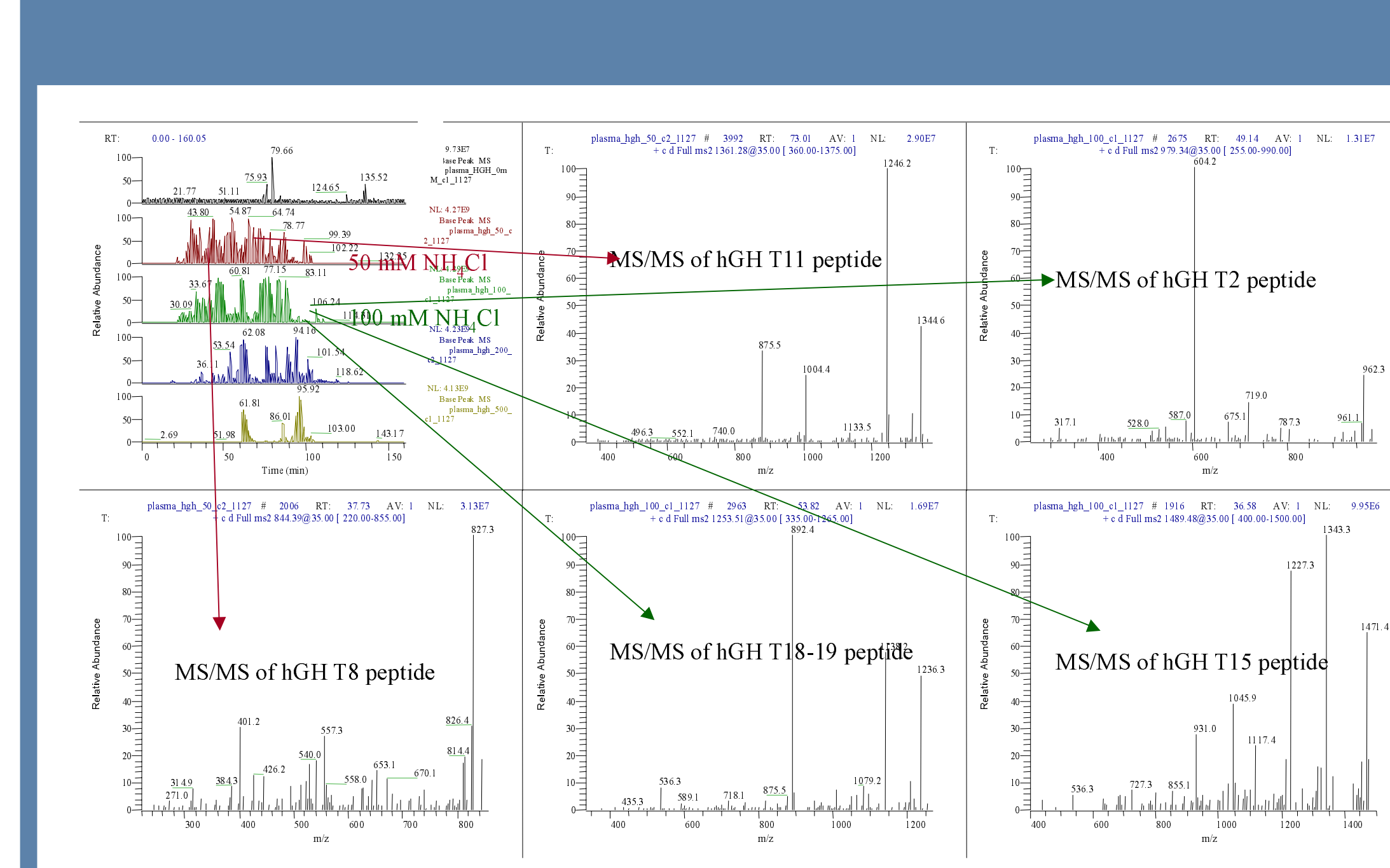


FIGURE 4. Using Data Dependent Mass Tagging for Automated MS/MS of D0 and D8 ICAT Peptide Pairs

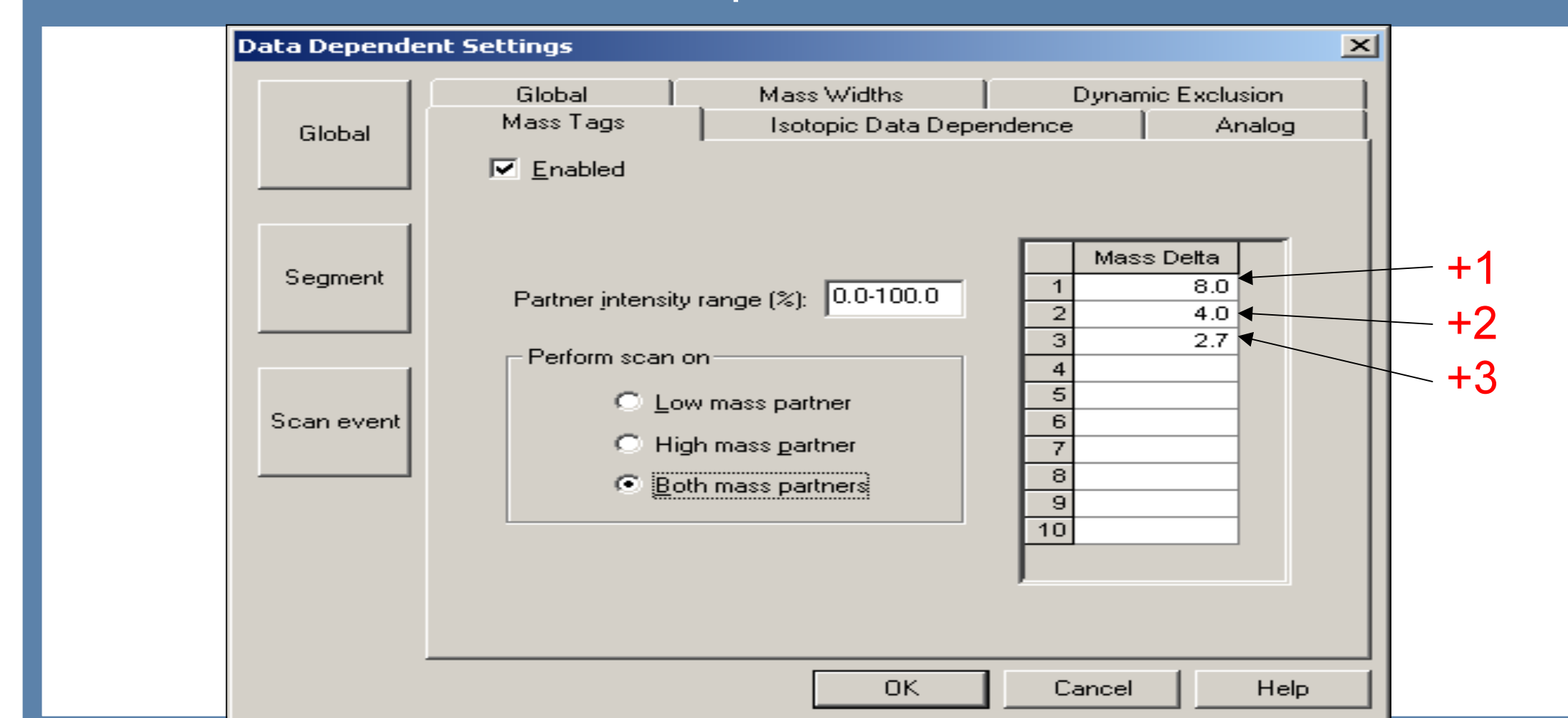


FIGURE 5. BioWorks and TurboSEQUENT Search Parameters

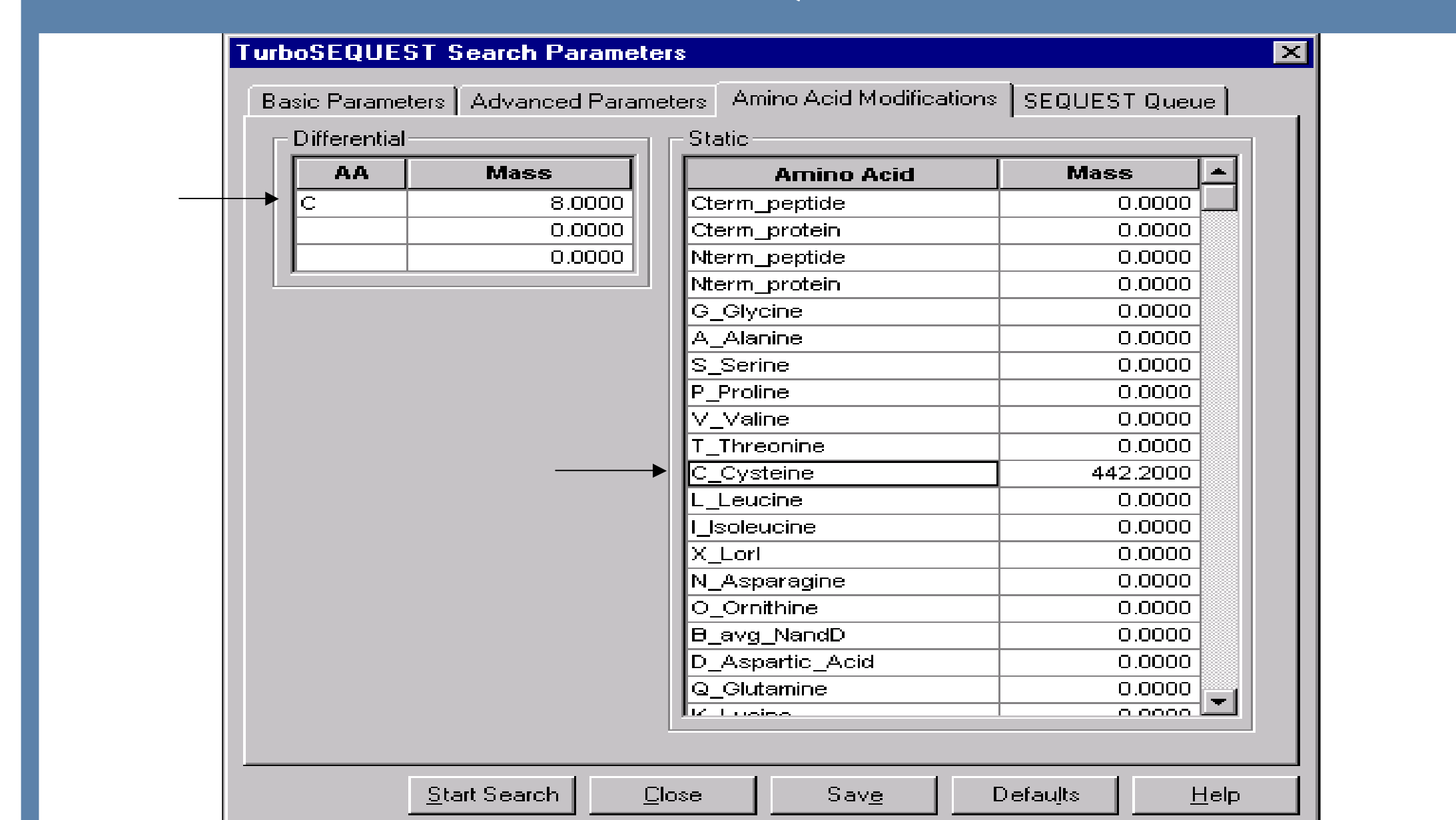


FIGURE 6. Differential Quantitation by BioWorks 3.0 (XPRESS) Software

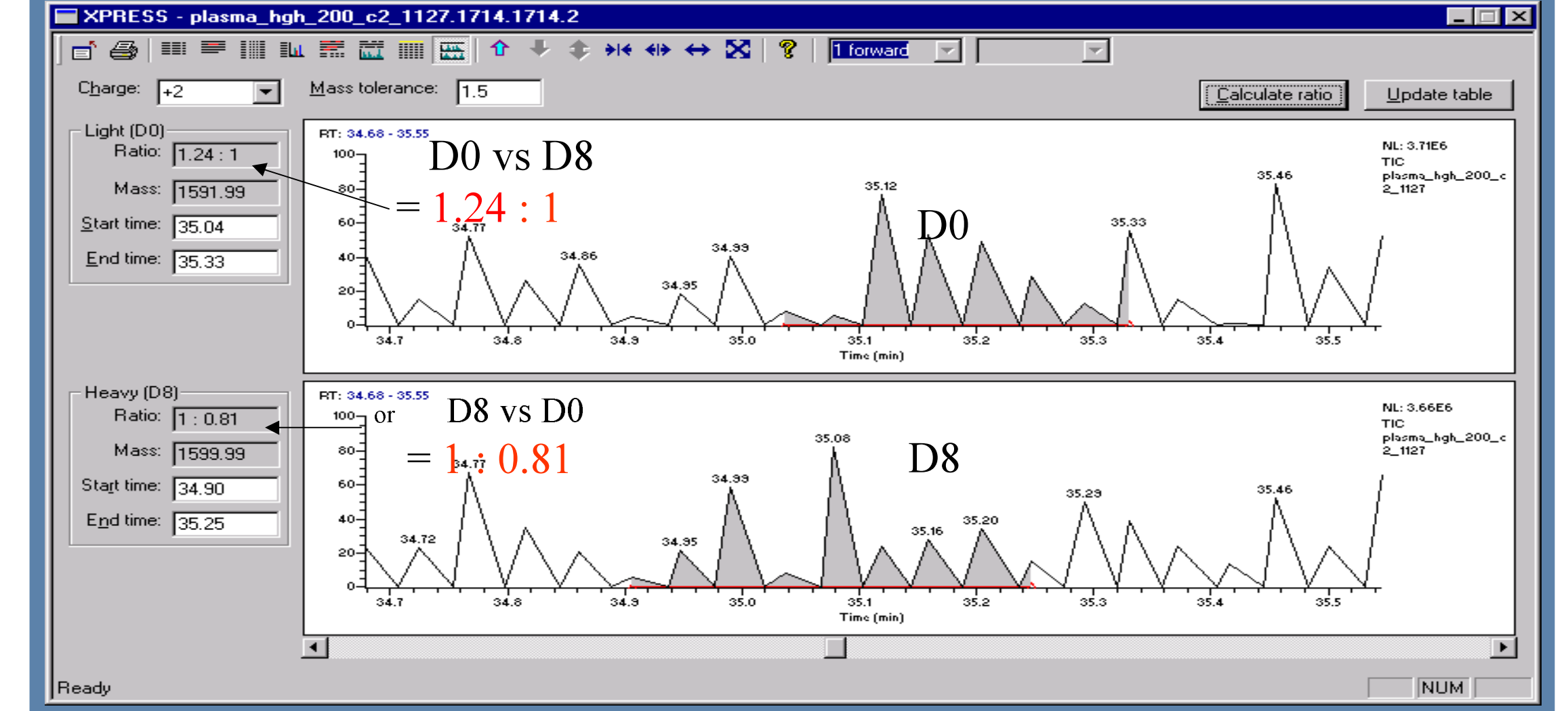
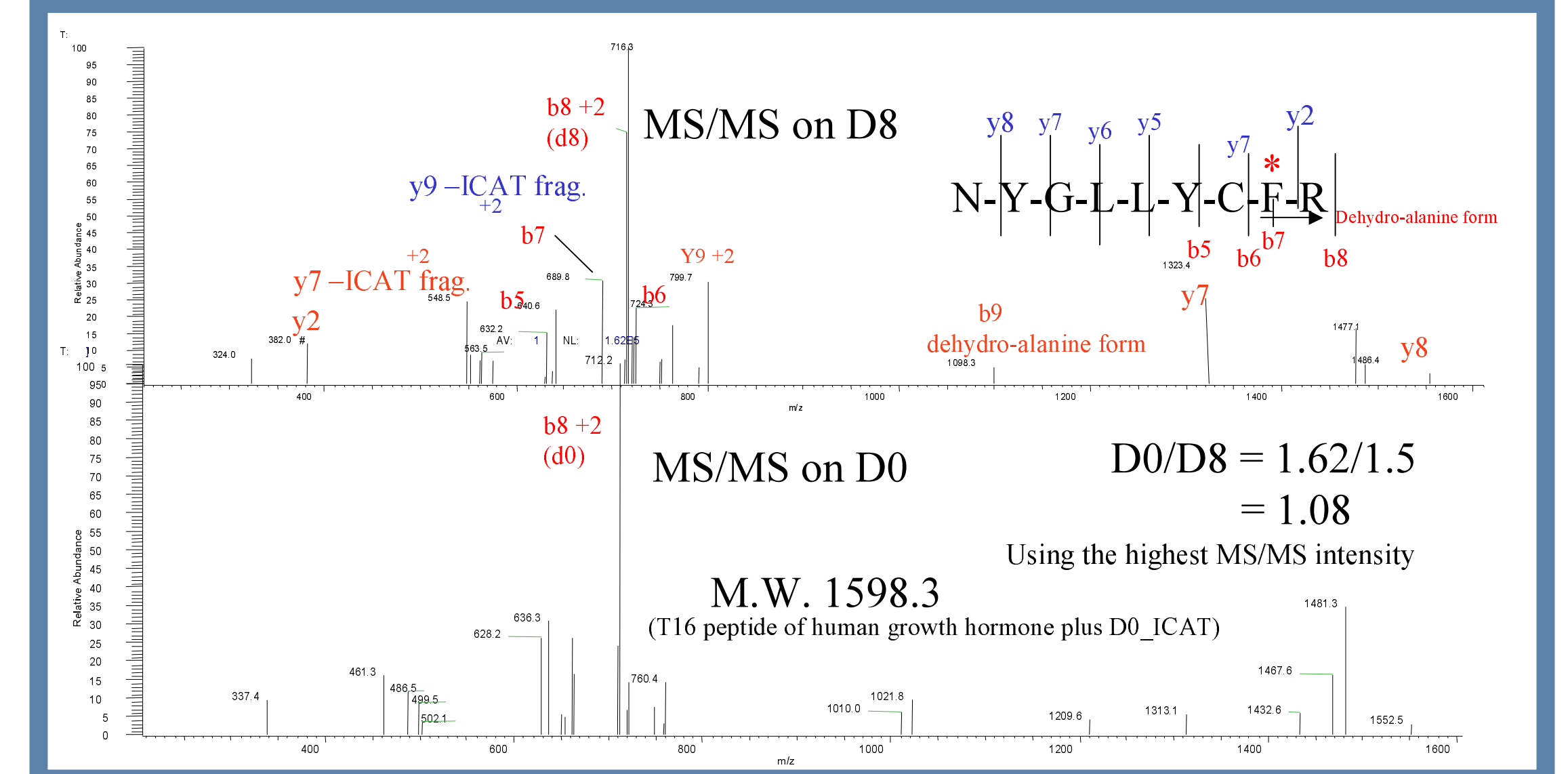


FIGURE 7. Differential Quantitation by SRM (daughter ions) Assisted by Xcalibur® 1.3 (Mass Tags) Software



hGH in 1st plasma pool : hGH in 2nd plasma pool

Theoretical 1 : 1  
Experimental 1.16 : 1  
D0/D8 = (1.08 (from MS/MS) + 1.24(from MS)) / 2 = 1.16

## Conclusions

- Automated 2D LC-MS/MS provides high resolution, high sequence coverage, and highly confident protein identification from a very complex sample.
- A low-level (femtomole) protein, hGH, was accurately identified and quantitated with this approach.
- Recommended ICAT ion exchange and avidin affinity clean-up steps are time consuming and may contribute to experimental error. They can be avoided using automated on-line 2D LC-MS/MS with strong cation exchange and reversed phase chromatography.