Proteomics


2. Conventional method: 2-dimensional electrophoresis (2-DE), mass spectrometry of trypsin-digested protein spots, database searching for identity or homology (for a list of pertinent references see Amersham Biosciences bulletin 80-6429-60, pp 89-93 (1998)).

3. Newer techniques include multidimensional chromatography of total protein digest followed by tandem mass spectrometry


   - chromatofocusing followed by reversed phase chromatography and tandem mass spectrometry
4. **Isotope-coded labeling of proteins, followed by trypsin digestion and analysis by tandem mass spectrometry**

**ICAT** – isotope coded affinity tag, labels sulfhydryl groups, e.g. thiol group of cysteine

**iTRAQ** - proprietary reagent, labels primary amine groups, e.g. epsilon amino group of lysine


---

Fig. 2 Gygi et al, Nature Biotechnology 17, 994-999 (1999)
Mass tag labeling of digested proteins

TMT (tandem mass tags)-labels lysine and peptide N-termini-Fisher Scientific
Quantification is from reporter mass
5. DIGE – 2-D Fluorescence Difference Gel Electrophoresis
- uses three fluorescent dyes, Cy2, Cy3 and/or Cy5
- dyes bind to the lysine residues

- standard labeled with Cy2
- protein extract 1 labeled with Cy3
- protein extract 2 labeled with Cy5

- mix the labeled extracts and run on a single gel
- image the gel with the Typhoon with excitation at three wavelengths
- images are merged and differences determined using DeCyder Differential Analysis Software
Advances in two areas

1. Electrophoresis
   - 2-dimensional electrophoresis, separation by pI in the first dimension, followed by molecular size in the second dimension; first used by O’Farrel in 1975, using metabolically labeled *E. coli* cells (O’Farrel, J Biol Chem, 250, 4007-4021)
   - development of immobilized pH gradients, increases resolution; amphoteric molecules, Immobilines, covalently co-polymerized with acrylamide (Gőrg, et al., Electrophoresis 21, 1037-1053 (2000))

2. Mass spectrometry
   - Ionization of large biomolecules, MS/MS
     - MALDI-TOF (Matrix assisted laser desorption/ionization time of flight mass spectrometry) (Karas and Hillenkamp, Anal Chem 60, 2299-2301 (1988))
     - ESI (Electrospray ionization) mass spectrometry (Fenn et al., Science 246, 65-71 (1989))
MALDI-TOF

Ashcroft, Univ. Leeds, An Introduction to Mass spectrometry (http://www.astbury.leeds.ac.uk/Facil/MStut/mstutorial.htm)
ESI

Ashcroft, Univ. Leeds, An Introduction to Mass spectrometry (http://www.astbury.leeds.ac.uk/Facil/MStut/mstutorial.htm)
Sample preparation-Basic protocol

1. Cell breakage—sonication, grinding, lysis
2. Protein solubilization and stabilization—buffer, protease inhibitors
3. Protein assay—Lowry protein assay
4. 1D SDS-PAGE
5. Isoelectric focusing in the first dimension—according to charge
6. SDS-PAGE in the second dimension—according to molecular size
7. Staining of gel—Sypro Ruby, Coomassie Blue
8. Spot quantification—Samespots, ImageQuant
9. Digestion of two protein spots and crude sample
10. Analysis of trypsin fragments by LC-MS/MS
11. Database search and analysis using Proteome Discoverer

Note: Always wear gloves, wash all glassware, tubes, etc., with methanol
Choice of sample- \textit{E. coli} or soybean meal

\textbf{Soybean meal procedure-water soluble proteins}

1. Weigh 1.0 g soy meal in vial
2. Add 10 mL of distilled water, mix with stirring rod
3. Stir for 10 min on magnetic stirrer
4. Adjust pH to 8.6 with 0.1 N NaOH, \sim 7 drops
5. Stir for 10 min and check pH, adjust to pH 8.6 if necessary.
6. Incubate overnight with stirring at 5\(^\circ\)C

\textit{Note: Use an overnight extracted sample from here on}

7. Transfer 1 mL into a microcentrifuge tube, and centrifuge at 14,000 \textit{x} g for 20 min.

8. Determine protein content using the Lowry procedure \cite{lowry1951} and from 260/280 ratio

\begin{equation*}
\text{Protein (mg/ml)} = [1.55A_{280} - 0.76A_{260}] \times \text{dilution factor}
\end{equation*}
E. coli-water soluble proteins

1. Add 1 mL of water to 1 g of E. coli DH5α cells.
2. Pipet ~2.0 mL of the suspension into a 15 mL centrifuge tube
   Note: Instructor will prepare suspension
3. Place tube in ice bucket and sonicate for 6 min at 60% power
   Note: the probe must be submerged at least ¼ inch beneath the surface of the suspension)
4. Transfer suspension to a microfuge tube and centrifuge for 20 min 14,000 x g
5. Determine protein content using the Lowry procedure

Lowry et al., J Biol Chem, 193, 265-275 (1951) and from 260/280 ratio Protein (mg/ml) = \[1.55A_{280}-0.76A_{260}\] \times dilution factor
Protein assay

1. We will use the Lowry procedure-less interference from nucleic acids, polysaccharides compared with the BioRad dye-binding assay for crude extracts.

2. Set up standard curve, using bovine serum albumin (1.0 mg/ml) as a standard; include blank (no protein) and use five sample volumes of the unknown.

3. Remember to take into account the initial dilution factor of your unknown.

Note: we are using single tubes in class; should use standards in triplicate.
Notes

1. For solubilization methods for various tissues check: Molloy, Anal Biochem, 280,1-10 (2000)

2. When samples are prepared in ‘lysis’ buffer or ‘rehydration buffer’, use: the Amersham Protein Quantification procedure 2-D Quant kit, cat. No. 80-6483-56 (or similar procedures from other manufacturers), to quantify protein in the presence of urea and detergents.

3. 2-D Sample Preparation on p. 152

4. 2-D Electrophoresis Principles and Methods, published by GE Healthcare, troubleshooting on p. 129
1D SDS-PAGE

1. To perform a successful 2-D gel, we recommend that a 1D SDS-PAGE gel be run first:
   - to ascertain that indeed enough protein is present
   - that the proteins have not degraded

2. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under reducing conditions:
   - protein is denatured with SDS: \([\text{CH}_3-(\text{CH}_2)_{11}-\text{CH}_2-O-\text{SO}_3^-] \text{Na}^+\)
   - the alkyl chain of SDS binds to the nonpolar amino acids and charged sulfate ion extends into the solvent, thereby unraveling the protein and conferring a uniform negative charge
   - protein disulfide bonds are reduced with mercaptoethanol \(\text{SH}-\text{CH}_2-\text{CH}_2-\text{OH}\) or dithiothreitol \(\text{SH}-\text{CH}_2-\text{CHOH-CHOH-CH}_2-\text{SH}\)
   - all proteins migrate through the gel towards the anode
   - protein migration through the gel is inversely proportional to its molecular size
Preparation of 12.5% gel

1. Assemble cassette as shown in the manual. Make sure it is leak proof by adding a small volume of water, then discard water and dry the cassette.

2. Mark the cassette plate with comb inserted at a level of 5 mm below the teeth to indicate level of running gel.

3. Prepare 12.5% acrylamide running gel

   *Note: unpolymerized acrylamide is a neurotoxin, wear gloves; allow left-over monomer to polymerize before disposal.*

   - Add all reagents a-e, swirl to mix or invert gently-prevent aeration
   - Add the ammonium persulfate
   - Immediately pipet acrylamide solution into the cassette, layer with water-saturated isobutanol to provide a level surface, and to exclude air.

   *Allow the isobutanol to run carefully down the edge of the cassette; avoid ‘bombing’ of the surface*

   - Allow to polymerize for 30 min
4. Prepare stacking gel, and pipet into cassette with the comb inserted at an angle to allow trapped air bubbles to escape; level comb making sure there is 5 mm below the teeth. Allow to polymerize.

5. In the meantime, calculate sample volume to be used to obtain 200 µg of protein in a volume of 100 µl final:

\[
200 \, \mu g/\text{sample conc (µg/µL)} = \text{vol sample (µl)}
\]

Dilute sample to 100 µL with water

6. Add equal volume (100 µL) of 2X sample buffer, denature at 100°C for 4 min.

7. Concentration of sample is now 1 µg/µL, need to load -5, 10, 20 and 30 µg per well in duplicate, therefore pipet 5, 10, 20, and 30 µL per well, in duplicate -lane 1, BSA sample -lane 2, molecular weight markers

Note: insert gel loading tip near well bottom underneath the buffer, then deliver sample slowly, avoid introducing air bubbles

8. Run for 45 min at 180 constant voltage
First dimension-IEF

1. Isoelectric focusing, IEF, will be used to separate proteins according to charge-
   - each protein has its own intrinsic charge due to its content of the acidic and basic amino acids, the \(-\text{COO}^-\) and \(-\text{NH}_3^+\) side chain functional groups

   - proteins will continue to migrate in the applied electric field until it reaches a zone of pH equal to its intrinsic pH or isoelectric point (pI), and the net charge of the protein will be zero

2. Procedure
   a. Calculate volume of sample to load, need 50 µg protein for a minigel

      \[
      50 \, \mu\text{g} \div \text{sample concentration (µg/µL)} = \text{Volume sample (µL) needed}
      \]

      Make up to 125 µL with Rehydration buffer (it contains the required amount of dithiothreitol)

   • Pipet sample-buffer solution between electrodes in the ceramic strip holder

   • Remove plastic backing from the IPG (Immobilized pH gel) strip, place gel-side down onto the sample, avoid trapping air bubbles, and with the (+) positive end towards the pointed end of the strip holder
d. Cover with 1 mL of cover fluid, snap lid in place

f. Place on IPGphor unit in the proper orientation, follow settings in the manual

g. Note the number on the strip holder, mark a 15-ml tube with name and strip holder number

h. After electrophoresis record voltage and volt/hours

i. the strips are stored in the tube at -20°C until used for the second dimension
542E-Introduction to proteomics

Lecture 3 & 4
Fall 2019
Lab period 3

1. Second dimension SDS-PAGE of 7 cm IEF strip
   - p. 20
   - 8,000 V
   - 59620 Vhrs
   - Soybean
   - 4000V
   - 43,819 Vhrs

2. 1\textsuperscript{st} dimension IEF using the 18-cm strip
2\textsuperscript{nd} dimension-7 cm strip/SDS-PAGE

1. Equilibration and reduction of disulfides with dithioerythritol using \textit{Equilibration buffer}, 15 min
2. Alkylation with iodoacetamide (omitted)
3. Remove tape from the bottom of the gel cassette, remove the comb and place in the clamp assembly
4. Rinse gel well with electrophoresis buffer
5. Place IEF strip in well allowing room for the MW application papers (place the anode(+) end of the strip all the way to the left on the gel).
6. Hold 1 piece of IEF sample application paper with the forceps, pipet 10 \textmu L MW marker onto the paper and place at the cathode (-) end.
7. Fill compartments with buffer
8. Run for 45-60 min at 180 constant volt
9. Remove gel, label staining box with your initials, fix gel in 10\% methanol, 7\% acetic acid, 30 min
10. Stain with Sypro Ruby overnight, rinse with water
Staining with Sypro Ruby

1. Staining with Sypro Ruby
   - sensitive ruthenium-based dye,
   - about 100-fold more sensitive than Coomassie Blue and equal to silver stain.
   - detects about 1 ng of protein
   - binds to lysine, arginine and histidine residues
   - it has a dynamic range of three orders of magnitude
   - absorption bands at 278 nm and 462 nm, and emission band at 610 nm

2. It is compatible with mass spec analysis of proteins and with protein sequencing
1. Calculate the amount of sample to load on the IEF strip. You will need 500 µg protein

\[ \text{Sample volume (µL)} = \frac{500 \, \text{µg}}{\text{Sample concentration (µg/µL)}} \]

Dilute sample with Rehydration buffer to 350 µL

Rehydration buffer contains the required amount of dithiothreitol

2. Pipet sample into strip holder between electrodes, remove plastic backing from the IEF strip, place gel-side down on sample, add cover fluid, snap lid in place and place strip holder on the IPGPhor unit.

3. Run the IEF program as shown in the manual

4. Record the strip holder number and your name on the 50-mL tube

5. Record voltage and volt-hours at the end of the run
1. Please sign up for a time for using the Typhoon scanner in the Protein Facility for October 10

2. There will be no lecture that day, read through section 4E before coming to the Protein Facility

3. On October 10, after you are finished scanning the gel with the Typhoon, place your gel in Coomassie stain for overnight staining
7 cm 1D gel compared to 7 cm 2D gel (stained with Sypro Ruby)
18 cm IEF
Volts: 8,000
Volt hours: 110,410
2\textsuperscript{nd} dimension 18 cm IEF/SDS-PAGE

1. For the large gels (23 x 19 cm) a Hoeffer DALT electrophoresis system is used; able to accommodate 10 gels.

2. Equilibrate strip with 10 mL of equilibration buffer containing 10 mg/mL of dithiothreitol for 15 min on a rocker.

3. Drain strip, rinse the well with electrophoresis buffer and center the strip in the well, leaving space for the MW application papers.

4. Hold 4 pieces of IEF sample application paper together with the forceps, pipet 40 μL MW marker onto the papers and place at the cathode (-) end.

1. Write your name with pencil on a piece of filter paper and place near anode (+).
5. Seal strip and filter paper in place with 0.5% agarose containing bromophenol blue

6. Connect electrodes and run for 15 min at 80V (to allow the protein to enter the gel), then overnight at 100 Volt

7. The gel will be stained with 0.1% Coomassie Blue/40% methanol/10% acetic acid/water) for 30 min

8. The gel is destained with 40% methanol/10% acetic acid/water for 1 to 3 hr.

Note: Steps 7 and 8 will be done by the facility
1. Gel scanning and documentation is done in the Protein Facility.

2. Please sign up for a specific time.

3. Lecture/tour on tandem mass spectrometry in the Proteomics Facility

4. There will be no lecture on Thursday, October 13, each student will go to the Protein Facility at their assigned time.
542E-Introduction to Proteomics

Lecture 6

Fall 2016
Methods for spot analysis

• Two methods will be used for 2-D gel spot analysis
  – Nonlinear Dynamics Progenesis SameSpots
  – ImageQuantTL from Amersham Biosciences
Spot analysis and differential spot analysis using SameSpots

1. Identify coordinates of spots on a 2D gel, e.g. pI and/or MW

2. Differential analysis of proteins comparing treatment to control
   – Up- and down-regulation of protein expression
   – Identification of disease markers, etc.

3. Detailed instructions are provided in Section 6E
Image format

- Any 16-bit grayscale tiff
- Image can be from
  - Typhoon Scanner
  - flatbed scanner with transparency adaptor
  - scanned photograph
  - digital camera
There are 10 basic steps for analysis of your gels using Progenesis SameSpots:

1. Image QC - used to determine if there are any problems with the gel image itself and can be used for cropping and manipulation of the gel image.

2. Reference Image Selection - the reference image will be the gel to align to when aligning each of the gels in the experiment.

3. Mask of Disinterest - used to choose areas of the gel to ignore during analysis.

4. Alignment - aligns the gels in the experiment to the reference image. Probably the most important step for a good analysis of a set of gels.

5. Prefiltering - used to remove spots that you do not want analyzed (small spots, spots at the edges of the gel, gel anomalies).

6. Experiment design setup - used to group the gels into groups you wish to compare (e.g. control versus treatment).

7. View Results - used to review the spot analysis and edit any spots if necessary. Also used to create tags to help with data mining.

8. Progenesis Stats - used to provide multivariate statistical analysis on tagged and selected spots.

9. Spot Picking - used to create a pick list for a spot picking robot.

10. Report - used to create an HTML report of the results.
1. Image QC
2. Reference Image Selection

Choosing a good reference image will help during the alignment stage. Ideally, the reference image should show a clear and representative spot pattern, and have a minimum of distortion.

For experiments such as time series or dose response, choosing the middle-point frames to give the best results.
3. Mask of Disinterest
4. Alignment
5. Prefiltering
5. Normalization
5. Prefiltering complete
6. Experiment Design Setup

Which experiment design type do you want to use for this experiment?

- Between-subject Design
  - Do samples from a given subject appear in only one condition? Then use the between-subject design.
  
  To set up this design, you simply group the images according to the condition (factor level) of the samples. The analysis calculation assumes that the conditions are independent and thereby gives a statistical test of whether the means of the conditions are all equal.

- Within-subject Design
  - Have you taken samples from a given subject under different conditions? Then use the within-subject design.
  
  Notice you must have a sample from every subject for every condition to use a within-subject design.

  For example, you would choose this type of design for a time series experiment where every subject has been sampled at each time point.

  To set up this design, you tell the software not only which condition (factor level) each image belongs to, but also which subject it came from. The software will then perform a repeated measures analysis.

  A standard analysis is not appropriate because the data violates the ANOVA assumption of independence. With a repeated measures ANOVA, individual differences can be interrelated or reduced as a source of between-condition differences (which helps to create a more powerful test).

  The within-subject design can be thought of as an extension of the paired-samples t-test to include comparisons between more than two repeated measures.
6. Experiment Design Setup
7. View Results
7. View Results-create tags
8. Progenesis Stats

**Question:**
Are there any outliers in my data?
Does my data cluster according to my experimental conditions?

**What's this?**
Principal Components Analysis produces a simplified, graphical representation of your multidimensional data.

![Progenesis Stats interface](image)

- Are there any outliers in my data?
- Does my data cluster according to my experimental conditions?
- Group my spots according to how similar their expression profiles are.
- How many replicates should I run?
- What is the power of my experiment?
- Where are the features on my gel image?
8. Progenesis Stats - power analysis
8. Progenesis Stats-principal components analysis
8. Progenesis Stats-dendogram
8. Progenesis Stats-features of the gel image
9. Spot Picking
9. Add Molecular weight markers
9. Spot picking
10. Report
10. Report

Spot present in student gels, absent in the standard image

Spot downregulated in the student gel compared to the standard gel
ImageQuantTL (GE Healthcare)

• Area quantitation--calculates the area under the curve that is created by a plot of pixel location versus pixel intensity along a linear object.

• Volume quantitation--calculates the volume under the surface created by a 3-D plot of pixel locations and pixel intensities.

• See detailed instructions beginning on p.76 Section 6-E of the manual.
Select gel and spot areas
Select and subtract background
Determine the protein amount of the spot

- Protein amount of spot = \( \frac{\text{volume of the spot}}{\text{volume of total protein}} \) × loading volume × protein concentration
542E-Introduction to Proteomics

Lecture 7
Fall 2019
Spot cutting and digestion

1. The advantage of 2D gel electrophoresis is that the protein complement of the cell is completely separated.

2. Single proteins will be available for analysis.

3. The 2D gel provides information on MW and pI, which helps in identifying the correct protein from the database search.

4. Trypsin catalyzes the hydrolysis of the peptide bond at the carboxyl-side of the amino acids lysine (K) and arginine (R):

\[
\text{H}_3\text{N}^+\text{-Gly-Gly-Ser-Lys-Lys-His-Pro-Ala-Arg-Arg-Arg-Thr-Gly-Ala-Leu-Val-COO}^-\n\]

\[\text{Peptide bond}\]
5. Spot cutting - remember to wear gloves at all times

- Clean a glass plate with methanol and deionized water
- Place a piece of filter paper on the glass plate
- Place gel on the filter paper
- Aspirate ~ 20 µL water and some air into syringe fitted with a blunt-cut needle
- Cut into the gel with a twisting motion, this should remove the gel plug containing the protein (or use forceps if plug was not removed from the gel)
- Deposit the BSA plug (from the 1-D gel) into a pre-assigned well of the red-colored 96-well reaction plate (designed for the automated digester)
- Deposit the protein plug (from the 2-D gel) into a pre-assigned well of the plate
- Remove a plug from a blank area of the gel (to serve as a control) and deposit into a pre-assigned well of the plate
- Proceed with the digestion process of the automatic digester

6. A manual procedure for in-gel digestion is provided

7. Digestion of the crude protein extract with trypsin/Lys-C

8. The digestion solution can then be analyzed either by peptide mass fingerprinting (PMF), tandem mass spectrometry (LC-MS/MS), or the peptides can be separated by HPLC and subjected to Edman degradation to determine internal sequence
In-gel digestion

1. Destaining of gel slice/spot

2. Reduction with dithiotheritol
   Breaks disulfide bonds and unfolds protein

3. Alkylation with iodacetamide
   Alkylation of the SH groups of cysteines to form carbamidomethyl cysteine

4. In-gel digestion with trypsin
   Typically 1µg per gel slice/spot (1:50 enzyme:sample)
   Overnight at 37ºC

5. Terminate digestion with formic acid

6. Extraction of the tryptic peptides
Protein identification from gel spots
Solution digestion with trypsin/Lys-C

1. Dilute sample

2. Reduction with dithiotheritol
   Breaks disulfide bonds and unfolds protein

3. Alkylation with iodacetamide
   Alkylation of the SH groups of cysteines to form carbamidomethyl cysteine

4. In-gel digestion with trypsin/Lys-C
   4 ug trypsin/Lys-C (1:25 enzyme:sample)
   Overnight at 37°C
   Trypsin misses few Arginine sites but typically misses some lysine sites
   Addition of Lys-C decreases the number of lysines missed

5. Terminate digestion with formic acid
Protein identification (with quantitation) from a crude extract
Protein Identification by Peptide Mass Fingerprinting

Reliable and unambiguous protein identification by PMF requires highly accurate mass measurement.

<table>
<thead>
<tr>
<th>Search m/z</th>
<th>Mass tolerance (Da)</th>
<th>#Hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1529</td>
<td>1</td>
<td>478</td>
</tr>
<tr>
<td>1529.7</td>
<td>0.1</td>
<td>164</td>
</tr>
<tr>
<td>1529.73</td>
<td>0.01</td>
<td>25</td>
</tr>
<tr>
<td>1529.734</td>
<td>0.001</td>
<td>4</td>
</tr>
<tr>
<td>1529.7348</td>
<td>0.0001</td>
<td>2</td>
</tr>
</tbody>
</table>


Disadvantages of using peptide mass fingerprinting

1. If more than one protein is present in the digest, peptide fragments are produced from each protein, complicating the data

2. If the protein is post-translationally modified- in this case MS/MS can resolve and identify the modification

3. If the protein is from an organism whose sequence in not in the database-in this case homologous sequences will be identified, if present
Overcome some of these disadvantages by using MS/MS

Nomenclature for Product Ions Arising from the Fragmentation of Protonated Peptides

<table>
<thead>
<tr>
<th>Site of backbone cleavage</th>
<th>if charge is retained on amino-terminal fragment</th>
<th>if charge is retained on carboxy-terminal fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>αC-C bond</td>
<td>a</td>
<td>x</td>
</tr>
<tr>
<td>C-N amide bond</td>
<td>b</td>
<td>y</td>
</tr>
<tr>
<td>N-αC bond</td>
<td>c</td>
<td>z</td>
</tr>
</tbody>
</table>


The most commonly observed are the b- and y- ion series. These arise from cleavage of the amide bond.

Sequence ion nomenclature for a protonated tetrapeptide.
542E-Introduction to Proteomics

Lecture 8
Fall 2019
Mass Spectrometry terms

m/z - mass per charge

Average mass - mass calculated by summing the average atomic mass of the constituent elements

\[ H = 1.00794 \]

Monoisotopic mass - mass calculated by summing the mass of the most abundant natural isotope of the constituent elements - usually used with low (< 2000 Da) molecular weight compounds. For example, for peptides only the mass of \(^{12}\text{C}\) is used.

\[ H = 1.007825 \]

Examples of average versus monoisotopic masses

<table>
<thead>
<tr>
<th>Name</th>
<th>Charge (n)</th>
<th>((M+nH)n^+) Average</th>
<th>((M+nH)n^+) Monoisotopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin (1-7)</td>
<td>+1</td>
<td>757.8605</td>
<td>757.3997</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>+1</td>
<td>1297.51</td>
<td>1296.6853</td>
</tr>
<tr>
<td>ACTH (clip 1-17)</td>
<td>+1</td>
<td>2094.46</td>
<td>2093.0867</td>
</tr>
<tr>
<td>ACTH (clip 18-39)</td>
<td>+1</td>
<td>2466.72</td>
<td>2465.1989</td>
</tr>
<tr>
<td>ACTH (clip 7-38)</td>
<td>+1</td>
<td>3660.19</td>
<td>3657.9294</td>
</tr>
<tr>
<td>Insulin (bovine)</td>
<td>+1</td>
<td>5734.59</td>
<td>5730.6087</td>
</tr>
<tr>
<td>Insulin (bovine)</td>
<td>+2</td>
<td>2867.80</td>
<td>2865.8083</td>
</tr>
</tbody>
</table>
Resolution
Resolution is expressed as \( m/\Delta m \) and can be defined in two different ways:

10% valley-a pair of adjacent ion peaks at \( m1 \) and \( m2 \) are resolved so that a valley of 10% is observed between them, relative to the least abundant ion of the pair, \( \Delta m \) is the main difference between \( m1 \) and \( m2 \)

full-width, half-maximum (FWHM)-the width of an ion at mass m, at 50% abundance is used to define \( \Delta m \)

Resolutions determined using FWHM are about twice the resolutions determined using the 10% valley method. Figure 1 shows the importance of resolution when analyzing small peptides.
FIGURE 8.4. Resolution: The hypothetical [M+H]^+ molecular ion clusters for angiotensin (molecular mass, 1283) (a), reduced insulin A chain (molecular mass, 2340) (b), and reduced insulin B chain (molecular mass, 3400) (c) are shown at various resolutions. Monoisotopic mass, peak top mass, and average mass are indicated by the dark blue, gray, and light blue lines, respectively.

FIGURE 8.5. Resolution: The hypothetical [M+H]^+ molecular ion clusters for bovine insulin (molecular mass 5734) (a), and horse heart myoglobin (molecular mass 16,952) (b) are shown at various resolutions. Monoisotopic mass, peak top mass, and average mass are indicated by the dark blue, gray, and light blue lines, respectively.

**Mass accuracy**

Mass accuracy can be expressed as either a percentage of the measured mass or in parts-per-million (ppm). For example, mass = 2,000 Da ± 0.01% or mass = 2,000 Da ± 100 ppm. Since this is expressed as a percentage, as the mass of the analyte increases, the absolute mass error will increase as well. This is shown in the table below:

<table>
<thead>
<tr>
<th>Percent</th>
<th>ppm</th>
<th>m/z</th>
<th>Mass Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01%</td>
<td>100</td>
<td>1,000 Da</td>
<td>0.1 Da</td>
</tr>
<tr>
<td>0.01%</td>
<td>100</td>
<td>5,000 Da</td>
<td>0.5 Da</td>
</tr>
<tr>
<td>0.01%</td>
<td>100</td>
<td>50,000 Da</td>
<td>5.0 Da</td>
</tr>
<tr>
<td>0.0005%</td>
<td>5</td>
<td>2000 Da</td>
<td>0.01 Da</td>
</tr>
</tbody>
</table>
Resolution and mass accuracy on the Q-Exactive
Mass range: 50-6,000 m/z
Resolving power: 140,000 (FWHM) @ m/z 200
Scan rate: up to 12 Hz at resolution 17,500 @ m/z 200
Mass Accuracy
  Internal Calibrant: < 1 ppm RMS
  External Calibrant: < 3 ppm RMS

The RMS (root mean square) error is calculated using the formula below

\[ RMS = \frac{\sum_{i=1}^{n}(E_{ppm})^2}{n} \]

\( E_{ppm} \) is the ppm error and \( n \) is the number of masses considered.
Overview of the Q Exactive

MS/MS measurements
1. Measure all ions present
2. Choose and isolate an ion for fragmentation (parent ion or precursor ion)
3. Transfer enough energy to fragment the ion
4. Measure the masses of the fragments that retained a charge (daughter ions or fragment ions)

Fragmentation methods
Collision Induced Dissociation (CID)
An ion/neutral process in which an ion is dissociated as a result of interaction with a neutral target species.

Higher energy Collision-induced Dissociation (HCD)
CID that occurs in the HCD cell of the Orbitrap. Produces triple-quadrupole-like product ions mass spectra.

Voltages
RF-Radio frequency, variable amplitude
DC-Direct current, variable voltage
Figure 3-13. Schematic of the Q Exactive and Q Exactive Plus MS

from Thermo Fisher Scientific
1. Ion source-includes ion transfer capillary and S-lens

2. Flatapoles-transmits ions from the source to the quadrupole, also pre-filters the ions and eliminates neutral ions

3. Quadrupole-filters ions based on m/z

4. C-trap-intermediate storage device

5. Collision cell for HCD (Higher Entergy Collisional Dissociation)

6. Orbitrap analyzer
Electrospray ionization (ESI) is a soft ionization technique that is used to transfer species from a liquid solution to the gas phase. The sample solution enters the ESI needle to which high voltage (3-5kV) is applied. The needle sprays the sample solution into a mist of fine droplets that are electrically charged on their surface. As the solvent evaporates, the electrical charge density at the surface of the droplets increases. At the critical point (Rayleigh stability limit) of the charge density, the droplets are divided into smaller droplets due to the electrostatic repulsion being greater than the surface tension of the droplets. This is repeated until the droplets are very small and highly charged. From these droplets, the sample ions are ejected into the gas phase by electrostatic repulsion and enter the ion transfer tube.
API Source

- Ion sweep cone-protects the ion transfer capillary
- Ion transfer capillary-assists in desolvation of the droplets
- S-lens-focuses the ions to the exit lens
Flatapoles

Ion focusing device
Bent flatapole-removes the neutral particles
TK lens-focuses the ion beam

Figure 3-13. Schematic of the Q Exactive and Q Exactive Plus MS

from Thermo Fisher Scientific
Quadrupole

The quadrupole is used as an ion transmission device with the ability to filter the ions according to their mass-to-charge ratios. By applying RF and DC voltages to the rods, the filter characteristics are changed.

- Four parallel rods create an electrostatic field that filters the ions by m/z
- The rods are aligned in pairs carrying equal and opposite charges
- A combination of RF (radio frequency, variable amplitude) voltage and DC (direct current, variable voltage) is applied to the rods.
- The ions travel between the rods and for a given ratio of RF-DC voltages the trajectory of only some ions will allow them to reach the detector. All the other ions will collide with the rods and will not reach the detector.
- This allows for the selection of a particular ion. A range of m/z ions can be detected by continuously changing the applied voltage

![Image of Quadrupole Diagram](image-url)
Curved linear trap (C-trap)
Ions are collected near the middle of the C-trap
RF is ramped down and DC voltage is applied
Ions are ejected to the Orbitrap entrance
As the ions enter the Orbitrap, they are concentrated for a short time
Higher-Energy Collisional Dissociation (HCD) Cell

Ions are passed through the C-trap directly to the HCD cell. Ions are accelerated to a higher energy kinetic state and then allowed to collide with the neutral nitrogen molecules. This results in bond breakage and fragmentation of the ions. Ions are transferred back to the C-trap.

Sequence ion nomenclature for a protonated tetrapeptide.
Orbitrap
Ion trap with no RF or magnetic fields
Moving ions are trapped around an electrode, potential barriers created by the end-electrodes confine the ions axially
The frequencies of the oscillations are controlled by shaping the electrodes

Figure 3-18. Schematic of Orbitrap cell and example of stable ion trajectory
By Fast Fourier Transformation (FFT) of the image current, the instrument obtains the frequencies of the axial oscillations and therefore the m/z of the ions.
Sample preparation for the Q Exactive

After digestion, all samples are dried down in a SpeedVac.

In-gel digestion samples:
1. Reconstitute samples in 1.25 µL Buffer B (0.1% formic acid in acetonitrile
2. Centrifuge
3. Add 23.75 µL Buffer A (0.1% formic acid in water)
4. Vortex and centrifuge
5. Transfer to sample vial with insert.
6. Cap sample vial.

Crude protein extract samples
1. Reconstitute in 95 µL Buffer A (0.1% formic acid in water)
2. Add 5 µL PRTC (250 fmol)
3. Vortex and centrifuge.
4. Transfer 20 µL to sample vial with insert.
5. Cap sample vial.
**Set up sequence run on the Q Exactive**

2 gel samples, 2 BSA controls, one crude extract

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Method setup on the Q Exactive

LC gradient using a pulled glass emitter packed with reverse-phase C18 material
Data-dependent method using a specific tune file
This method will run a full MS scan followed by 20 MS2 scans.

Total cycle time is 1680 ms
- **MS**: 80 ms, 5e5 AGC target
- **MS2**: 80 ms, 2e5 AGC target

Each scan will run for either 80 ms or until the number of ions reaches the AGC target. The next scan will start once the AGC target is achieved or the scan time reaches 80 ms, whichever occurs first.

Peptide match enables the QE to select ions with peptide-like isotopic distributions.
542E-Introduction to Proteomics

Lecture 9-10
Fall 2019
Post-run processing

This shows the total ion count (TIC) for the MS scans (top pane). The bottom pane shows the total ion count for the MS2 scans.
Full MS showing m/z 777.83 and m/z 435.77. These will trigger an MS2 scan.
Averaged MS2 of 777.83, retention time is 13.96-16.50

Averaged MS2 of 435.77, retention time is 13.33-16.50
MS2 of 777.83 labeled with b and y ions series
MS2 of 777.83 with amino acid sequence (y-ions)

Sequence is DDPHACYYSTVDDDK
Data analysis using Proteome Discoverer
Processing Workflow

Spectrum Files -> Spectrum Selector
Mascot - search engine
Sequest HT - search engine
Percolator - validator
Database searching with Mascot and Sequest HT

Cleavage rules are applied to each entry in a protein sequence database. This can be compared with the MS/MS data produced from a trypsin digestion of an unknown protein.

If the data matches the entry, the protein can be identified. If the database does not contain the unknown protein’s sequence, proteins with the closest homology will be found.

Protein sequence data can be added to the local Mascot server’s database.

Modifications:
- Static: carbamidomethyl Cys (from iodoacetamide)
- Dynamic: oxidation of Met and deamidation of Asn and Gln

From the data produced on the Q Exactive, ASCII files containing the MS/MS peak lists can be exported for Mascot searches using the internet.

Each MS/MS peak list contains a pair of mass and intensity values and each peak list is delimited by BEGIN IONS and END IONS.
MS-Digest of BSA

- MS-Digest of a known protein will yield the expected peptides produced from a digest with the specified enzyme

MS-Digest Search Results

Parameters

Database: User Protein
Considered modifications: [Acetyl (Protein N-term) | Gln->pyro-Glu (N-term Q) | Met-loss (Protein N-term) M | Met-loss+Acetyl (Protein N-term) M | Oxidation (M) ]
Digest Used: Trypsin
Max. # Missed Cleavages: 1
User AA Formula: C2 H3 N1 O1
Minimum Digest Fragment Mass: 800
Maximum Digest Fragment Mass: 4000
Minimum Digest Fragment Length: 5

Index Number: 1
pl of Protein: 5.8
Protein MW: 69294
Amino Acid Composition: A48 C35 D40 E59 F30 G17 H17 I15 K60 L65 M5 N14 P28 Q20 R26 S32 T34 V38 W3 Y21

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Consensus Workflow

Filters and processes the results from the database search
Quantitation Processing Workflow

Additional nodes:
- Minora Feature Detector
Quantitation Consensus Workflow

Additional nodes:
- Feature Mapper
- Precursor Ions Quantifier
- Peptide in Protein Annotation
Process the gel spots
Process the crude extract digestion
From the job queue open the consensus files for your samples
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- Protein FDR confidence: level of confidence for the protein
- Master: indicates whether the protein is the master protein of a protein group
- Accession: unique identifier from the FASTA database
- Description: name of the protein
- Sum PEP score: protein score calculated as the negative logarithms of the PEP values of the connected PSMs
- Coverage: percentage of the protein sequence covered by the peptides
- # Peptides: number of distinct peptide sequences in a protein group
- # PSMs: total number of identified peptide sequences (peptide spectrum matches, PSMs), including redundant peptides
- # Unique Peptides: total number of peptide sequences unique to the protein group
- # Protein Groups: number of protein groups the protein belongs to
- # AAs: number of amino acids in the protein
- MW [kDa]: molecular weight of the protein
- calc. pl: pi of the protein
- Score Mascot: sum of the scores of the individual peptides from the Mascot search. It is the cumulative protein score based on summing the ion scores of the unique peptides identified. If a peptide is redundant, the highest-scoring peptides is used.
- # Peptides Mascot: number of distinct peptide sequences in a protein group from the Mascot search
- Score Sequest HT: sum of the scores of the individual peptides from the Sequest HT search. It is the sum of all the peptide Xcorr values above the specified score threshold. It is calculated by:
  \[
  0.8 + \text{peptide_charge} \times \text{peptide_relevance\_factor}
  \]
  Where peptide_relevance_factor is a parameter set in the Sequest HT node with a default value of 0.4
- # Peptides Sequest HT: number of distinct peptide sequences in a protein group from the Sequest HT search
Coverage

Protein card information
### Peptide Groups

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• Sequence: the sequence of amino acids that compose the peptide.
• Modifications: static and dynamic identifications found in this peptide
• Quality PEP: posterior error probability is the probability that the observed PSM is incorrect. Also called local FDR.
• Quality q-value: q-value for the peptide group
• # Protein Groups: number of protein groups in which this peptide is found
• # Proteins: number of proteins in which this peptide is found
• # PSMs: total number of peptide sequences for the protein, including redundant peptides
• Master Protein Accessions: accession of the master protein
• # Missed Cleavages: the number of cleavage sites in the peptide that the cleavage reagent or enzyme did not cleave
• Theo MH+[Da]: protonated, monoisotopic mass of the peptide
• Confidence Mascot: confidence icon for the Mascot search; green for high confidence, yellow for medium confidence, red for low confidence
• Ions Score Mascot: the probability that the observed match is a random event. The score is reported as \(-10 \times \log_{10}(P)\), where P is the absolute probability. A higher score indicates less chance of it being incorrect.
• Confidence Sequest HT: confidence icon for the Sequest HT search; green for high confidence, yellow for medium confidence, red for low confidence
• XCorr Sequest HT: scores the number of fragment ions that are common to two different peptides with the same precursor mass and calculates the cross-correlation score for all candidate peptides queried from the database.
Peptide Spectrum Match

Sequence: DTHSKEASRF, Charge: +3, Monoisotopic m/z: 398.5307 Da (-0.32 amu, 0.53 ppm), MW: 1193.6014 Da, RT: 2.1801 min.
Identified with Mascot (v2.4.1), Score: 75, Precursor: q/-Val-2, Precursor PEP: 0.001, Auto matched by search engine: 37.
Fragment mass tolerance used for search: 0.9 Da
Fragments used for search: b, y, H2O, NH4.

Fragment Match:
- m/z: 398.5307 Da
- Mass: 1193.6014 Da
- Charge: +3
- Score: 75
- Precursor: q/-Val-2
- Precursor PEP: 0.001

Peptide Spectrum:
- Mass range: 398.5307 Da
- Matched: True
- Tolerance: 0.05 Da
- Match confidence: 99.99%

Peptide Spectrum Match Identification Details:
- Mass tolerance: 0.05 Da
- Use search settings
- Select precursor: q/-Val-2
- Match tolerances: Mass: 0.05 Da, Mass tolerance: 0.05 Da

Fragment Report:
- b, y, H2O, NH4
- Mass: 398.5307 Da
- Matched: True
- Tolerance: 0.05 Da
- Match confidence: 99.99%
Crude extract digestion results
Filter for PRTC peptides

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Filter Groups
4 of 1058 Peptides chosen (244 filtered out)