

In-Solution Digestion using the Investigator™ ProGest™

Proteomic Technical Note

Introduction

Although 1D & 2D PAGE remain prominent techniques of protein separation & analysis; liquid chromatography (LC) methods are being employed with increasing frequency. Relative advantages in speed, amenability to automation and the ability to avoid gel-specific problems mean that LC-based separations are likely to continue growing in popularity.

One such approach utilizes LC column separation to fractionate proteins based upon their electrostatic charge and/or according to their hydrophobicity. Another method employs pre-packed pipette tips or columns for crude separations, a gel packed column to separate according to size and/or an ion exchange column for charge-based separations.

These and other new methods demonstrate the need for new procedures to automate digestion of protein samples in liquid phase, as well as in gel plugs. Some studies have suggested that in order to identify the maximum number of proteins in any given sample, both LC & electrophoresis approaches should be used in combination.

In this brief note we present a method for liquid phase digestion (In-Solution Digestion) which will illustrate how the automation benefits previously available for use with gel plug samples may now also be successfully applied to proteins in solution.

Materials & Methods:

Sample Preparation & Concentration

Bovine Serum Albumin (BSA) solutions were prepared from a vial of 2mg/ml Albumin Standard available from Pierce. The concentrations used for reaction were 1.0 pmol, 750 fmol & 500 fmol of BSA in 20 µL of final volume.

Genomic Solutions ProGest Blue Microtiter Plates (Part #: PRO10005)

Genomic Solutions ProGest Trypsin Digestion Kit (Part#:0080-0210)

Hardware

Investigator ProGest sample digestion workstation (Digilab Inc.), equipped with software version 2.01.15 was used to carry out the liquid phase digestion. (Figure 1)

Mass spectrometric Analysis was performed on an ABI DE-STR Voyager (Applied Biosystems Inc.)

Software

Methods' Editor v1.1.0.29 was used to create & modify the method used on the ProGest. (Figure 2)

In-Solution Digestion using ProGest Page 2

ProFound, a part of the Knexus suite (Digilab Inc.) was used for protein identification. Other components of the Knexus package were used for spectra analysis, data visualization and analysis.



Figure 1: Investigator ProGest sample digestion robot

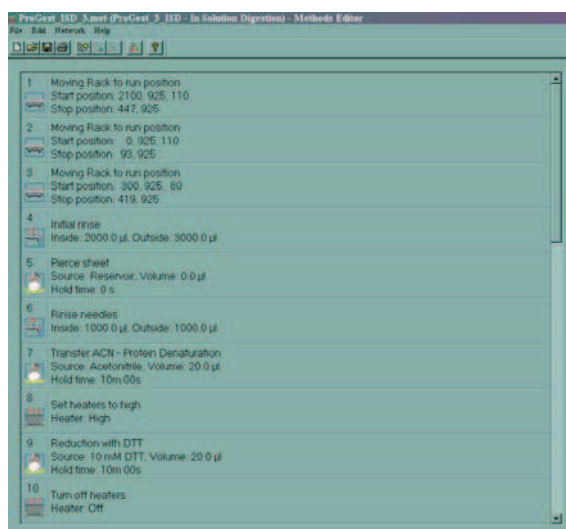


Figure 2: Methods' Editor, a program to create and amend methods for the ProGest & the ProMS

Method for Liquid Phase Digestion

This method was adapted from one normally used with the ProPrep II sample preparation station. Listed below are the main steps of the Liquid Phase digestion protocol:

- 1) Denature the protein (20 µL in aqueous solution) in a blue plate with 20 µL of acetonitrile for 20 minutes.
After addition of acetonitrile, each sample well should hold 50% acetonitrile.
- 2) Reduce the denatured protein with 20 µL of 10 mM Dithiothreitol (DTT) for 20 minutes at 55°C.
- 3) Alkylate the sulfhydryls with 20 µL of 50 mM Iodoacetamide for 20 minutes.
- 4) Trypsin Activation: The concentration of the trypsin stock solution in 0.1% formic acid is 20 µg/200 µL (0.1 µg/µL). The stock solution is diluted with 25 mM ammonium bicarbonate to produce the 8.0 ng/µL (working solution). Addition of the ammonium bicarbonate also raises the pH from < 2.0 to approximately pH = 8.5.
- 5) Add 20 µL of trypsin (working solution) to samples.
- 6) Add 20 µL of 25 mM ammonium bicarbonate.
- 7) Incubate for 4 hours at 37°C.

In-Solution Digestion using ProGest Page 3

Materials & Methods (con't):

Spotting

Add 0.3% formic acid to acidify the peptide solution. Elute the samples with matrix solution onto a Voyager mass spectrometer (ABI 100). A saturated matrix solution (10 mg/mL) was diluted 1:2 to prepare the elution solution. The samples were allowed to dry on the target plate of the MALDI target. From each sample a MALDI-TOF mass spectrum was recorded.

Results:

Three concentrations of BSA were digested in a total of 20 μ L volume for each sample. For all concentrations, the peptide coverage ranged between 25%-35%. The low peptide coverage is attributed to non-detected peptides with masses less than 750 Da. which is the cutoff for detection of the mass spectrometer. Theoretical digestion with PAWS (Protein Analysis Work Sheet) shows a number of peptides with masses in the range of 150-750 Da. These small peptides are not accounted for when the value of peptide coverage is calculated. (See Appendix 1)

The expectation values for the proteins identified with ProFound were below 10^{-5} , which indicates that these results were valid and not generated from random hits. (See Appendix 1)

For the sample with 500 fmol of BSA (See Figure 3), the searches with ProFound yielded BSA or BSA precursor. The peaks were strong and isotopically resolved.

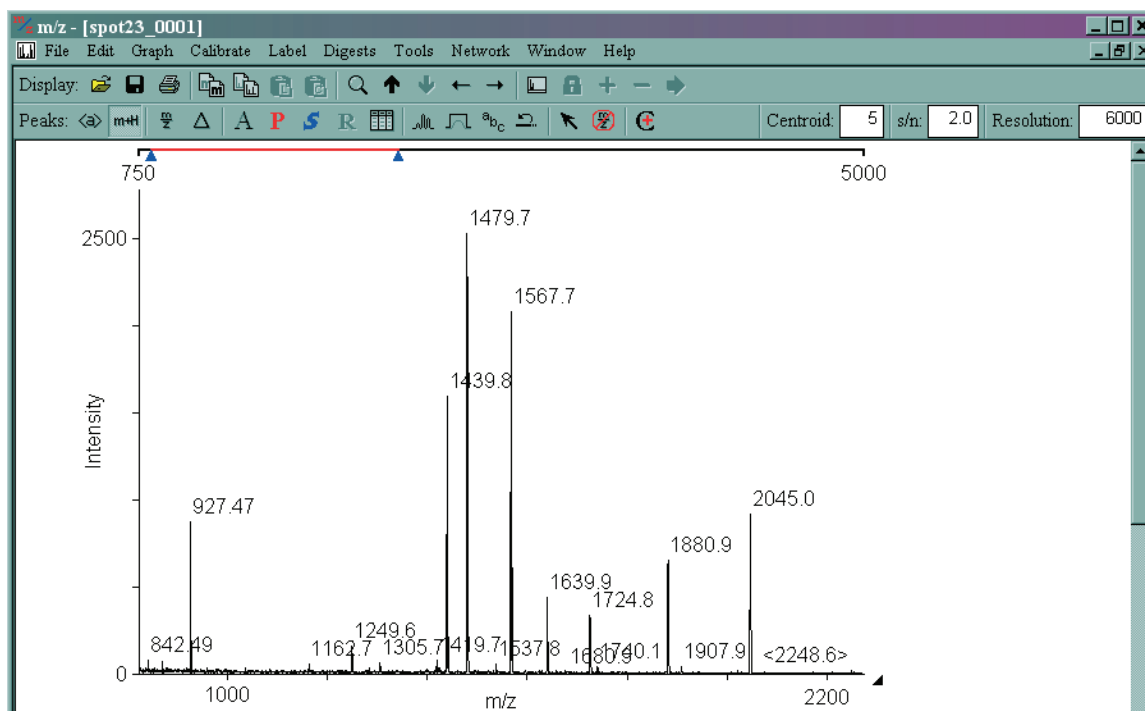


Figure 3: MS spectrum of spot 23 (500 fmol)

In-Solution Digestion using ProGest Page 4

Results (con't):

The amount of trypsin used was sufficient to perform the digestion. In this case, however, a higher concentration of trypsin would have been more helpful. Increasing the amount of trypsin used would have increased the ratio of trypsin to BSA resulting in more intense, consistent autolysis peaks for use as internal standards.

Conclusions:

The results demonstrate that the Investigator ProGest is well suited to automating liquid phase digestion. Furthermore, the adaptability of the ProPrep makes it easy to alter any of the existing protocols. This added flexibility allows the freedom to vary the amount of any reagent to yield better results.

Appendix 1

Snapshots of reports generated for sample 2 using PAWS and Knexus

Knexus is a software suite designed for analysis & manipulation of mass spectral data. Knexus is comprised of 4 software programs:

Enterprise M/Z is used for peak detection & analysis. It has a novel signal-to-noise based peak finding routines that allow it to be applied to strong or weak signals, without user intervention.

Profound is a search engine for searching protein sequence databases using information from mass spectral peptide maps.

Sonar MS/MS is a software tool for identifying proteins from MS/MS data. It contains quality control scoring and a brand new graphic delivery for MS/MS data.

PAWS (Protein Analysis Work Sheet) has numerous tools to improve analysis of acquired mass spectral data. Theoretical calculations can be used to identify cleavage of target proteins with various proteases and chemistries to obtain a better understanding of the resulting peptides. Also, searches can be conducted to identify possible peptide sequences that would correspond to unmatched spectral peptide peaks.

In conjunction with Enterprise M/Z, PAWS can be used to prepare graphics (see Figures 1-3) to associate actual data with theoretical calculations. PAWS also generates reports that detail the results of searches conducted via Profound and/or Sonar (Figures 4-7). These reports and graphics allow researchers to present their experimental conclusions with greater clarity.

In-Solution Digestion using ProGest Page 5

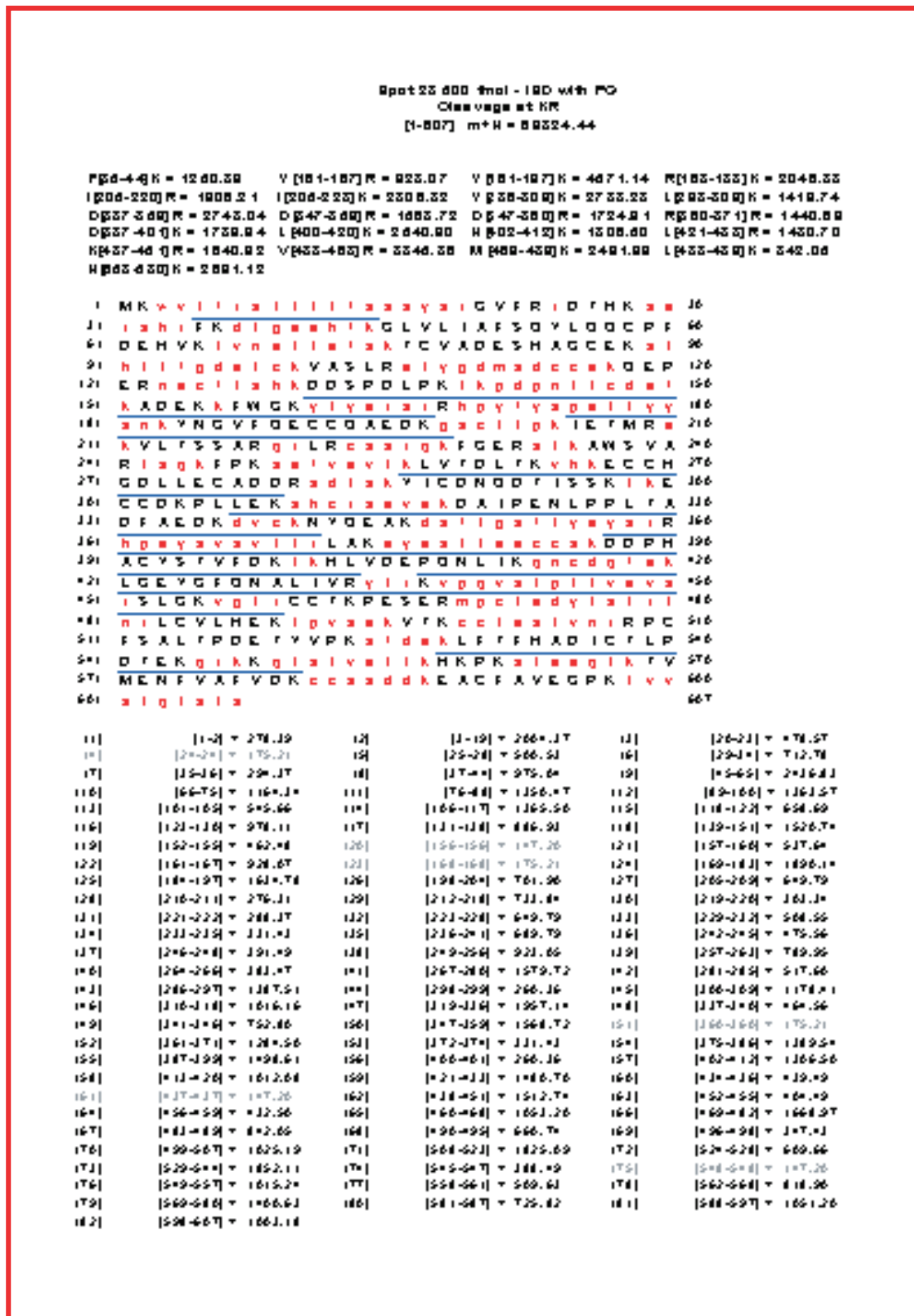


Figure 1

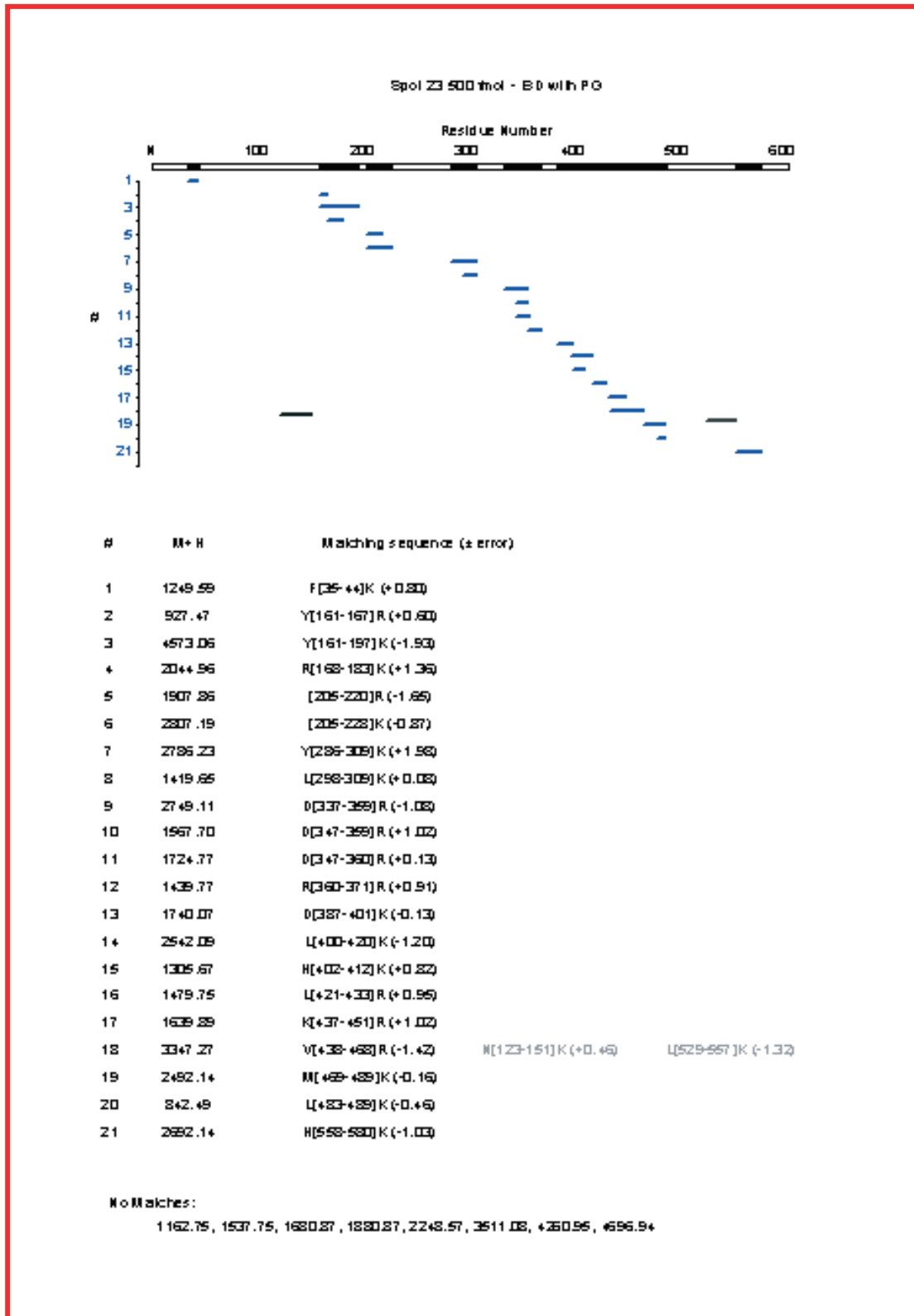


Figure 2

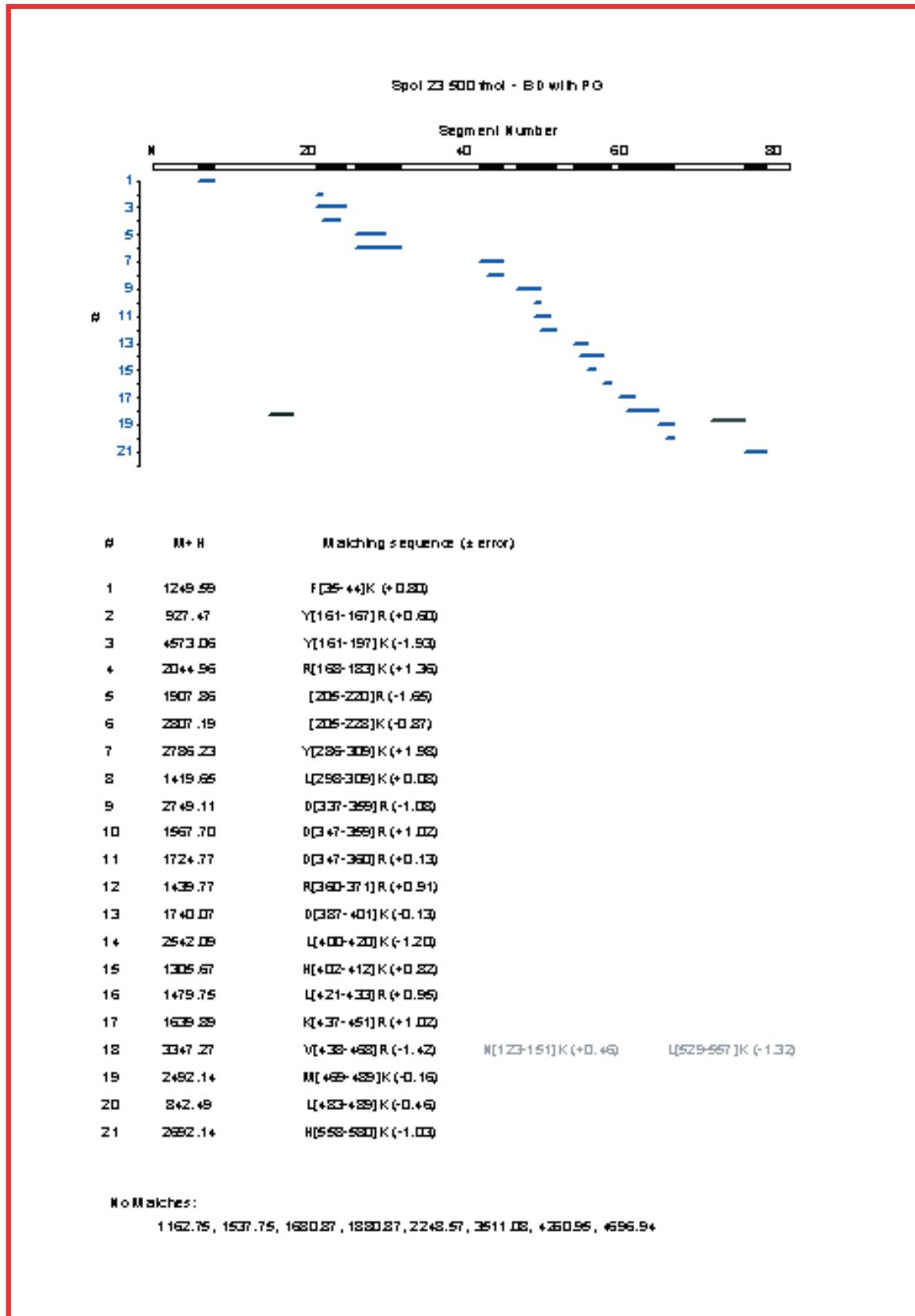


Figure 3

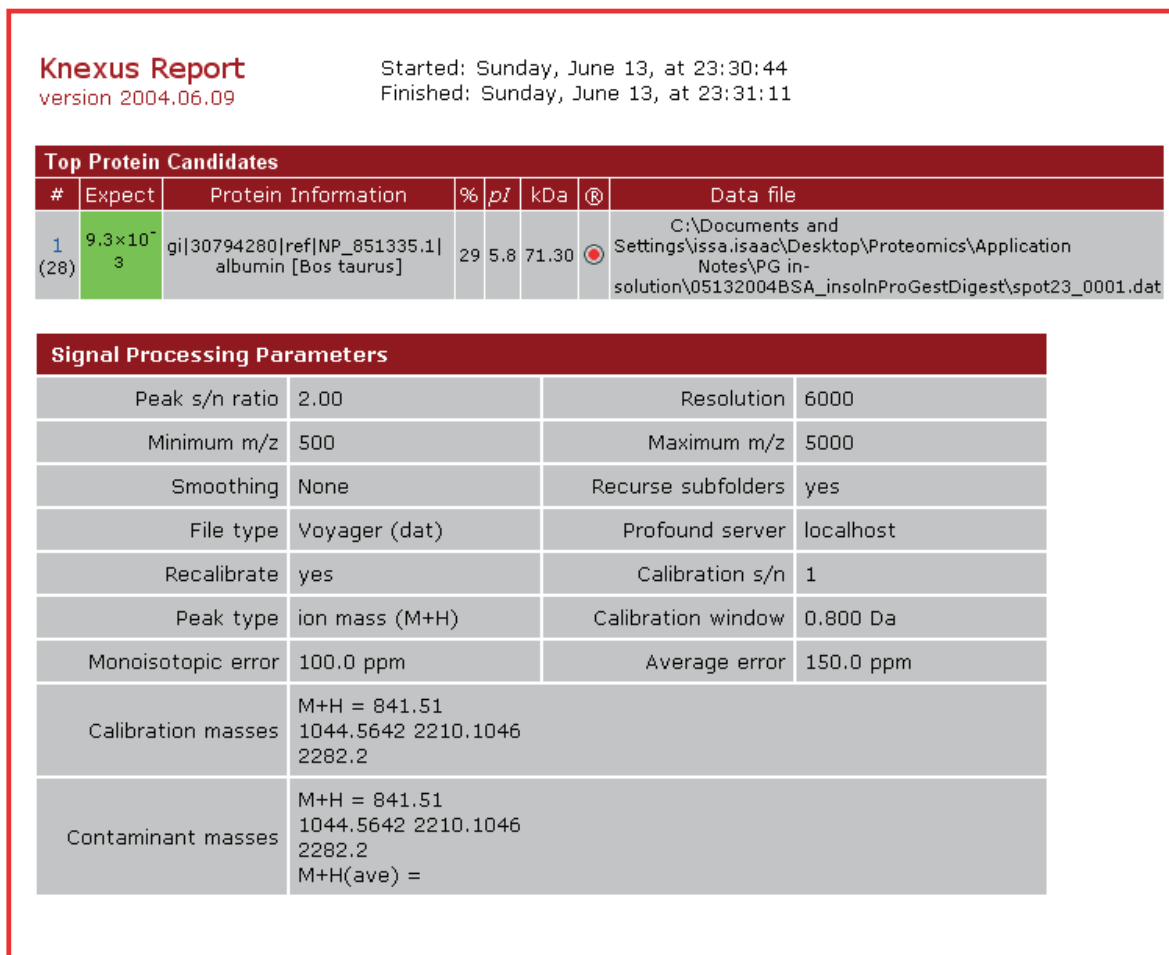


Figure 4

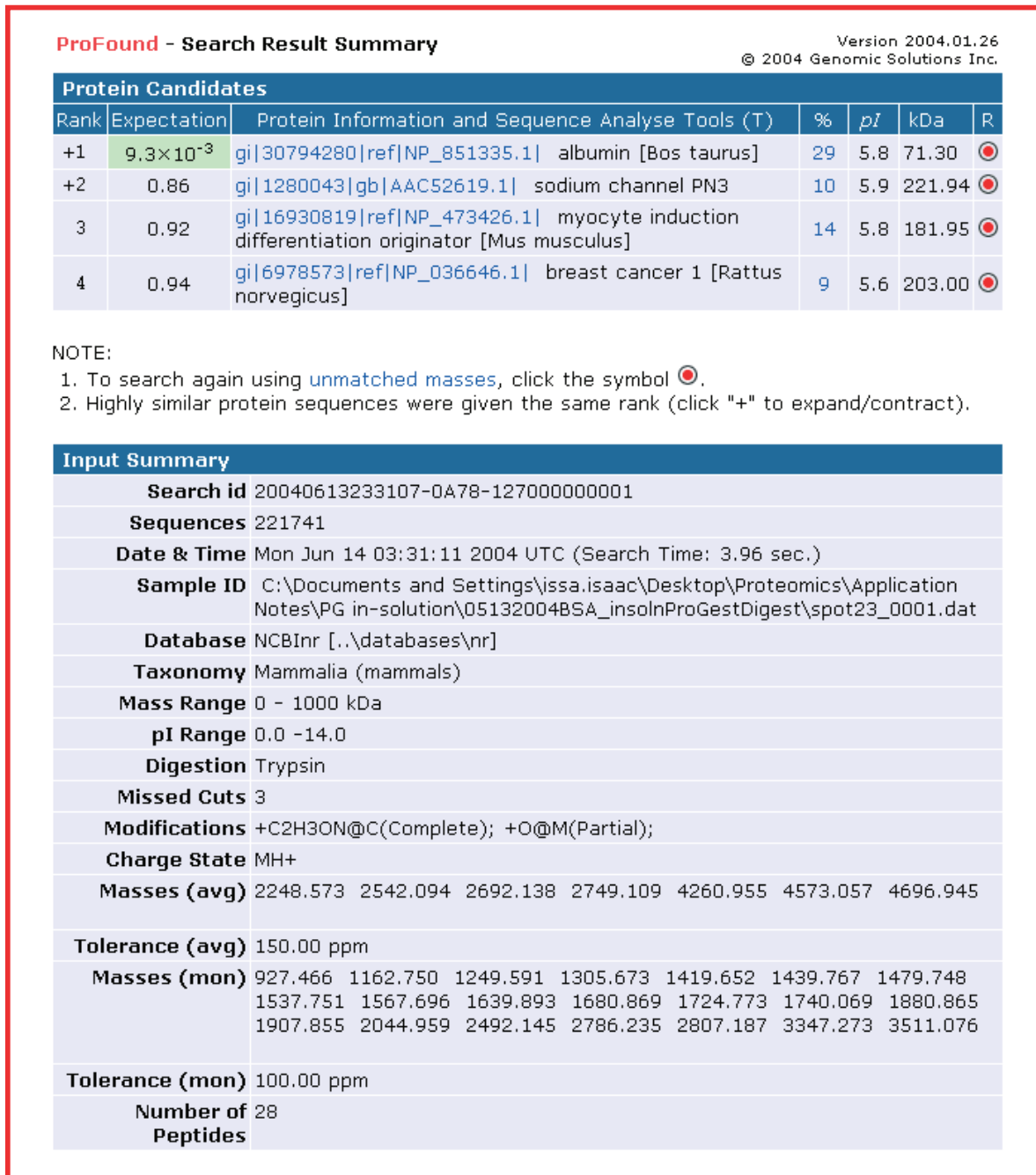


Figure 5

ProFound - Search Result Summary

Version 2004.01.26
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Protein Candidates						
Rank	Expectation	Protein Information and Sequence Analyse Tools (T)	%	pI	kDa	R
+1	9.3×10 ⁻³	gi 30794280 ref NP_851335.1 albumin [Bos taurus]	29	5.8	71.30	⊙
	-	gi 1351907 sp P02769 ALBU_BOVIN Serum albumin precursor (Allergen Bos d 6)	29	5.8	71.27	⊙
	-	gi 418694 pir ABBOS serum albumin precursor [validated] - bovine	27	5.8	71.25	⊙
	-	gi 229552 prf 754920A albumin	25	5.8	67.78	⊙
+2	0.86	gi 1280043 gb AAC52619.1 sodium channel PN3	10	5.9	221.94	⊙
3	0.92	gi 16930819 ref NP_473426.1 myocyte induction differentiation originator [Mus musculus]	14	5.8	181.95	⊙
4	0.94	gi 6978573 ref NP_036646.1 breast cancer 1 [Rattus norvegicus]	9	5.6	203.00	⊙

NOTE:

1. To search again using [unmatched masses](#), click the symbol ⊙.
2. Highly similar protein sequences were given the same rank (click "+" to expand/contract).

Input Summary	
Search id	20040613233107-0A78-127000000001
Sequences	221741
Date & Time	Mon Jun 14 03:31:11 2004 UTC (Search Time: 3.96 sec.)
Sample ID	C:\Documents and Settings\jissa.isaac\Desktop\Proteomics\Application Notes\PG in-solution\05132004BSA_insolnProGestDigest\spot23_0001.dat
Database	NCBIInr [..\databases\nr]
Taxonomy	Mammalia (mammals)
Mass Range	0 - 1000 kDa
pI Range	0.0 -14.0
Digestion	Trypsin
Missed Cuts	3
Modifications	+C2H3ON@C(Complete); +O@M(Partial);
Charge State	MH+
Masses (avg)	2248.573 2542.094 2692.138 2749.109 4260.955 4573.057 4696.945
Tolerance (avg)	150.00 ppm
Masses (mon)	927.466 1162.750 1249.591 1305.673 1419.652 1439.767 1479.748 1537.751 1567.696 1639.893 1680.869 1724.773 1740.069 1880.865 1907.855 2044.959 2492.145 2786.235 2807.187 3347.273 3511.076

Figure 6

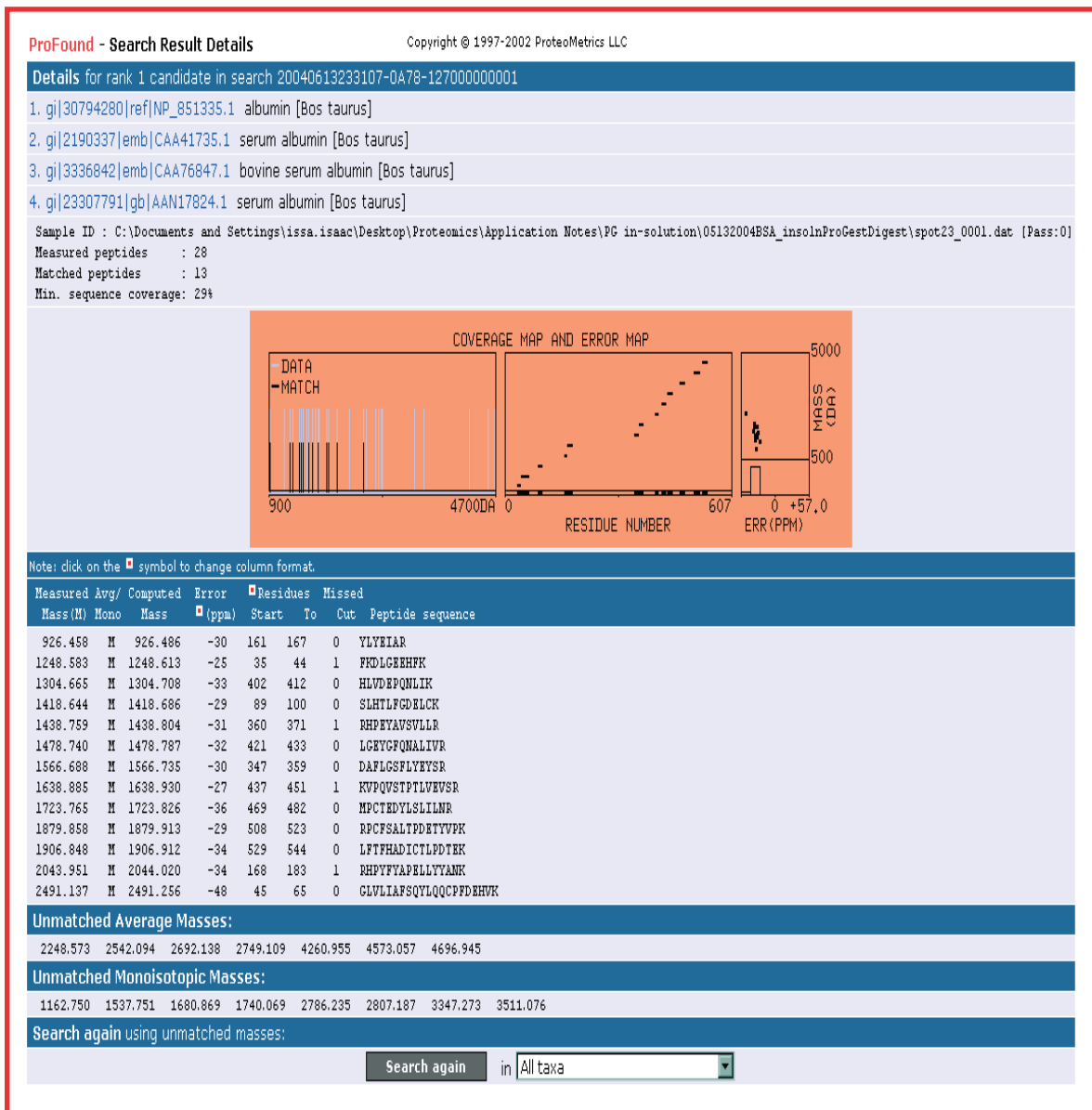


Figure 7

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