High Pressure Direct Protein Extraction from Tissue – Trypsin Digest with Pressure Cycling Technology (PCT)

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What is PCT?

The application of cycling high and low hydrostatic pressures to a solid or liquid sample to extract proteins, oligonucleotides, or smaller molecules.

Suzuki C, Suzuki K. *J Biochem* 1962; **52**: 67.

Ruan K, Lange R, Bec N, Balny C. *Biochem Biophys Res Commun* 1997; **239**: 150.

Chicon R, Belloque J, Recio I, Lopez-Fandino R. *J Dairy Res* 2006; **73**: 121.

Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. *J Biomol Tech* 2006;**17**: 173.

Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA. *Electrophoresis* 2007; **28**: 1022.

Pevsner P, Vecchione D, Stall B, Remsen T, Anand S, Stern A. 2007; .

Pevsner P, Vecchione D, Remsen T, Kessler P, Levers N, Yang P, Stern A, Samuels H. 2007; .

Pevsner P, Vecchione D, 1 Remsen T, Kessler P, Momeni M, Duddempudi S, Francois F, Stern A, Anand S. 2007; .

PCT Barocycler

PCT Application a

- Direct tissue extraction
  - Protein
  - Oligonucleotides
  - Small molecules

Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. *J Biomol Tech* 2006; **17**: 173.

Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA. *Electrophoresis* 2007; **28**: 1022.

Pevsner P, Vecchione D, Stall B, Remsen T, Anand S, Stern, A. British Mass Spectrometry Society Annual Meeting, Edinburgh, Scotland, September 9-12, 2007

Pevsner P, Vecchione D, Remsen T, Kessler P, Levers N, Yang P, Stern A, Samuels H. British Mass Spectrometry LCMS Meeting, Robinson College, Cambridge, UK, December 8-12, 2007.

Pevsner P, Vecchione D, 1 Remsen T, Kessler P, Momeni M, Duddempudi S, Francois F, Stern A, Anand S. British Mass Spectrometry LCMS Meeting, Robinson College, Cambridge, UK, December 8-12, 2007

PCT Application b

- Trypsin Digest

Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG,2nd, Smith RD. *J Proteome Res* 2008; **7**: 3276. DOI: 10.1021/pr7008077.

Pevsner PH, Melamed J, Remsen T, Kogus A, Francois F, Kessler P, Stern A, Anand S. *Biomarkers Med* 2009; **3**: 55.

Introduction a

- PCT reduces trypsin digest time from hours to minutes.
- Current methods often use heat and prolonged digest intervals.
- Samples digested in minutes with PCT give virtually the same results as those digested for one hour at 55℃.

Introduction b

- Samples for this study
  - Trypsin digest of protein calibrant mixture for matrix-assisted laser desorption ionization mass spectrometry (MALDI)
  - Trypsin digest of tissue extract for liquid chromatography mass spectrometry (LCMS)
    - normal mouse tongue and heart
    - irradiated mouse tongue and heart

Why Trypsin?

- Member of the serine peptidase class of proteolytic enzymes used to digest proteins into various-sized peptides for scientific analysis.
- Cleaves peptides at the n-terminus side of lysine and arginine (unless followed by a proline)

Rodriguez J, Gupta N, Smith RD, Pevzner PA. *J Proteome Res* 2008; **7**: 300.

Why pressure? a

- Denaturation takes place when the protein-protein and protein-solvent interactions are interrupted. Applying pressure causes an increase in the number of water-water and protein-water hydrogen bonds, while the number of protein-protein hydrogen bonds decrease, resulting in denaturation of the protein.

Silva JL, Weber G. *Annu Rev Phys Chem* 1993.

- High pressure exposes a protein's hydrophobic groups to water which allows molecules of water to penetrate the protein and increase the internal pressure, destabilizing the hydrogen bonds that are responsible for the secondary and tertiary structure of the protein. As a result, the protein is destabilized and unfolds.

Ryan K, Lange R, Bec N, Balny C. *Biochem Biophys Res Commun* 1997; **230**: 150

Why pressure? b

- Pressures between 29,000- 145,000 PSI (2-10 kbar) are adequate to denature protein, however, pressures >35,000 PSI will denature trypsin.

  Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG, Smith RD. *J Proteome Res* 2008; **7**: 3276.

- Under high pressure, non-native salt bridges, hydrophobic, and electrostatic interactions are disrupted within the protein. The application of pressure has also been shown to increase protein charge which increases electrostatic repulsions. These repulsions favor a conformational change within the protein (i.e. unfolding).

  Balny C. *Journal of Physics: Condensed Matter* 2004; **16**: S1245.

The role of temperature

- Temperature plays a significant role in a protein's susceptibility to proteolytic digestion.
- Depending on the stability of a given protein, temperatures up to 60℃ can be used.

Sim TS, Kim EM, Joo HS, Kim BG, Kim YK. *Lab Chip* 2006; **6**: 1056.

The role of time

Long digest intervals have been cited as a major disadvantage in protein studies and prompted a search for viable time-saving alternatives, such as PCT.

Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG, Smith RD. *J Proteome Res* 2008; **7**: 3276.

Trypsin Auto-digestion

- Trypsin auto-digestion often occurs in longer digest intervals and at higher temperatures, resulting in inaccurate MALDI analysis.
- To minimize excessive auto-digestion, the trypsin-substrate ratio is kept very low (~1:50), and the temperature below 55 ºC.
  - Ratio determines the extended length of time needed for digestion to occur.
  - A low ratio diminishes the noise present in MALDI spectra by reducing trypsin autodigestion peptides.

Sim TS, Kim EM, Joo HS, Kim BG, Kim YK. *Lab Chip* 2006; **6**: 1056.

Methods a

- Matrix-assisted laser desorption (MALDI) mass spectrometry
- Trypsin digest with PCT
  - 16 samples of BSA
  - 16 samples of Cytochrome-C
- Trypsin Digest Control (55℃ for one hour)

Methods b

- Materials
  - HPLC-grade water
  - Powdered BSA and Cytochrome-C
  - Trypsin- mass spectrometry grade treated with TCPK, a protease inhibitor
  - Peptide Calibrants
    - Angiotensin I-1296.68 (M+H)+ monoisotopic,
    - Neurotensin-1672.92 (M+H) + monoisotopic,
    - ACTH (clip 1-17)-2093.09 (M+H)+ monoisotopic
  - Protein Calibrants
    - Insulin 5733.49 Da
    - Cytochrome c 12,361.96 Da
    - Apomyoglobin 16,952.27 Da
    - Aldolase 39,211.28 Da
    - Bovine serum albumin 66,429.09

Methods c

- **Trypsin Preparation**
  - Trypsin (1µg/µL) dissolved in 100 mM ammonium bicarbonate.
- **Calibrant Preparation**
  - Calibrants dissolved in 0.1% TFA/Acetonitrile (ACN), 30/70.

Methods d

- BSA
  - 1158 µg (10 µg/ µL ammonium bicarb 100 mM) /trypsin 2 µg (1 µg/ µL ammonium bicarb 100 mM), total160 µL
- Cytochrome-C
  - 1158 µg (10 µg/ µL ammonium bicarb 100 mM) /trypsin 2 µg (1 µg/ µL ammonium bicarb 100 mM), total160 µL
- Sixteen samples of BSA and trypsin (158 µL /1158 µg BSA and 2 µL/2 µg trypsin), and sixteen samples of Cytochrome-C and trypsin (158 µL/ 1158 µg Cytochrome-C and 2 µL/2µg trypsin) were placed in individual microtubes.

PCT Protocol

- Experimental Protocol
  - Four samples of each protein barocycled at ambient temperature and 35,000 PSI for
    - 1 cycle (20 seconds)
    - 5 cycles
    - 10 cycles
    - 20 cycles
- Control Protocol
  - Four samples of each protein were digested with trypsin for one hour at 55° C.

MALDI preparation

- MALDI Matrix Preparation
  - 70% ACN/ 30% CHCA (cyano-4-hydroxycinnamic acid) matrix solution, 5 mg/mL, prepared for MALDI TOF-TOF.
- MALDI Calibration Wells
  - Calibration wells created by micropipetting 1 µL of each of the 2 peptide mixes in between sample wells and covering them with 2 µL of matrix.
- MALDI Plate Preparation
  - Two µL of each BSA and Cytochrome-C samples micropipetted onto separate wells of a stainless steel MALDI conduction plate for mass spectrometric analysis.
  - The samples were covered with 2 µL of matrix and then dried in an oven at 55 ℃.

MALDI Spectrometric Analysis

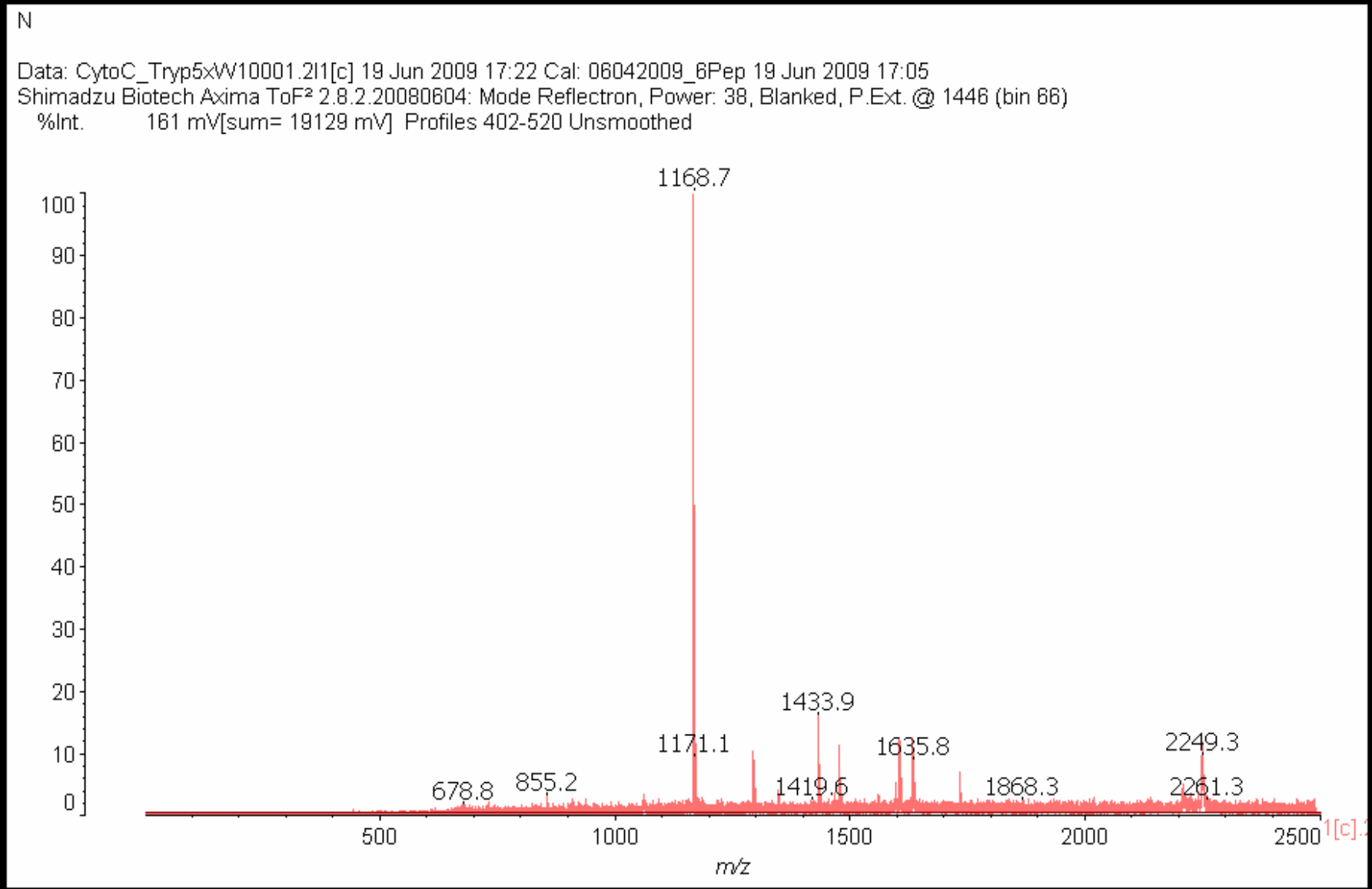
- Results were obtained using a Shimadzu Axima MALDI TOF-TOF.
- Analysis was conducted in reflectron mode.

Matrix Science MASCOT Search Parameters

- Peptide mass fingerprint (PMF) mode.
- Search enzyme set to trypsin.
- Variable modifications set to Oxidation (M) (methionine)
- Mass values set to monoisotopic
- Protein mass unrestricted
- Peptide mass tolerance set at ±1.5Da.
- Peptide charge state set at 1+.
- Search set to permit a maximum of 2 missed cleavages.

Results (MALDI)

- Analysis resulted in the positive identification of Cytochrome-C and BSA.
  - All MASCOT scores were significant matches.
  - Numbered scores represent exact matches.



Cytochrome-C – 5 Cycles

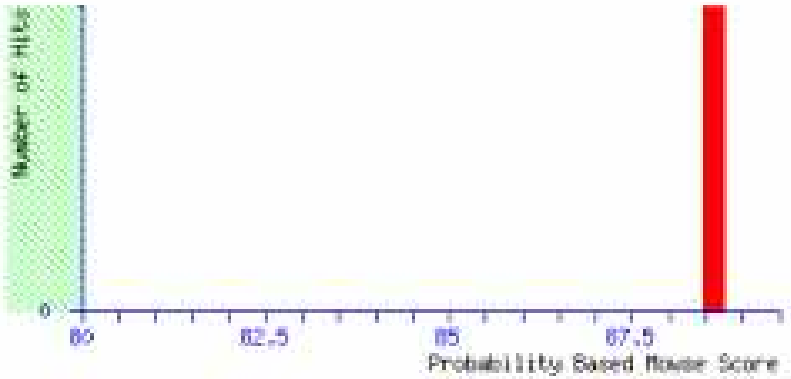
Mascot Score : 89

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : Cyto C Trypsin 5 cycles W1
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 21:20:25 GMT
Top Score : 89 for CYC_HORSE, Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



Concise Protein Summary Report

Format As: Concise Protein Summary Help

Significance threshold p= 0.05 Max. number of hits 5

Re-Search All Search Unmatched

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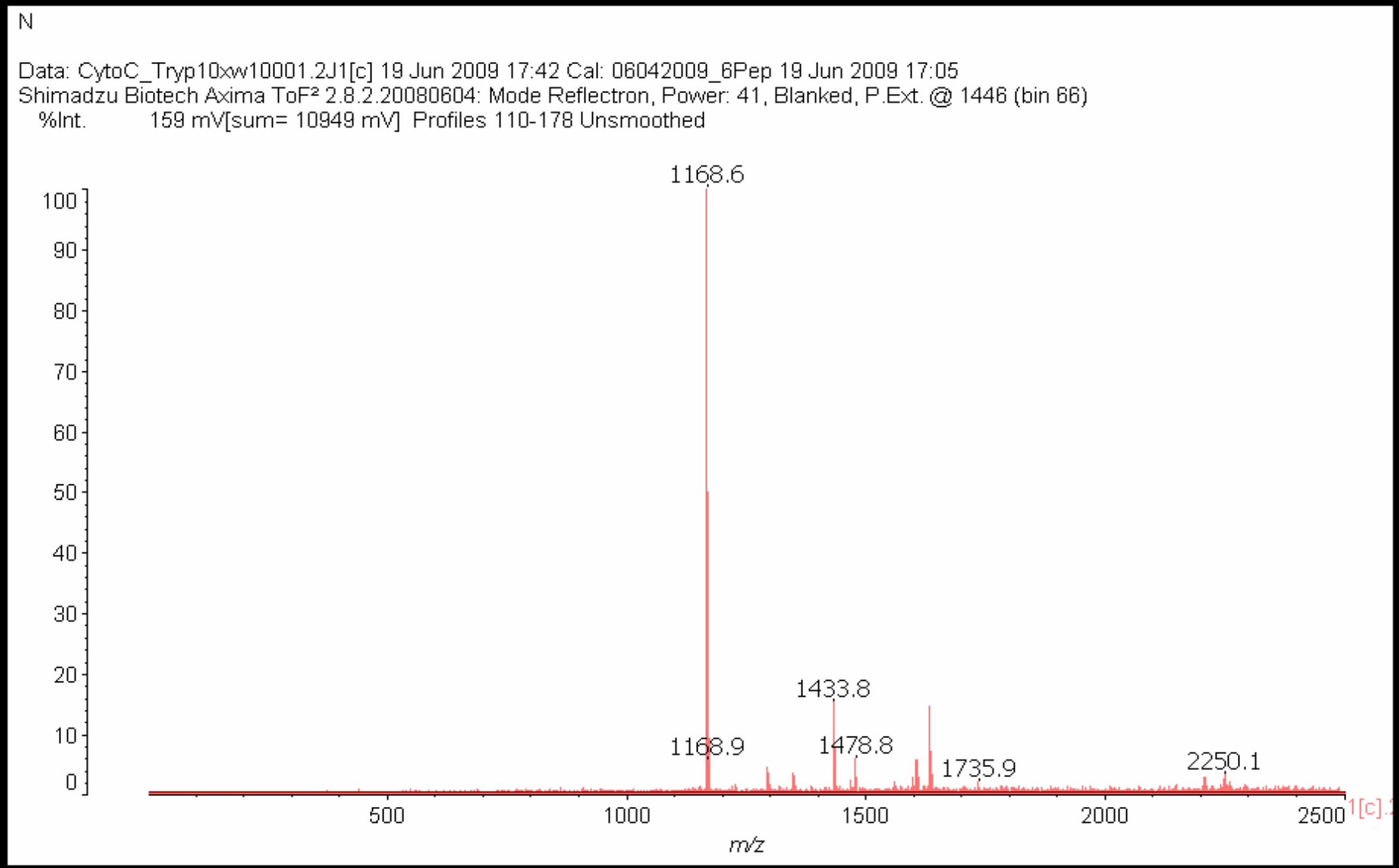
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2



Cytochrome-C – 10 Cycles

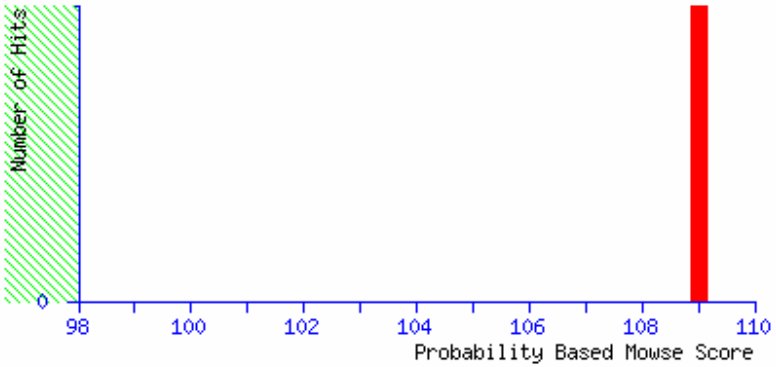
Mascot Score : 109

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : Cyto C Trypsin 10x w1
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 21:34:58 GMT
Top Score : 109 for CYC_HORSE, Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 5

Re-Search All | Search Unmatched

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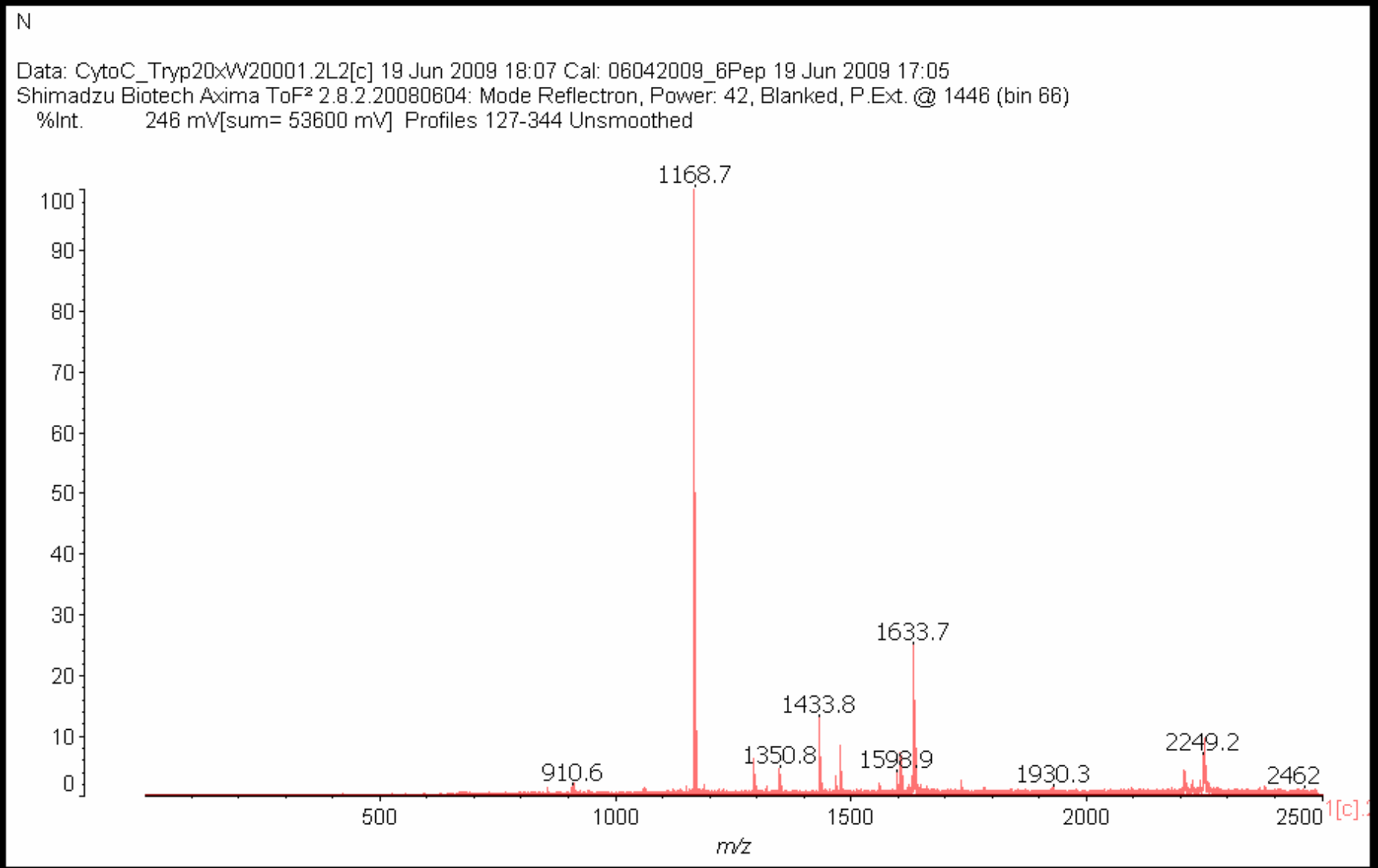
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2



Cytochrome-C – 20 Cycles

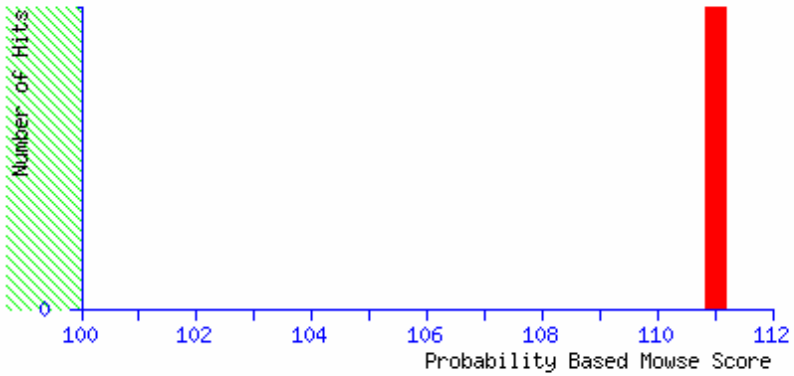
Mascot Score : 111

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : Cyto C Trypsin 20xW2
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 22:01:13 GMT
Top Score : 111 for CYC_HORSE, Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).



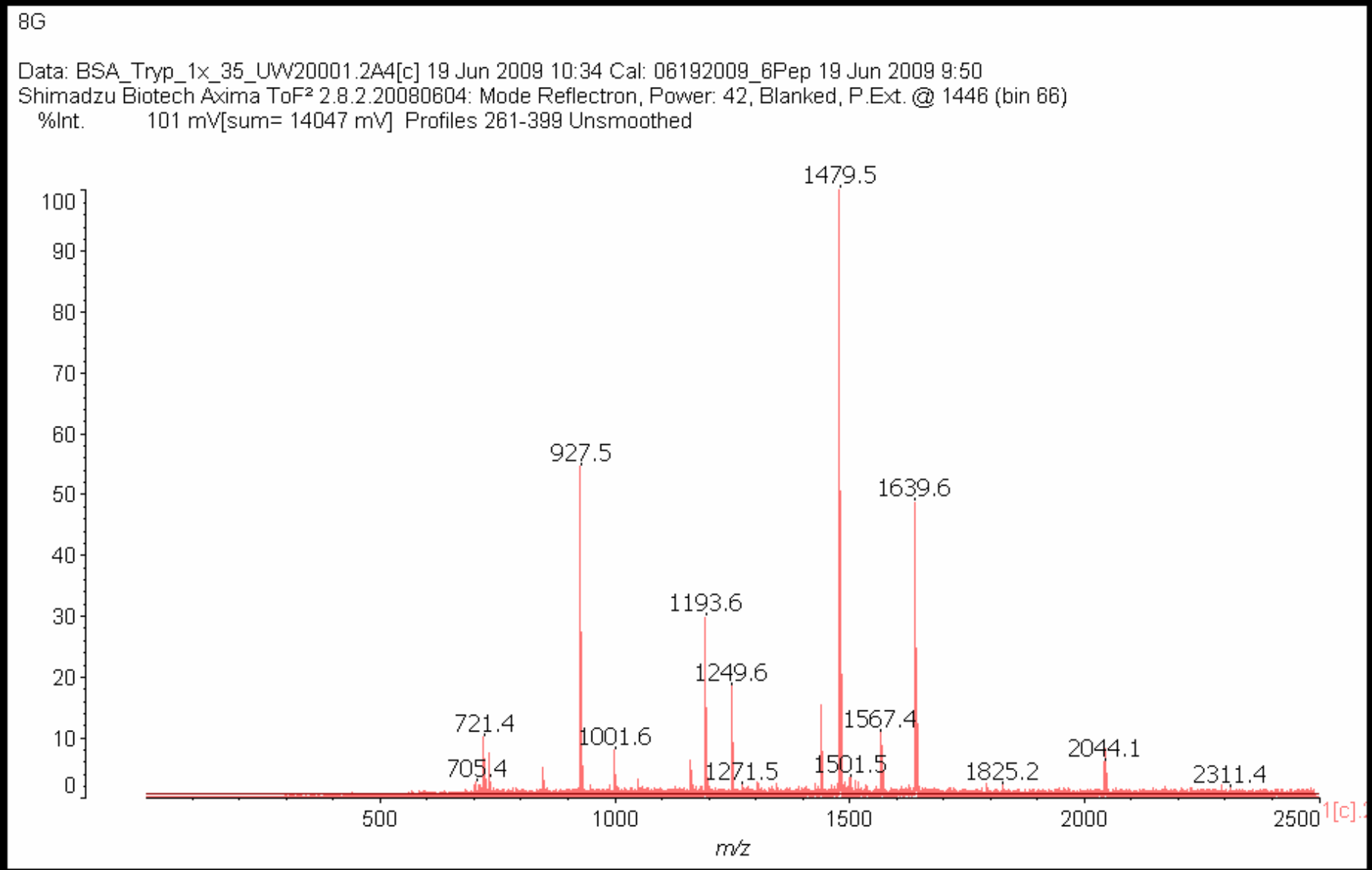
Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits 5

Re-Search All | Search Unmatched

1. CYC_HORSE Mass: 11825 Score: 111 Expect: 3.7e-06 Queries matched: 11
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2



BSA – 1 Cycle

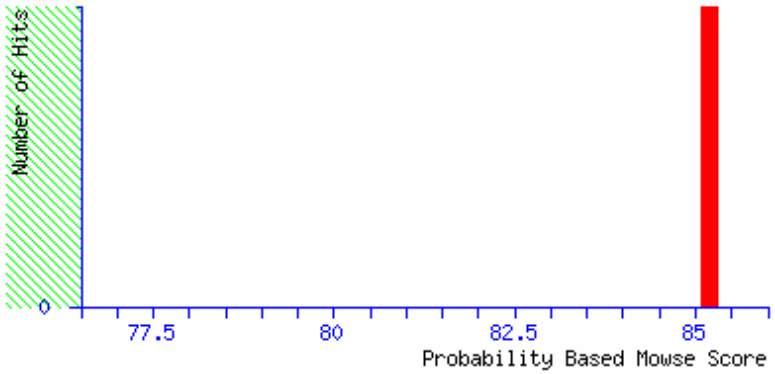
Mascot Score : 85

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : BSA Trypsin 10 cycles2MSMS
Database : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp : 19 Jun 2009 at 14:28:34 GMT
Top Score : 85 for ALBU_BOVIN, Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).

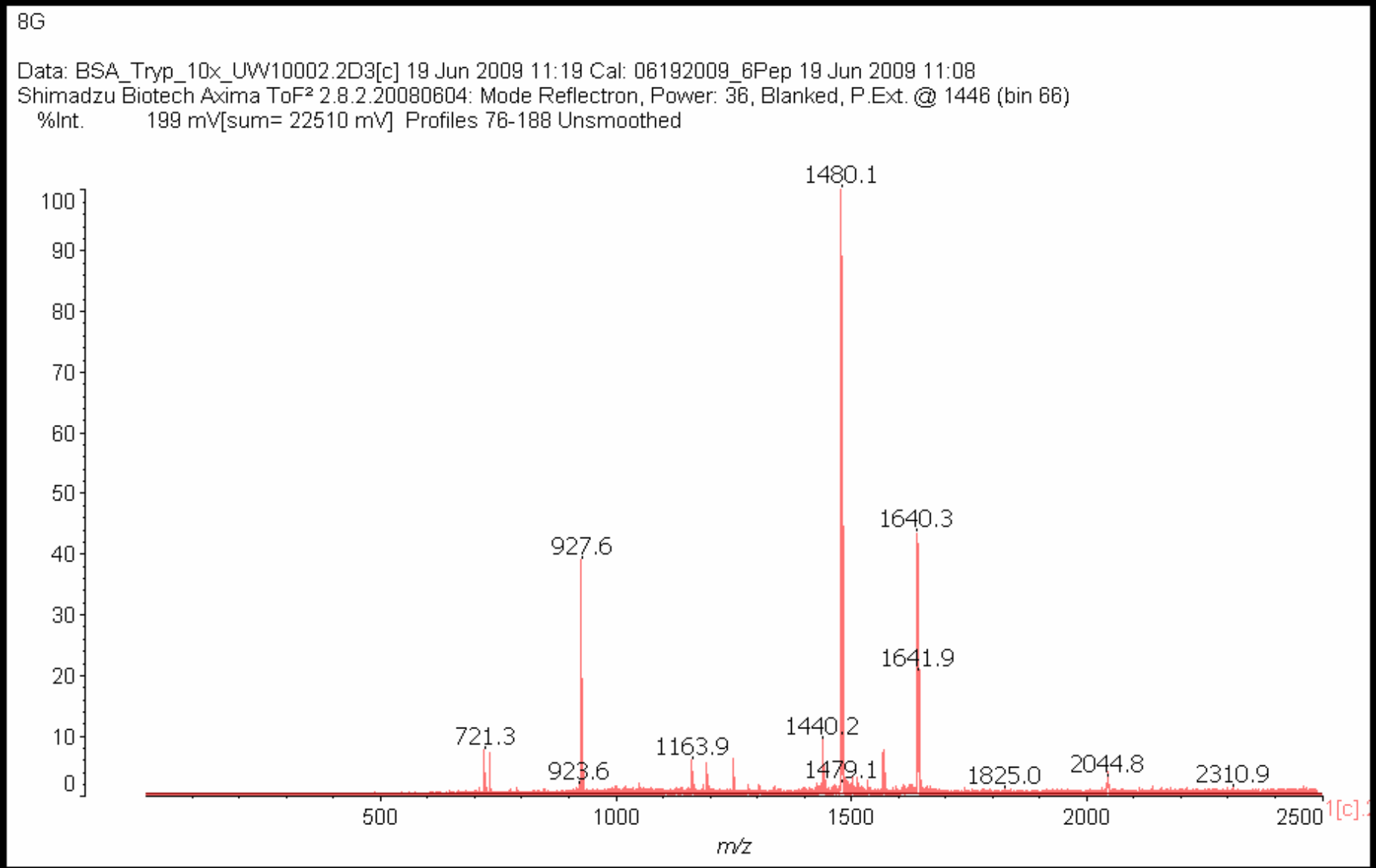


Concise Protein Summary Report

Format As: Concise Protein Summary — Help
Significance threshold p< 0.05 — Max. number of hits 5

Re-Search All | Search Unmatched

1. ALBU_BOVIN Mass: 69248 Score: 85 Expect: 0.0014 Queries matched: 14
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4



BSA – 10 Cycles

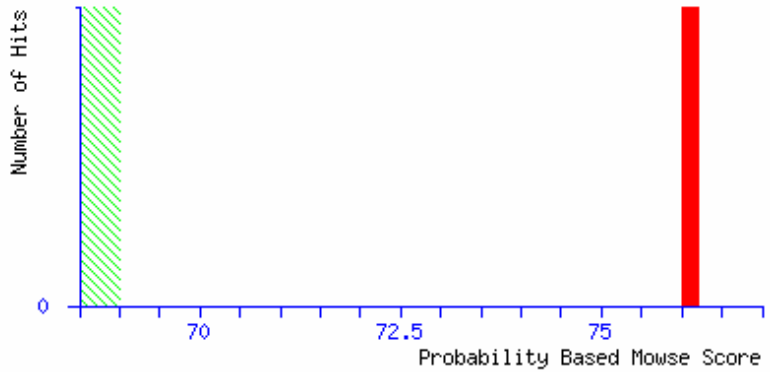
Mascot Score : 76

{MATRIX SCIENCE} Mascot Search Results

```
User            : Administrator
Email           :  jennifer.oprihory@nyumc.org
Search title    : BSA Trypsin 10 cycles2MSMS
Database        : SwissProt 57.3 (468851 sequences; 166149756 residues)
Timestamp       : 19 Jun 2009 at 15:12:38 GMT
Top Score       : 76 for ALBU_BOVIN, Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 69 are significant (p<0.05).

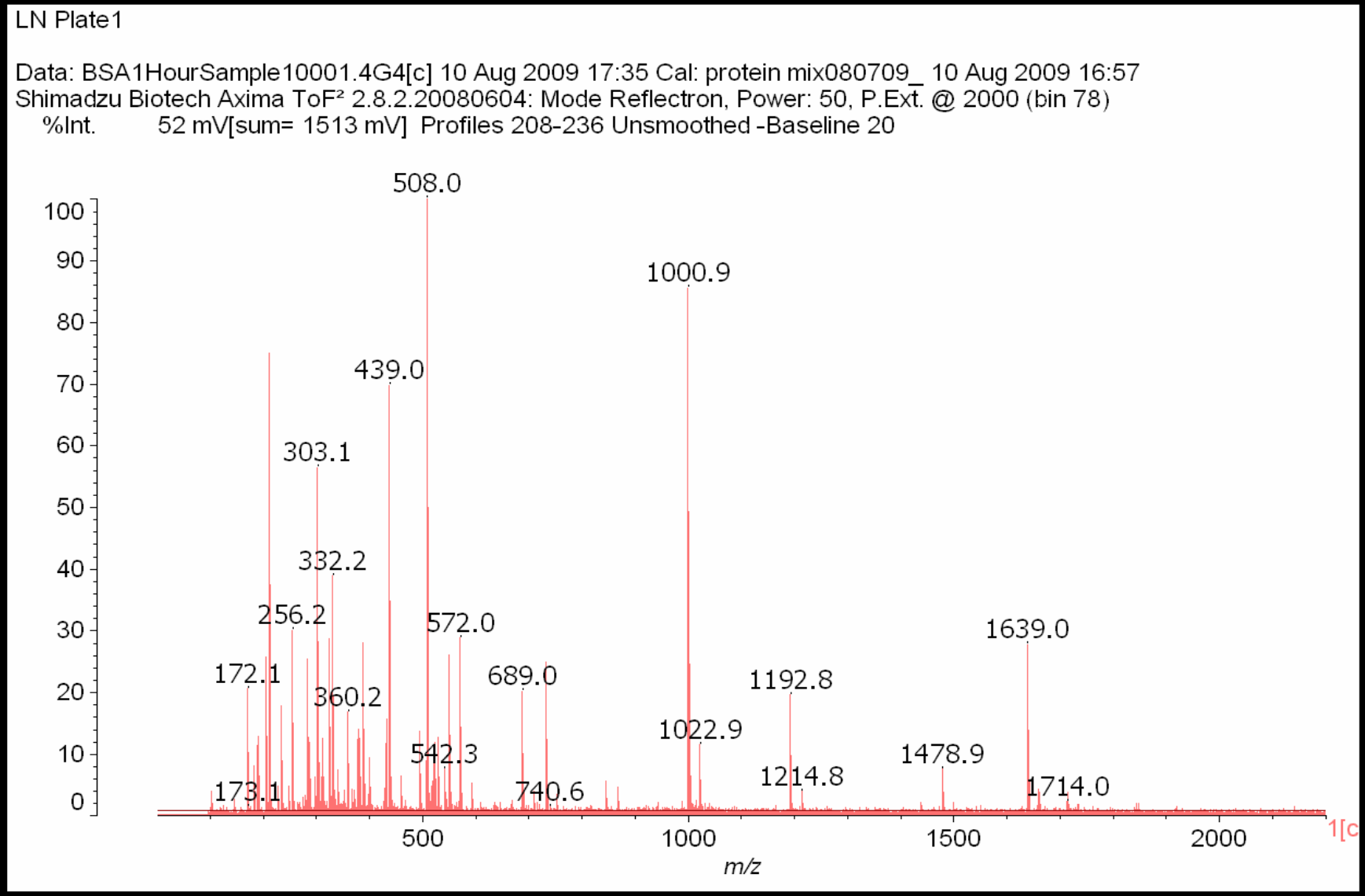


Concise Protein Summary Report

Format As	Concise Protein Summary	Help
	Significance threshold p< 0.05	Max. number of hits 5

Re-Search All | Search Unmatched

1. ALBU_BOVIN **Mass:** 69248 **Score:** 76 **Expect:** 0.012 **Queries matched:** 13
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4



1 Hour Digests

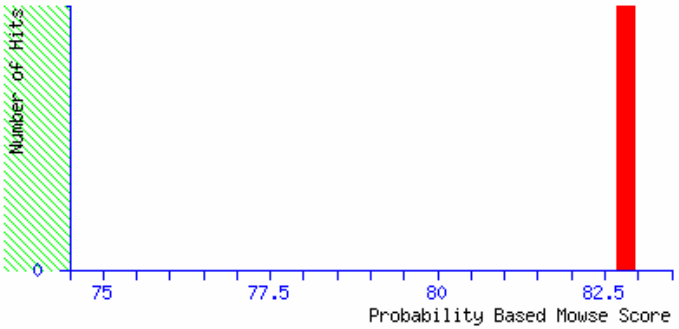
MASCOT Score: 83

Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : BSA2Jenn
Database : NCBInr 20090806 (9363125 sequences; 3207829265 residues)
Taxonomy : Mammalia (mammals) (728013 sequences)
Timestamp : 10 Aug 2009 at 21:43:39 GMT
Top Score : 83 for gi|76445989, serum albumin [Bos indicus]

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 71 are significant (p<0.05).

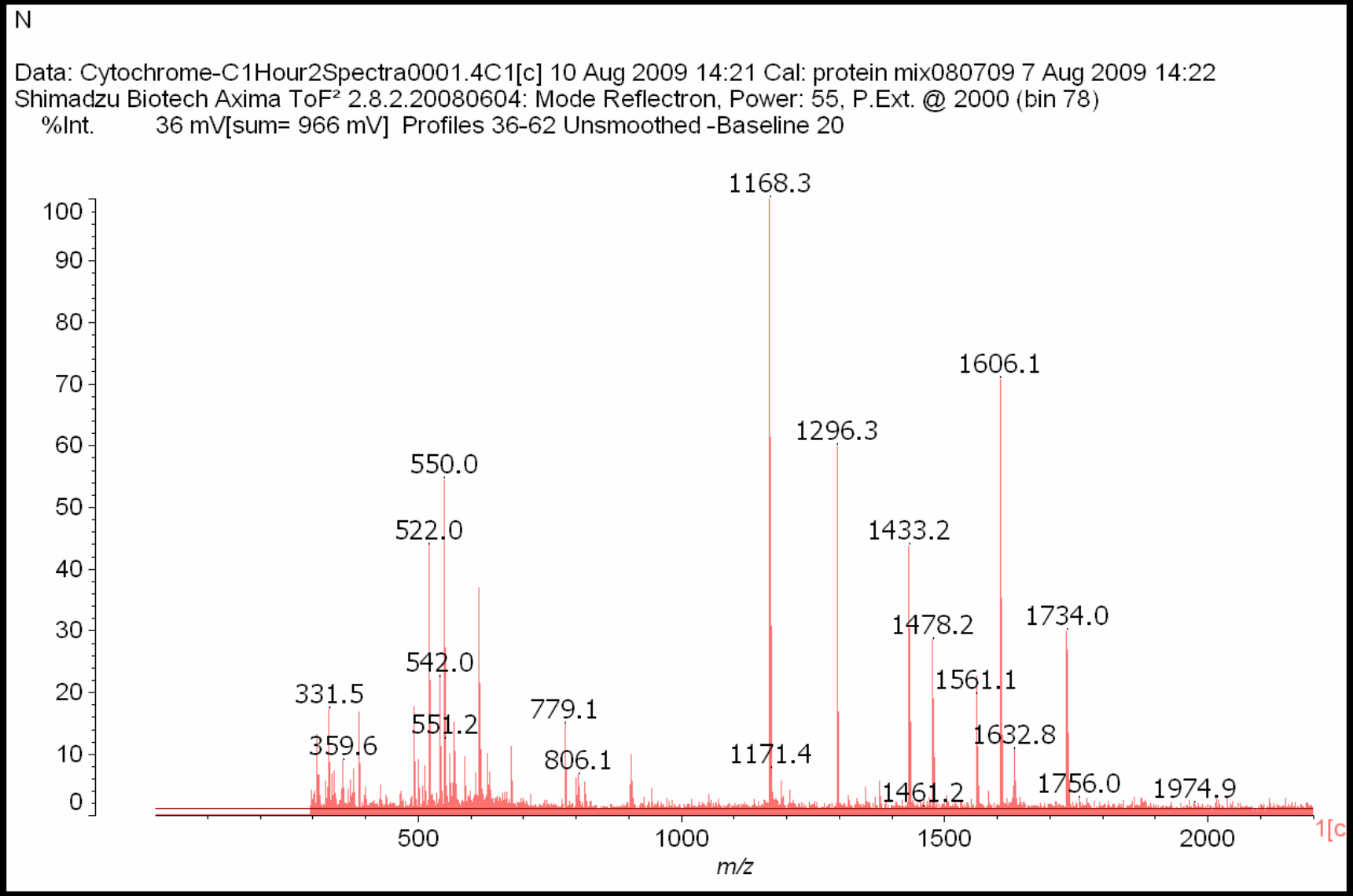


Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|76445989 **Mass:** 53890 **Score:** 83 **Expect:** 0.0038 **Queries matched:** 10
serum albumin [Bos indicus]
gi|30794280 **Mass:** 69278 **Score:** 68 **Expect:** 0.11 **Queries matched:** 10
albumin [Bos taurus]



1 Hour Digests

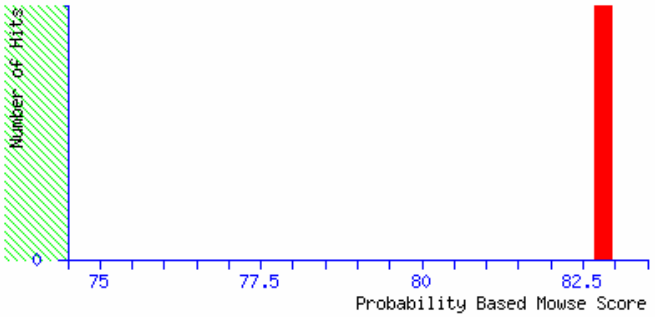
MASCOT Score: 83

MATRIX SCIENCE Mascot Search Results

User : Administrator
Email : jennifer.oprihory@nyumc.org
Search title : BSA2Jenn
Database : NCBInr 20090806 (9363125 sequences; 3207829265 residues)
Taxonomy : Mammalia (mammals) (728013 sequences)
Timestamp : 10 Aug 2009 at 21:43:39 GMT
Top Score : 83 for gi|76445989, serum albumin [Bos indicus]

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 71 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 20

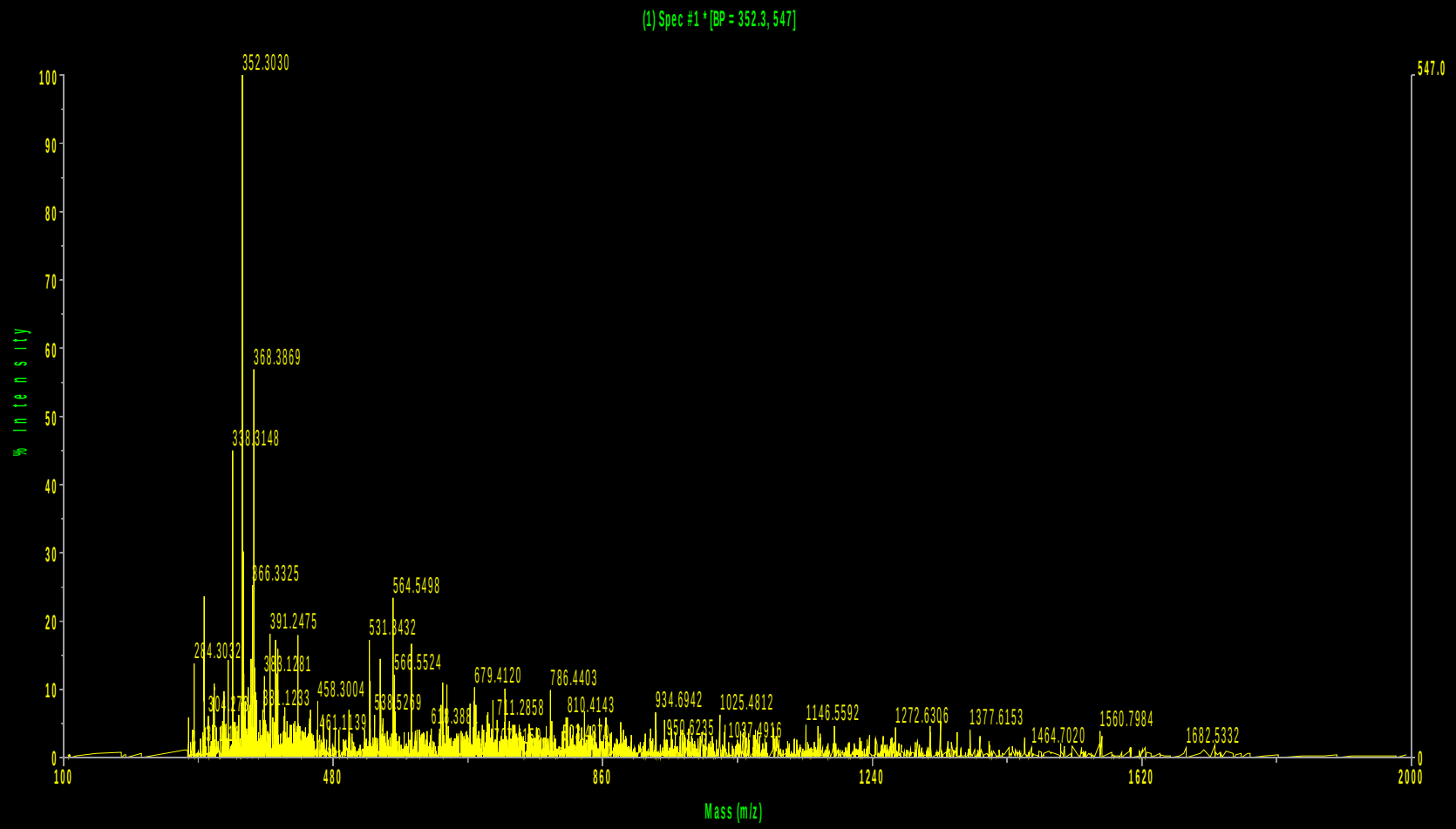
Re-Search All | Search Unmatched

1. gi|76445989 **Mass:** 53890 **Score:** 83 **Expect:** 0.0038 **Queries matched:** 10
serum albumin [Bos indicus]
gi|30794280 **Mass:** 69278 **Score:** 68 **Expect:** 0.11 **Queries matched:** 10
albumin [Bos taurus]

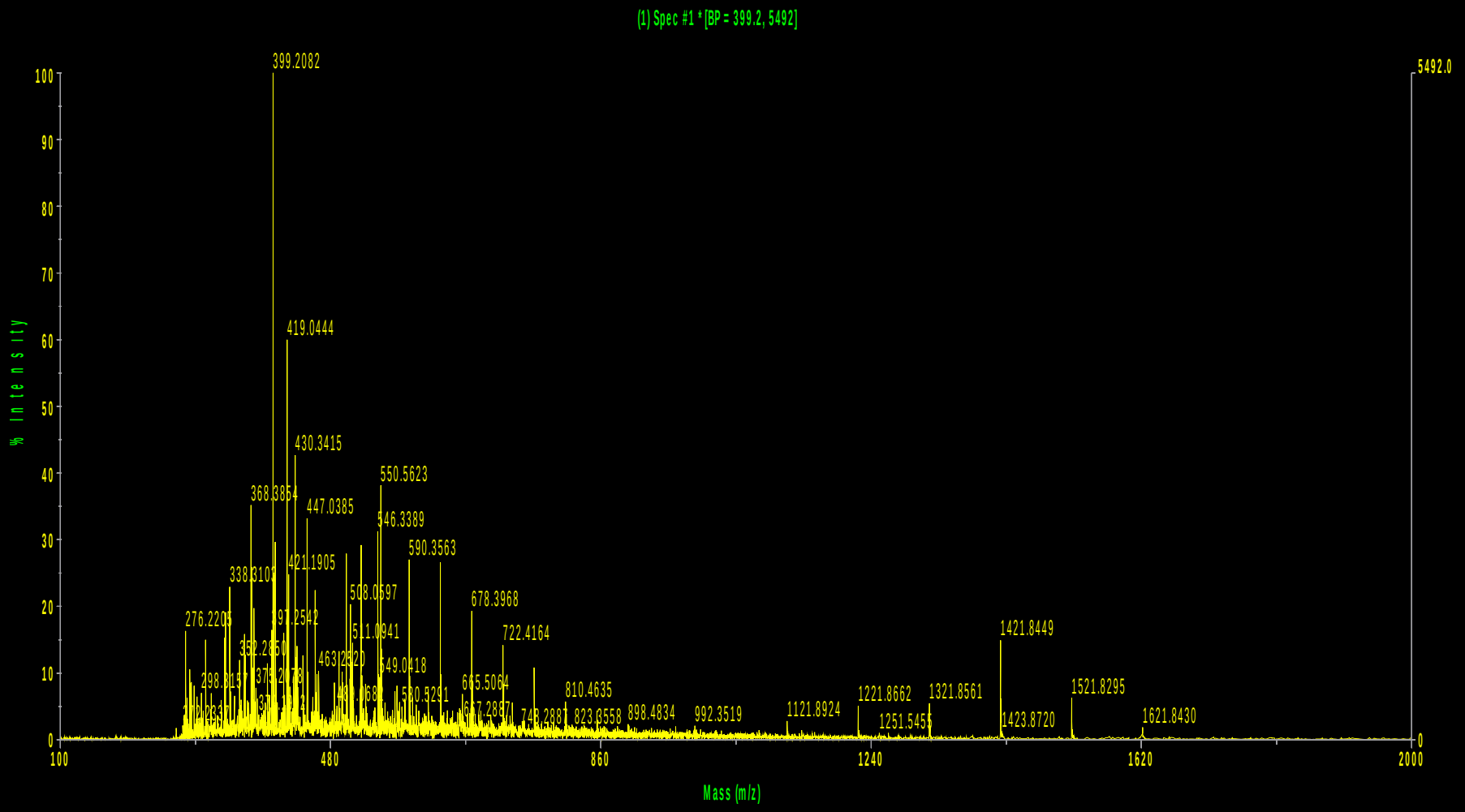
Results (LCMS)

- Successful identification of proteins from mouse tongue and heart
  - Tongue
    - Post 1 Gy, hemoglobin subunit α was identified; post 2 Gy, fatty acid-binding protein adipocyte was identified and post 3 Gy, hemoglobin α chains was identified. Albumin was identified in post 2 and 3 Gy.
  - Heart
    - Actin, alpha cardiac muscle 1 OS=Mus musculus was identified.

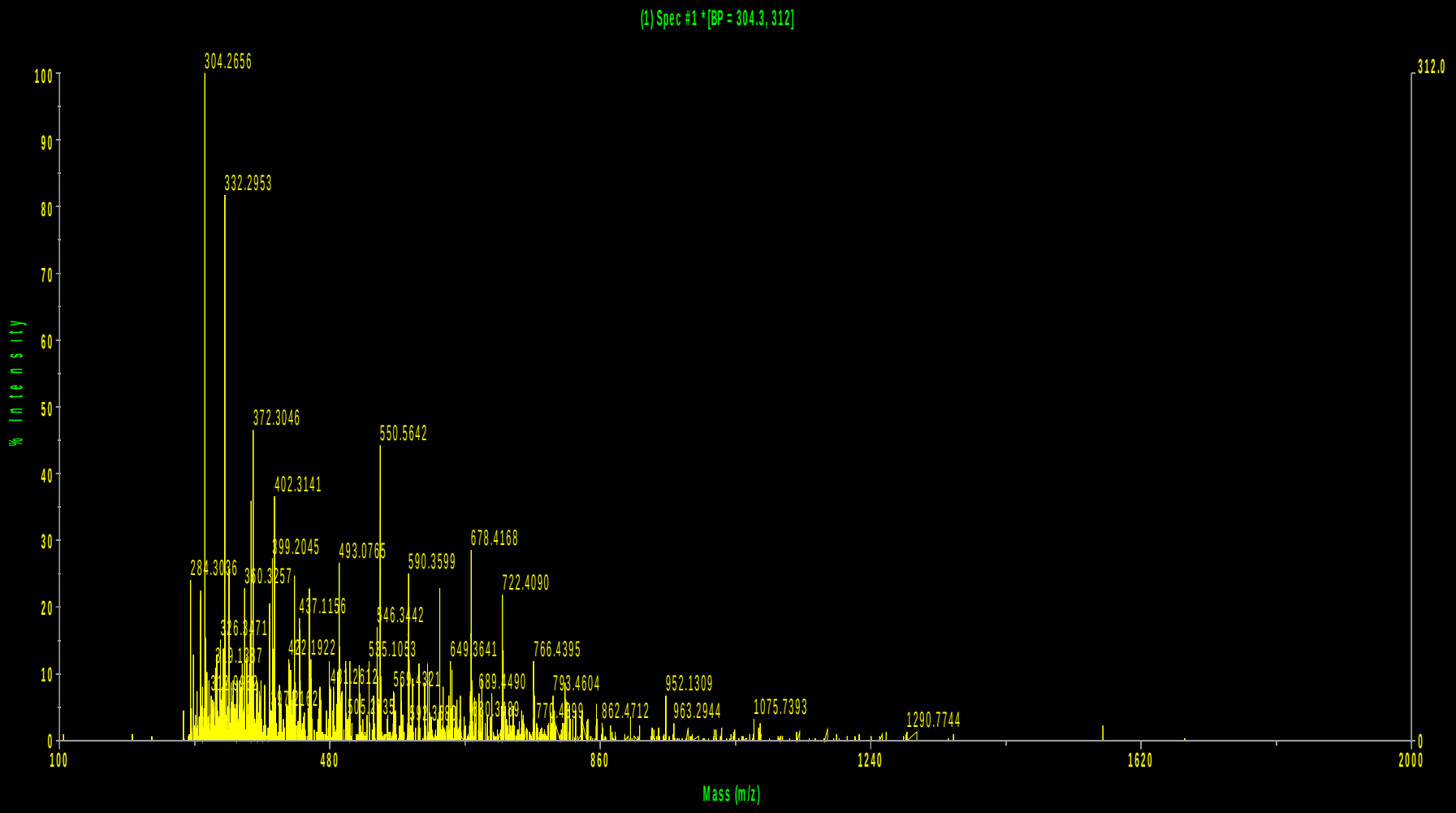
LCMS spectrum tongue normal



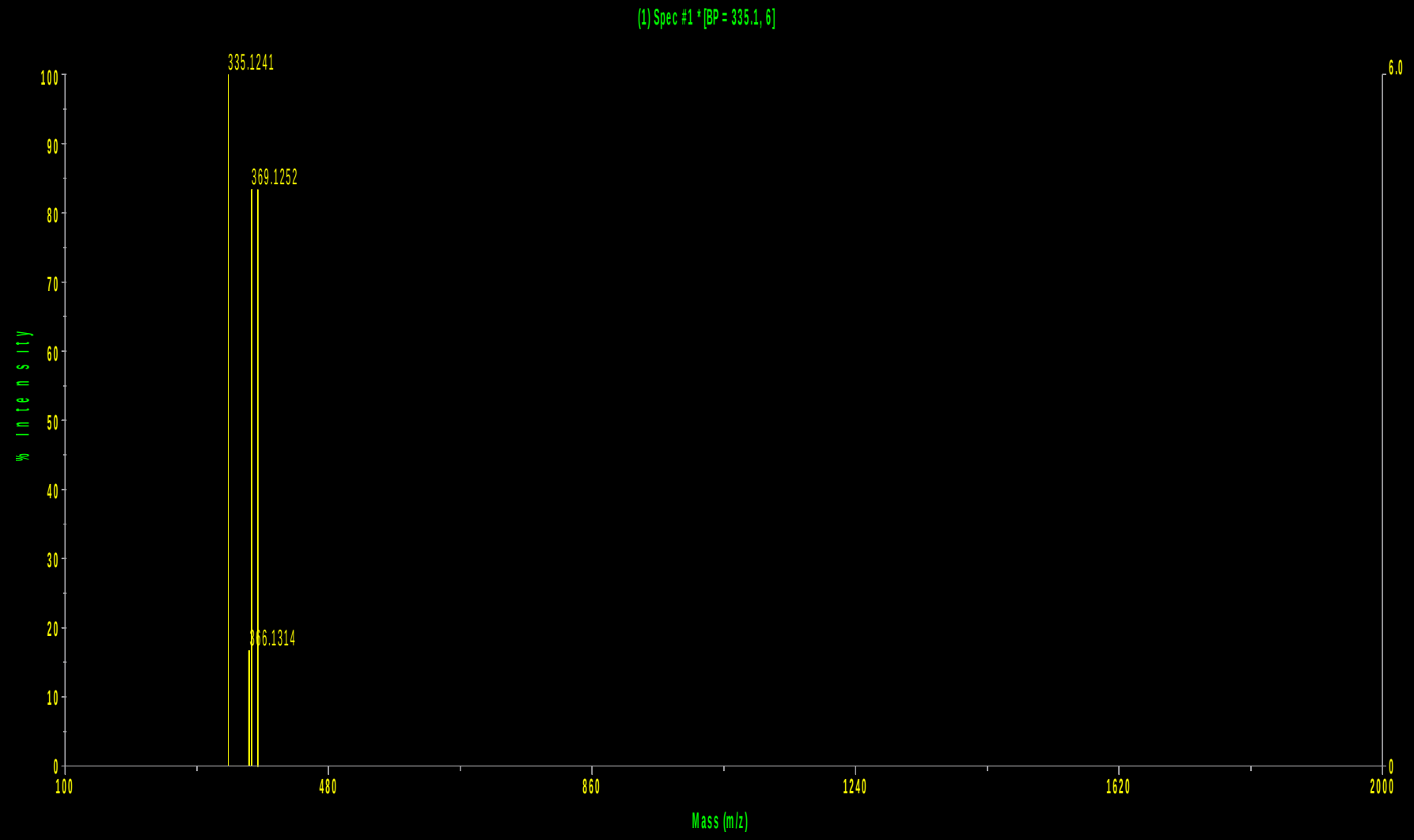
LCMS spectrum tongue post 1 Gy



LCMS spectrum tongue post 2 Gy



LCMS spectrum tongue 3 Gy



Discussion

BSA and Cytochrome-C were used to demonstrate proof of principle that trypsin digest of these proteins could be achieved in minutes at ambient temperature.

Conclusion

Trypsin digestion with PCT cycling at short intervals of high and low pressure produces rapid trypsin digestion comparable to longer intervals at ambient pressure and elevated temperature.