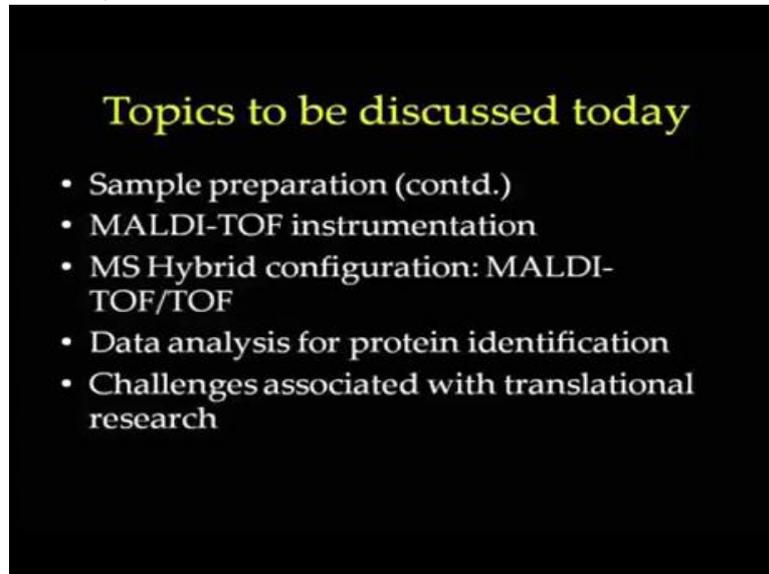


Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Mod 05 Lecture Number 21

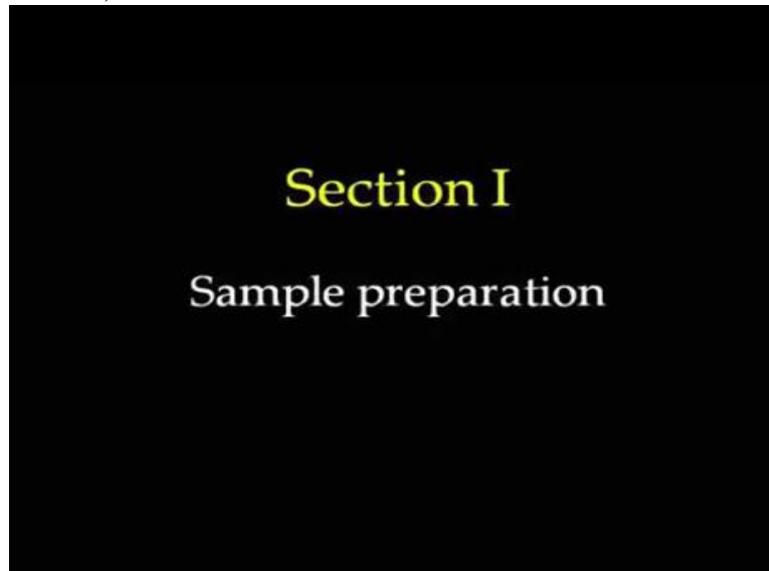
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Topics to be discussed today

- Sample preparation (contd.)
- MALDI-TOF instrumentation
- MS Hybrid configuration: MALDI-TOF/TOF
- Data analysis for protein identification
- Challenges associated with translational research

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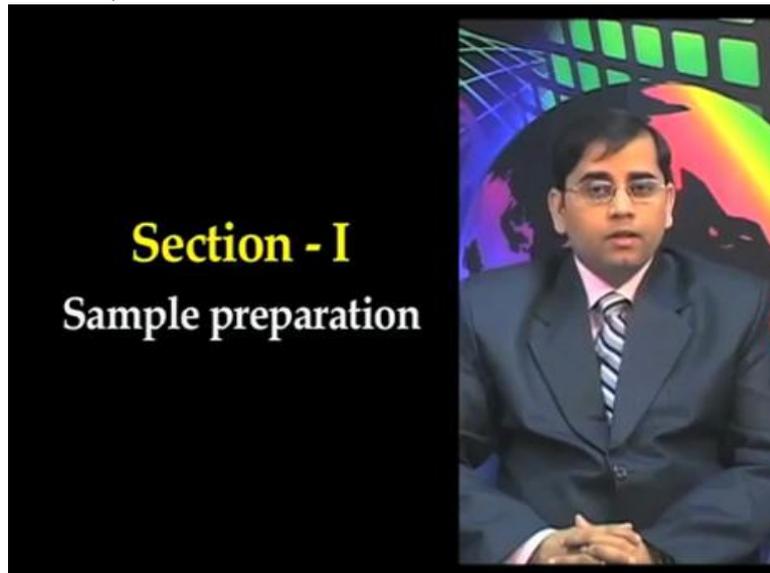


Section I

Sample preparation

So the in-gel digestion of the proteins isolated by the gel electrophoresis remains a core area in the mass spectrometry or in any of the proteomics applications.

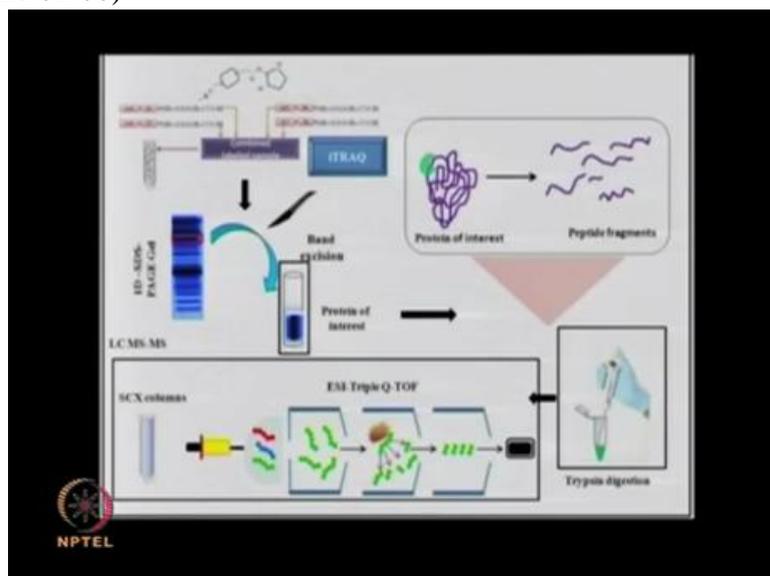
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The following video which we are going to watch is going to provide the broad guideline for the in-gel digestion. However the recipe is very flexible and it varies from lab to lab to meet the specific requirements of particular proteomic experiments.

The in-gel digestion procedure is compatible with the down-stream mass spectrometry analysis whether you want to continue with the MALDI-TOF or you want to do the LC-MS-based mass spectrometry analysis

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In-gel digested protein for MS Analysis Video

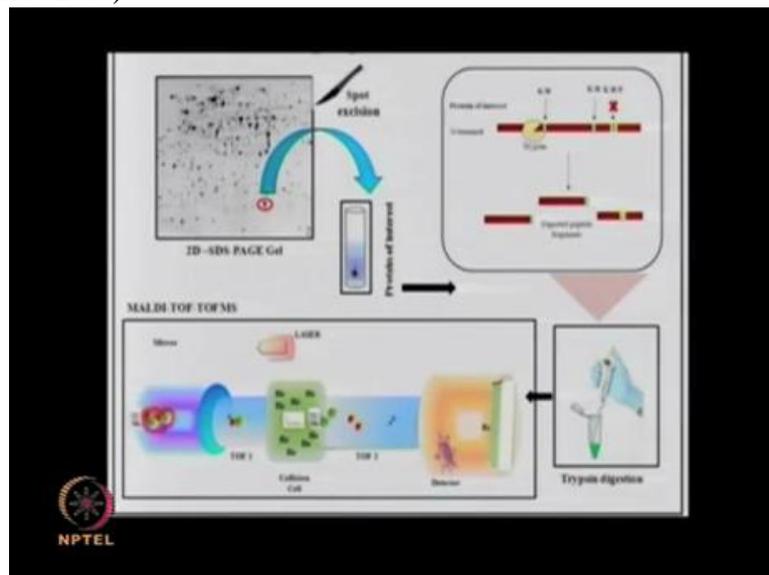
Often one- or two-dimensional gel electrophoresis is applied for separation of complex mixtures of proteins prior to mass spectrometric analysis. In-gel proteolytic digestion of

separated proteins is performed to cleave the proteins of interest present within the polyacrylamide matrix.

You can see the overview of various steps involved in this process. One can use one dimensional electrophoresis or take the protein samples directly from the mass spectrometry based experiments, separate those on the SDS PAGE or 2D gel and then excise the band of interest or spot of interest.

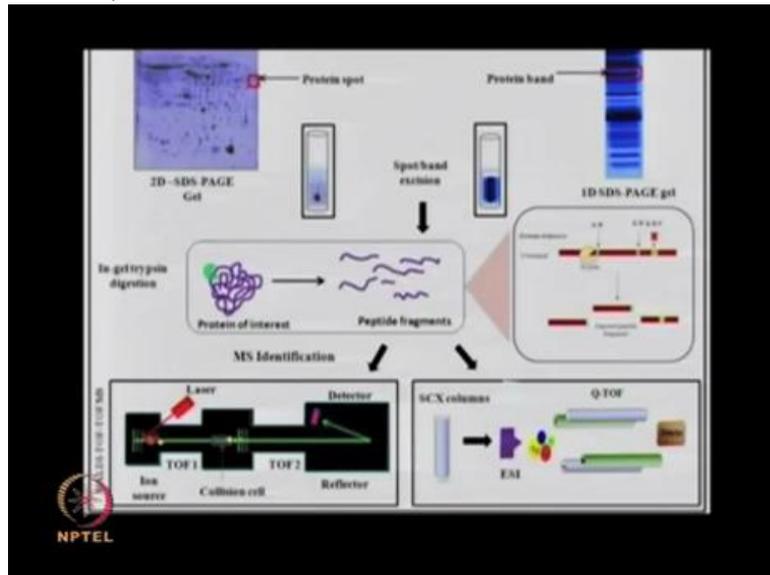
The gel-based techniques increases the dynamic range of analysis since they involve sequential separation of proteins based on molecular weight; lower to higher molecular weight. The mass spectrometric identification of the target protein greatly depends on the efficacy of the in-gel digestion process.

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That generates mixture of peptides from the target protein through proteolytic digestion.

(Refer Slide Time 02:44)



This slide gives you an overview of various steps involved in the in-gel digestion process and also how various of samples can be analyzed by using this method, whether you have 1D SDS PAGE gel, 2D SDS PAGE gel.

In either way, you have to do the in-gel peptide digestion. Once the peptide fragments are generated, then those can be analyzed using LC-MS/MS or MALDI-TOF/TOF or different types of hybrid mass spectrometry techniques.

So in-gel digestion, it is a multi-step procedure which remains central to the proteomic application. In-gel includes spot selection, spot excision, stain removal, reduction, alkylation, proteolytic cleavage and finally extraction of the peptides,

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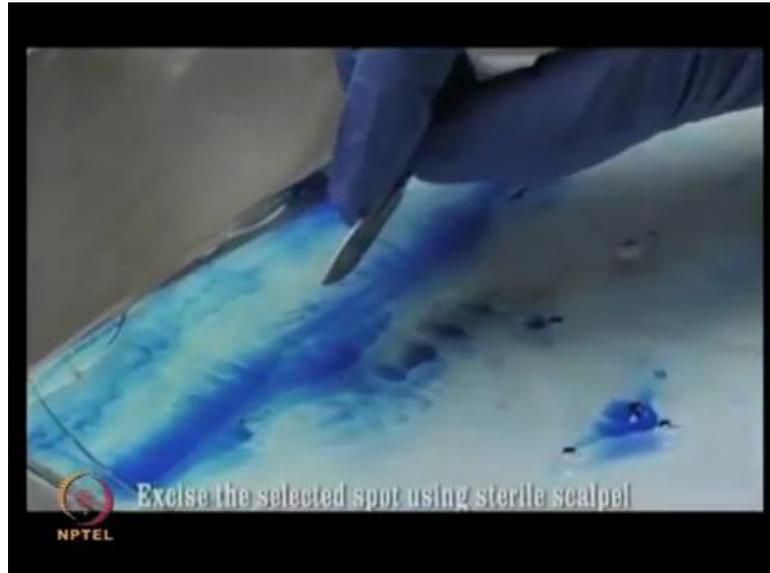
So let's say you have run a gel. First of all, you need to rinse the entire gel with water for few hours with intermittent changing of the water.

(Refer Slide Time 04:11)



Now you would like to excise the band of interest or the spot of interest.

(Refer Slide Time 04:15)



So keep a glass plate inside a laminar hood and clean the surface carefully Excise protein spot with a clean sterile scalpel

(Refer Slide Time 04:37)



... and place the gel slice into Eppendorf tube It is possible you have excised the large spot or large band.

(Refer Slide Time 04:46)



Then cut the slice into cubes

(Refer Slide Time 04:51)



... while avoiding too small pieces as they can clog pipette tips

(Refer Slide Time 05:00)



So this is the larger spot. You cannot take directly for the in-gel digestion. You need to excise that into small cubes.

(Refer Slide Time 05:13)



Now keep the small gel pieces into sterile micro-centrifuge tube.

(Refer Slide Time 05:23)



(Refer Slide Time 05:26)



Now you can add 50 to 100 micro liter of stain removal solution.

(Refer Slide Time 05:30)



For large gel slices,

(Refer Slide Time 05:36)



Take enough liquid to cover it completely. You can adjust the volume depending on the size of your spot or the band.

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After addition of the stain remover solution,

(Refer Slide Time 05:54)



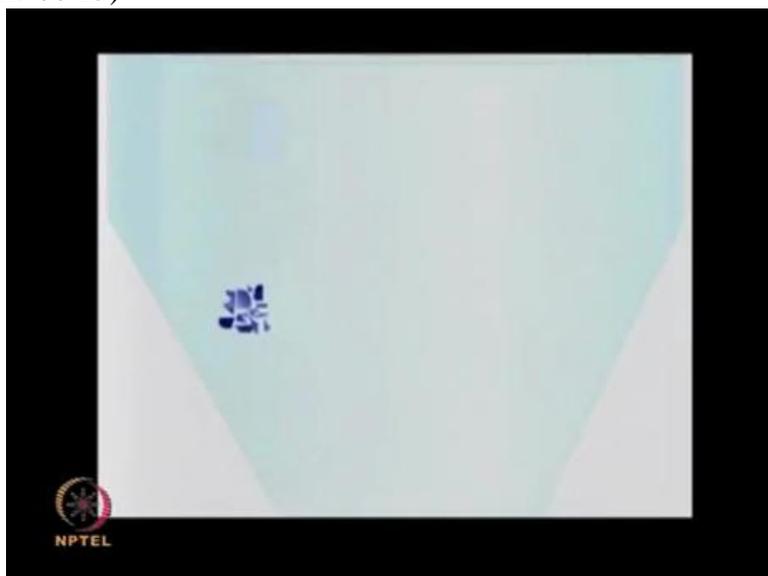
Rotate it on a shaker for 30 minutes at room temperature for complete removal of the stain from the gel pieces.

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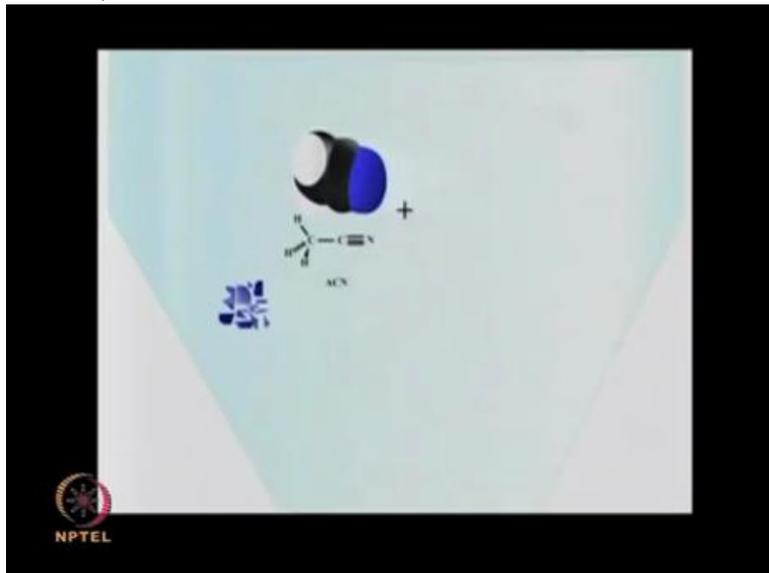


It is recommended that you change the solution after every 10 minutes.

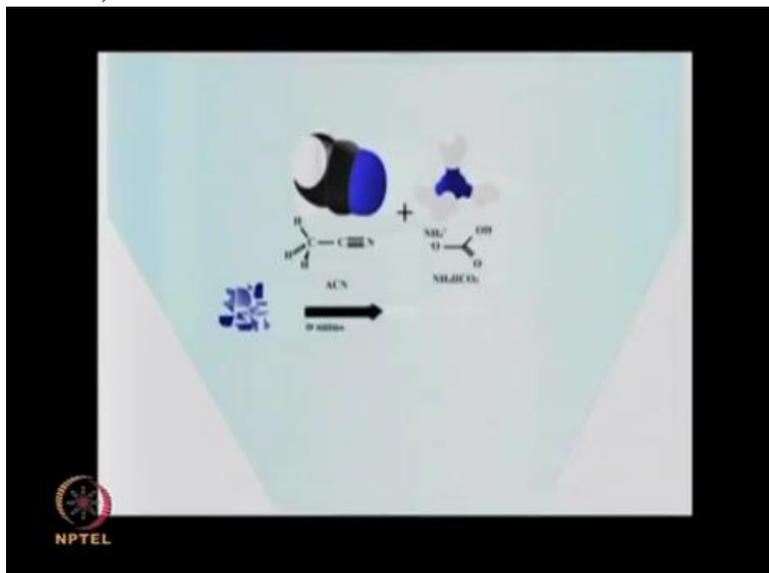
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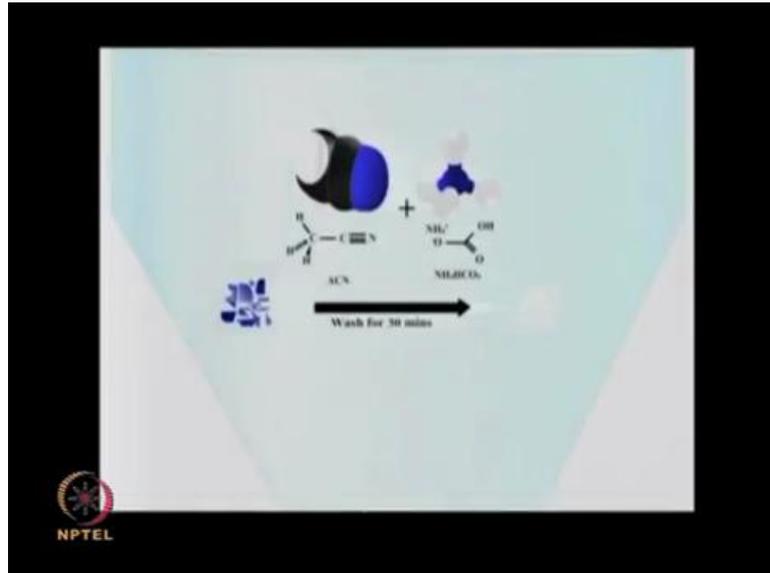
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(Refer Slide Time 06:29)



(Refer Slide Time 06:30)



By changing the solution and removing it after every 10 minutes, the Coomassie Brilliant Blue gel pieces, they become colorless. You have seen the animation. Now once the staining is removed,

(Refer Slide Time 06:43)



Then you can add dehydration solution. Add 50 to 100 micro liter of dehydration solution and rotate that on the room temperature

(Refer Slide Time 06:54)



You need to ensure that you have to change the solution after every 10 minutes so that gel pieces become white and they stick together.

(Refer Slide Time 07:07)



Spin gel pieces down at

(Refer Slide Time 07:18)



.... 1000g for 30 seconds

(Refer Slide Time 07:28)



Once centrifugation is complete then remove all the liquid.

(Refer Slide Time 07:33)



(Refer Slide Time 07:37)



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After removing the solution completely,

(Refer Slide Time 07:49)



Then add reduction solution Add 30 to 50 micro liter of the Reduction solution

(Refer Slide Time 07:59)



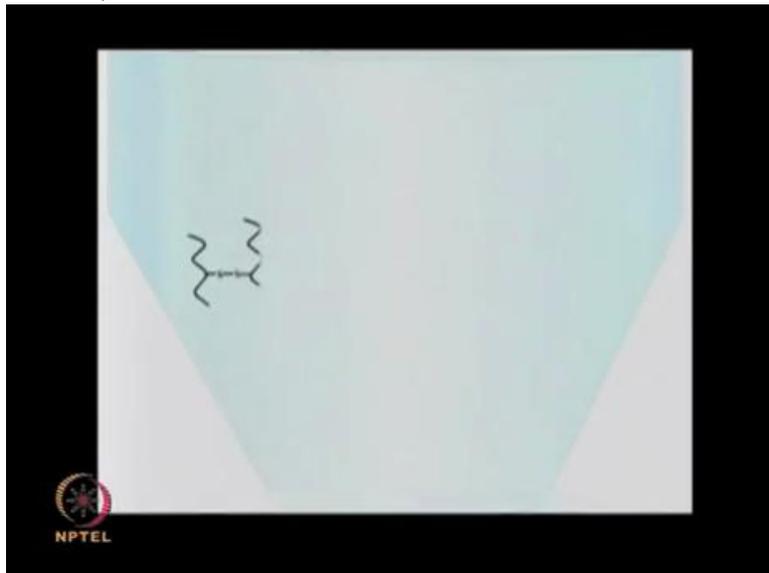
To completely cover gel pieces

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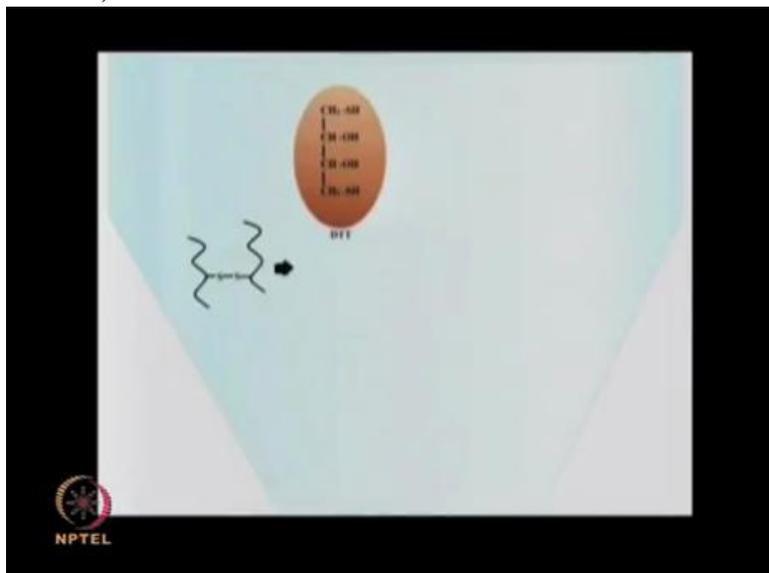


Incubate it for 30 minutes at 56 degrees Centigrade.

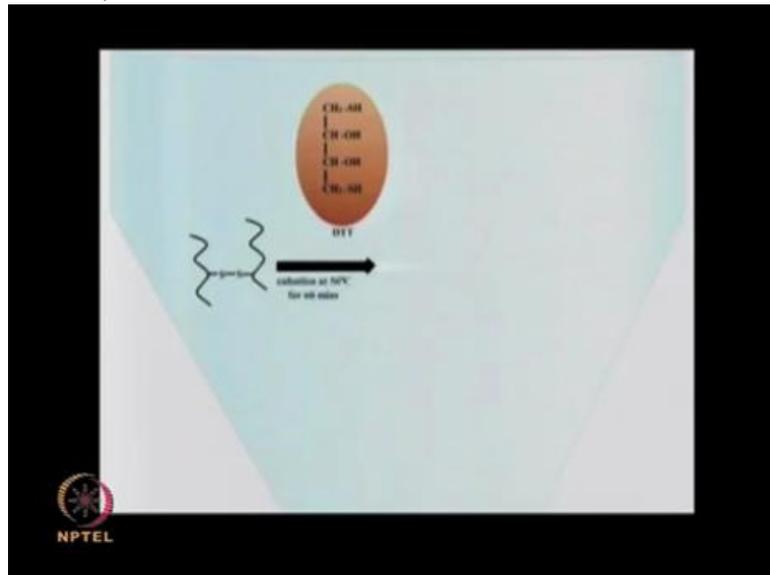
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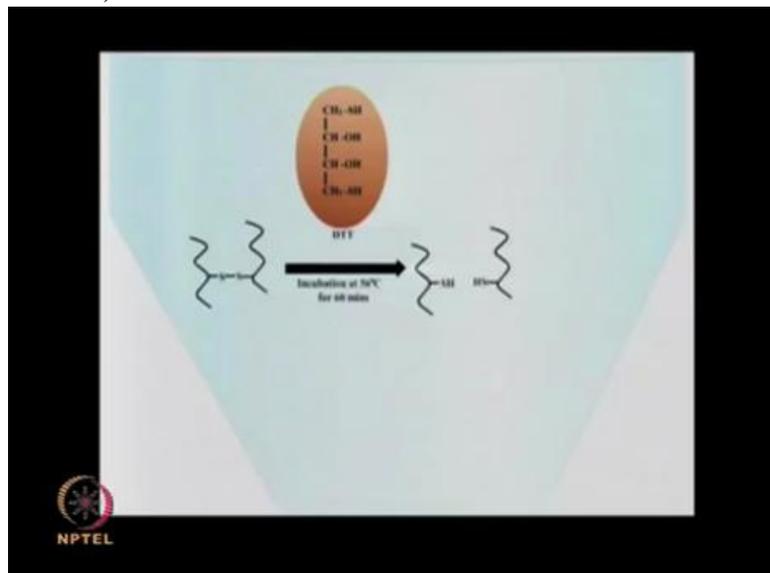
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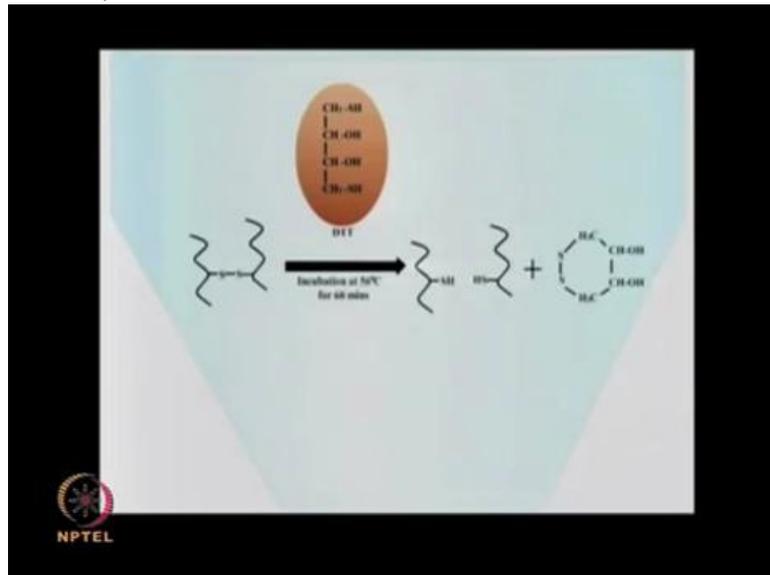
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(Refer Slide Time 08:17)



(Refer Slide Time 08:19)



For treatment of the protein residues with Dithiothreitol breaks the disulfide bonds.

(Refer Slide Time 08:22)



(Refer Slide Time 08:24)



(Refer Slide Time 08:30)



Chill down the tubes to room temperature. Add 50 micro liter of dehydration solution, mix it properly

(Refer Slide Time 08:44)



And incubate for 10 minutes

(Refer Slide Time 08:49)



Now remove all the liquids

(Refer Slide Time 09:01)



Then add the alkylation solution.

(Refer Slide Time 09:06)



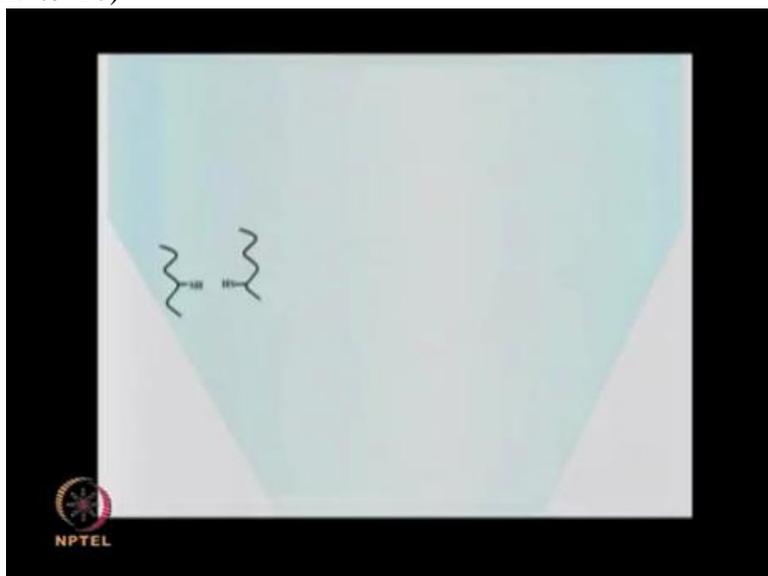
Add 30 to 50 micro liters of the alkylation solution

(Refer Slide Time 09:20)

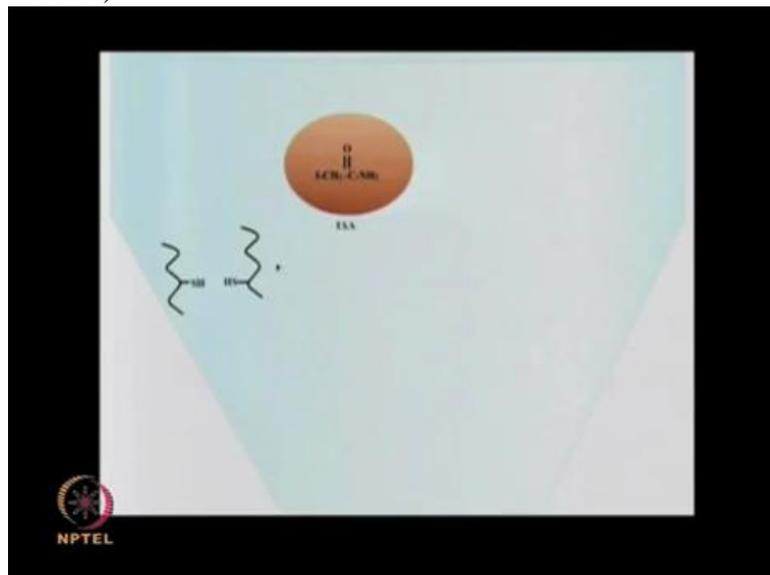


And incubate it for 20 minutes at room temperature in dark condition.

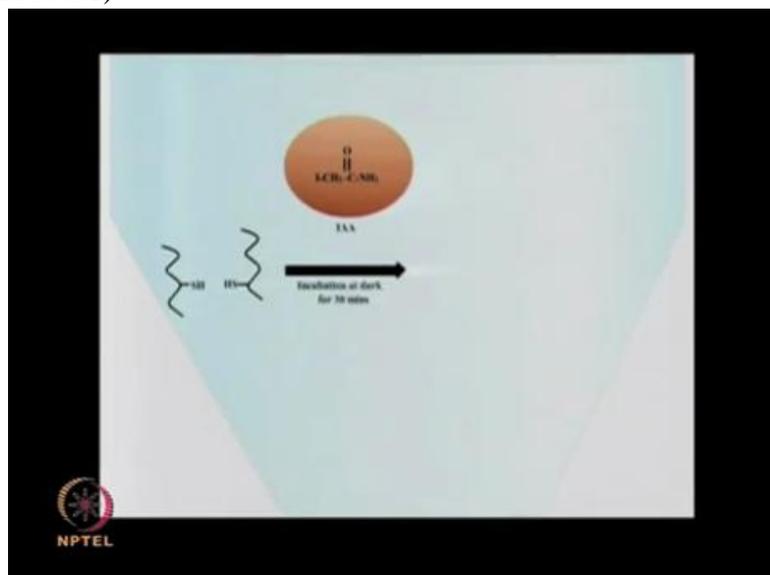
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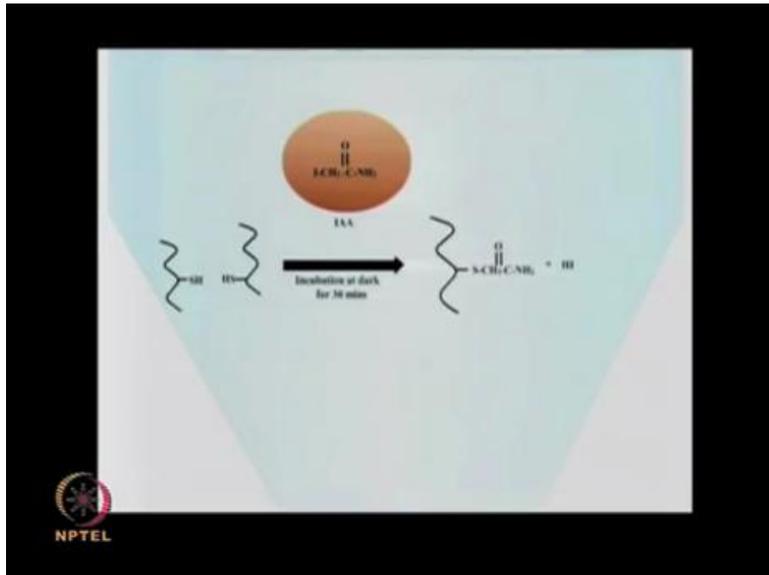
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(Refer Slide Time 09:31)

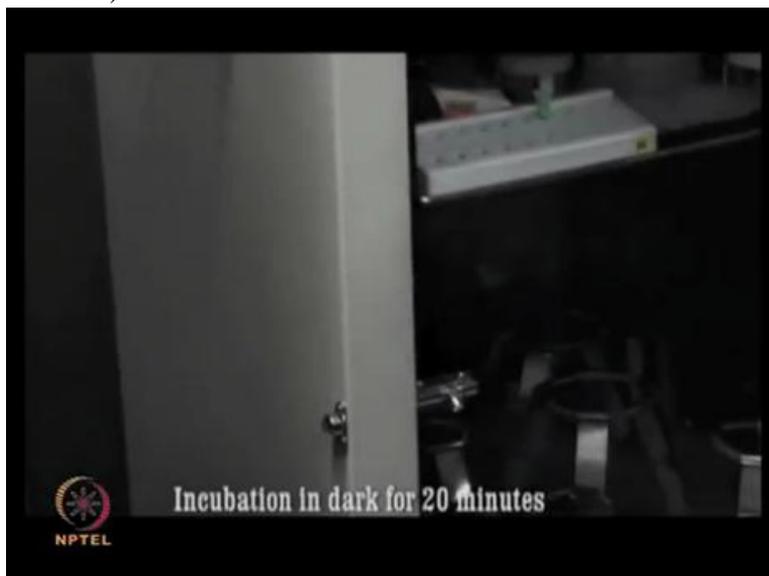


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Iodoacetamide prevents the reformation of disulfide bonds. It is an alkylating agent.

(Refer Slide Time 09:38)



It adds iodoacetoamide group to the sulfhydryl group and prevents the disulfide bond formation. Now your incubation is over. So you can remove the tubes.

(Refer Slide Time 09:52)



And now remove the solution.

(Refer Slide Time 10:00)



Now

(Refer Slide Time 10:02)



Add 50 micro liters of dehydration solution,

(Refer Slide Time 10:09)



Mix it properly by vortexing and incubate for it 10 minutes

(Refer Slide Time 10:17)



(Refer Slide Time 10:24)



Again you need to remove all the solution.

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Air-dry the gel pieces. And then add 25 micro liter of Trypsin solution, around 500 nanograms

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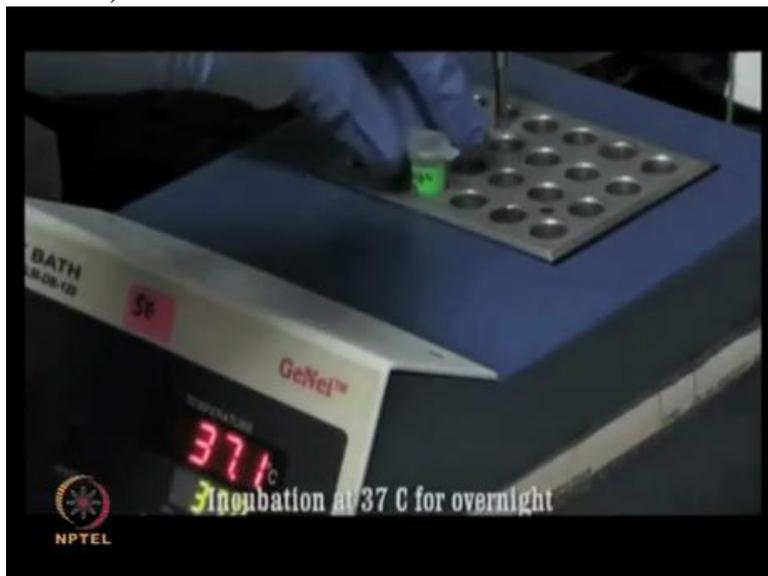
Add Trypsin to the dry gel pieces

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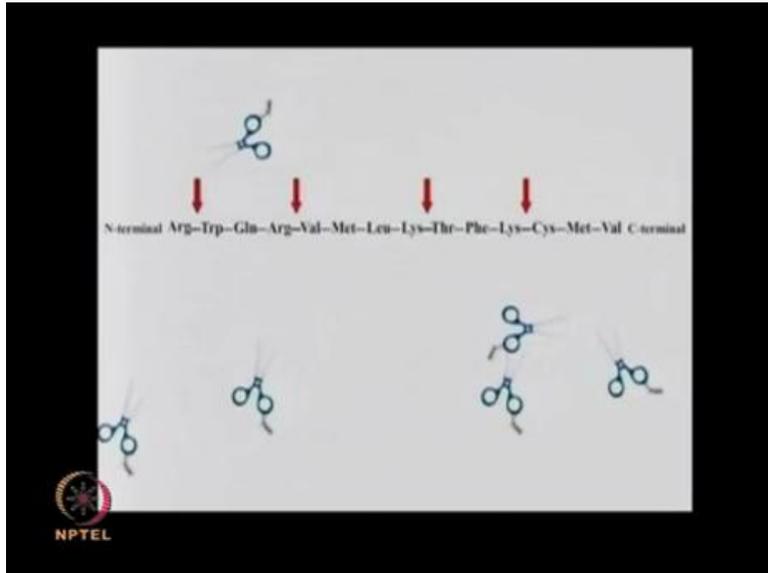
And keep on ice for 30 minutes

(Refer Slide Time 10:52)



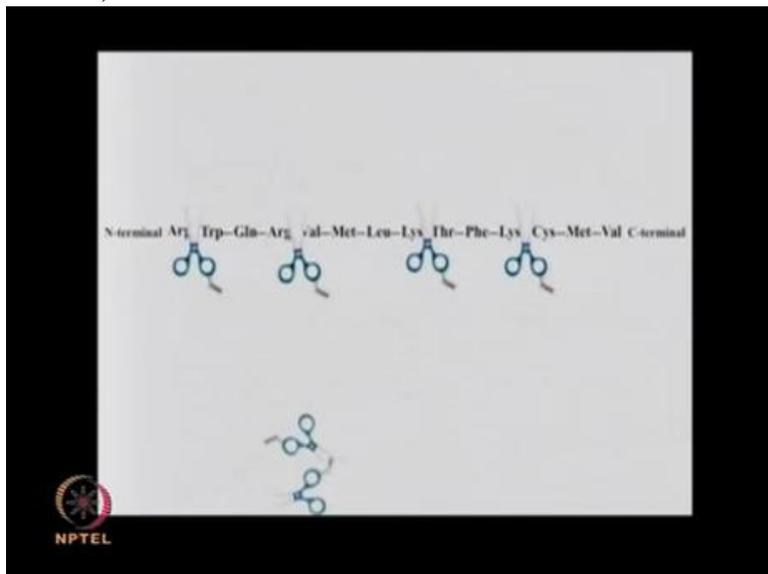
For absorption of enzyme by the gel pieces add 25 micro liter of ammonium bicarbonate buffer

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It is same buffer in which Trypsin is prepared; and incubate at 37 degrees C for overnight, 12-16 hours

(Refer Slide Time 11:15)



For proper proteolytic cleavage so prior to MS identification, proteins are digested to generate peptides. Several proteolytic enzymes are available. Chymotrypsin, trypsin, pepsin are some of the enzymes commonly used for proteolysis.

(Refer Slide Time 11:34)



After the overnight step we have to stop the reaction by keeping the reaction mixture in ice.

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After overnight incubation, the peptides generated through proteolytic digestion can be extracted using extraction buffer.

(Refer Slide Time 12:07)



Containing 0.1% Formic acid or Trifluoroacetic acid in 50% of Acetonitrile solution

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Now collect the supernatant in small aliquots and extracted samples can be stored in these small aliquots. Efficient extraction process is essential to ensure the release of peptides from gel-matrix to the solution

(Refer Slide Time 12:52)



All the solution volumes described in the protocol are recommended volumes but depending upon the experimental requirement, you can optimize and change these volumes and the incubation time according to your experimental requirements.

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Now these samples can be stored and used for the further mass spectrometric analysis

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In-gel digested protein samples are further processed by using ZipTip pipette tips

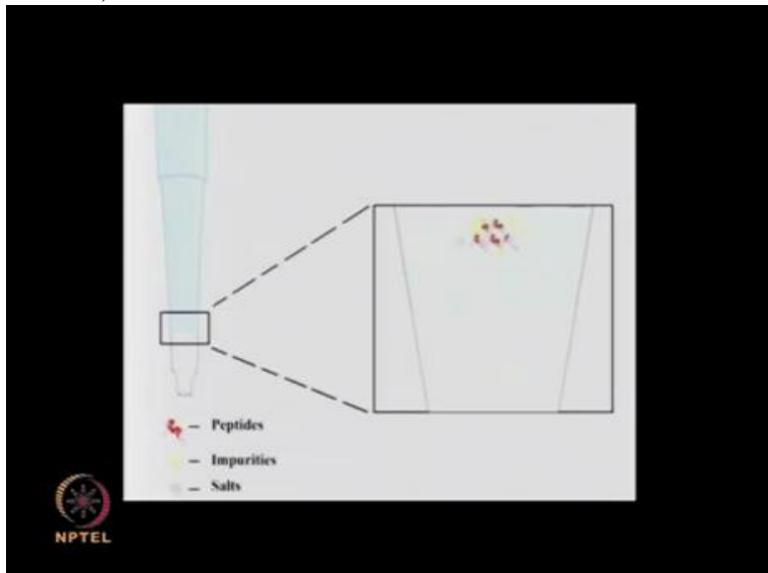
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... which contains C18 or C4 media for enrichment of peptides prior to MS analysis

ZipTip pipette tip is a 10 micro liter pipette tip with a bed of chromatography media fixed at its end. It is used for concentrating and purifying peptides as well as removing salts, detergents and interfering agents.

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So first, you need to attach the ZipTip pipette tip on the top of a suitable micropipette

Condition the ZipTip with 10 micro liter of acetonitrile.

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Perform the wash step three times.

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Wash it with 0.1% trifluoroacetic acid or TFA.

(Refer Slide Time 14:46)



After watching this video, now you are very clear about the in-gel digestion process how various steps are important to perform these experiments.

Now once you have done the in-gel digestion, you can directly use this Trypsin digest for further mass spectrometry analysis but it is often recommended that you add one more step, which is sample cleanup.

You do not want your columns or your MALDI instrument to get clogged due to the salt or some other interfering residues present in the mixture.

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Sample clean-up

- In-gel digested protein samples are processed further using ZipTip pipette tips containing C18 or C4 media for enrichment of peptides
- Salts and interfering agents, detergents are washed and finally samples are eluted in a very small volume of solvent



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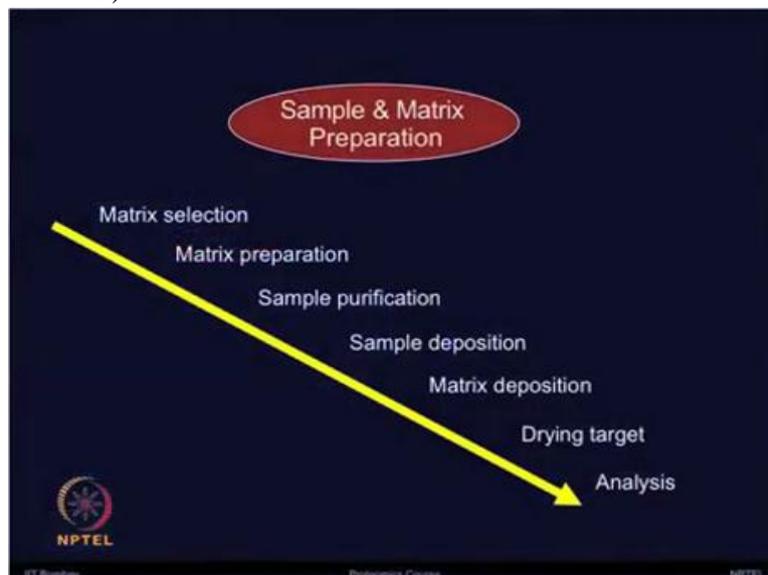
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So it is recommended that one should use the cleanup step in between. In-gel digested protein samples can be cleaned up by processing further ZipTip pipette tips which contain C18 or C4 media for enrichment of the peptides

Salts and interfering agents the detergents are washed and finally the samples can be eluted in a very small volume of the solvent.

So now you know how to perform the cleaning step by using ZipTips. Now you have the sample ready and you have selected the matrix. So now let me show you the various steps involved before you can actually start the MALDI experiment.

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You need to select the matrix. You need to prepare the matrix. You have already done the sample purification. Now sample need to be deposited on the MALDI plate. Either you can mix it with matrix or you can do this separately.

There are various combinations one can try and then once both sample and matrix are deposited on the MALDI target plate then you are ready to do the drying and the plate can be used in MALDI-TOF instrument for further analysis.

Let's first talk about matrix selection. So the important step in MALDI-TOF analysis is the selection of appropriate matrix for the sample. The matrix selection mostly depends on the

molecular weight of the target to be analyzed and often the type of application which you intend to do by using these instruments.

These matrices are low molecular weight organic compounds with low vapor pressure and volatile nature. Most of the matrices are acidic in nature so it can easily excite the photon and ionize the analyte for the analysis. However there are few basic matrices are also available.

In the slides I am giving you an overview of few matrices and some of their properties. But there are many more properties which are not mentioned here. But just to give you certain major features of these matrices commonly used for various applications.

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The slide is titled "Matrix selection" in yellow text on a black background. It contains two sections of text and two chemical structures. The first section, on the left, says "Peptides less than 5000 daltons, lipids and nucleic acids" and is accompanied by the chemical structure of α -cyano-4-hydroxycinnamic acid (labeled as α -cyano). The second section, below the first, says "Peptides and proteins having higher than 5000 daltons and sometimes also use for lipids" and is accompanied by the chemical structure of Sinapinic acid. At the bottom left is the NPTEL logo and the text "IT Bombay". At the bottom center is "Pharmaceutics Courses" and at the bottom right is "NPTEL".

One is alpha-cyano-4- hydroxycinnamic acid. When you have peptides less than 5000 Daltons or lipids and nucleic acids one can use this matrix.

One can also use sinapinic acid if peptides and proteins are having more than 5000 Dalton and it can also be sometimes used for the lipids.

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Matrix selection

<p>Small molecules and peptides which are not ionized by other matrices</p>	<p>2,5-Dihydroxybenzoic acid (DHB)</p>
<p>Used for small nucleotides and phosphorylation studies on proteins</p>	<p>Trihydroxyacetophenone (THAP)</p>
<p>Generally used for nucleotides</p>	<p>Picolinic acid</p>

Then you have options such as 2-5-dihydroxybenzoic acid also known as DHB. Small molecules and peptides which are not ionized by other molecules can be analyzed by using this matrix.

Trihydroxyacetophenone, THAP is used for small nucleotides. It is also used for phosphorylation and specialized applications. Then we have picolinic acid which is generally used for nucleotides.

So these are only few representative matrices. As you can see there are many options available for selecting the matrix depending upon the molecular weight and the type of applications.

But regardless of this, these matrices absorb energy from the laser source and convert both matrix and analyte into the gaseous phase. Matrix can also analyze analyte molecule for providing energy which comes from the laser bombardment.

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Sample and Matrix Deposition

Matrix preparation is done by mixing matrix into a suitable solvent and vortex for few minutes to dissolve it properly



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Now once you have selected the matrix, matrix can be prepared by mixing it in a suitable solvent and vortex it for few minutes so that it can dissolve properly. Now you are ready with both analyte the protein which you want to analyze as well as the matrix which we have selected for your application.

Now, one needs to think how to deposit sample on the MALDI target plate. So there are many ways of deposition of sample and matrix onto the MALDI plate. Mostly sample and matrix are mixed in an Eppendorf tube and then mixture is directly deposited by using a micropipette onto the MALDI plate. But one can also try various combinations.

In one approach the sample is directly deposited on to the MALDI plate followed by the matrices deposited above it and then it is properly mixed before drying process can happen.

Other way of doing it is to apply that with the sandwich-based method in which first a small amount of matrix is deposited on the plate and then you add the protein sample and again the matrix is spotted on top of it so that you have enough matrix below and above of the analyte.

So, one can try different combinations of placing the matrix and the analyte. And then once you have placed all of this sample of interest on MALDI plate then you are ready to dry the target plate.

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Points to ponder

- After in-gel digestion, samples cleaned with C18 or C4 columns
- This removes salts, detergents and other interfering agents
- Samples deposited on plate with suitable matrix
- Matrix selection based on the sample type

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Section II

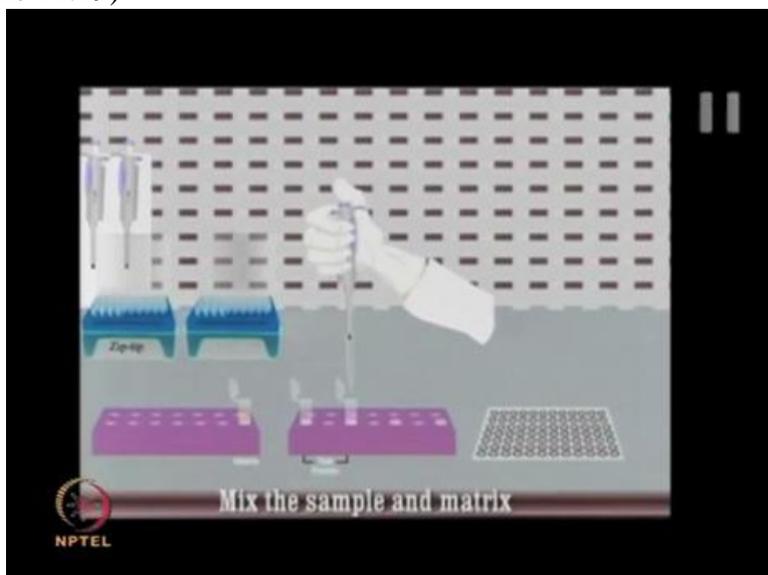
MALDI TOF instrumentation

(Refer Slide Time 21:47)



After spotting is done and the MALDI plate is dried almost 30 minutes, then the instrument can be turned on and MS analysis can be performed. Now there are various types of configuration of these instruments available as well as there are various types of commercial software which help to operate the hardware. It's not possible to go into individual detail but I am going to show you generic steps in the following video of MALDI-TOF instrumentation

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MALDI is performed in two steps. In first step, the compound for the analysis should be dissolved in a solvent containing small organic molecules, known as matrix.

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This mixture is dried before analysis and liquid solvent used in the preparation of the solution is removed.

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So in this video by depicting the matrix preparation as well as instrumentation we will try to give you the overview of MALDI-TOF instrumentation.

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Spot the mixture on the MALDI plate. How uniformly you can place these mixtures on the MALDI plate ensures your good spectra and data quality later on.

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(Refer Slide Time 23:31)



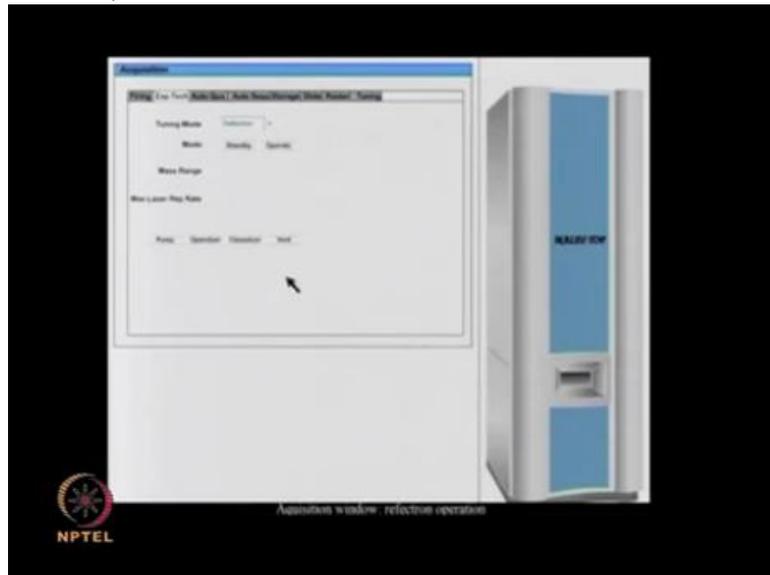
... completed on the MALDI plate, the samples are allowed to dry for 30 minutes after which the instrument is switched on

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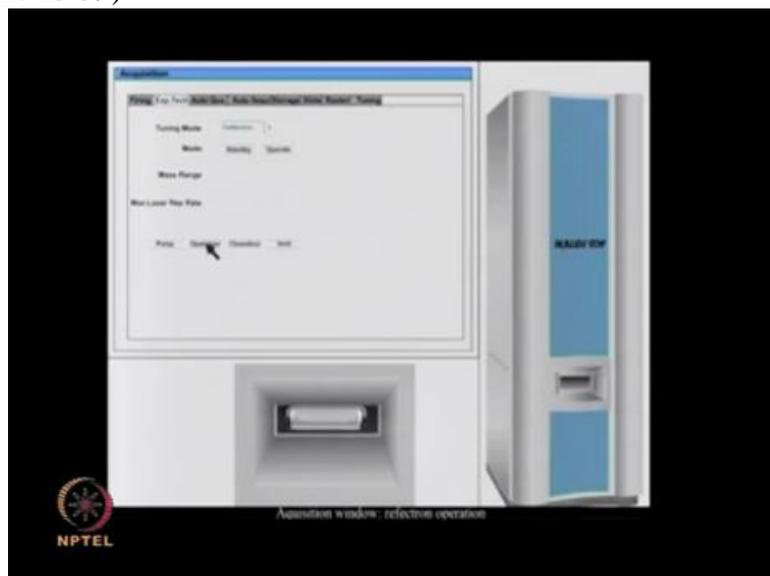
And the MS analysis can be performed. While these steps are happening

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You need to ensure that the instrument is on.

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So click on the software and

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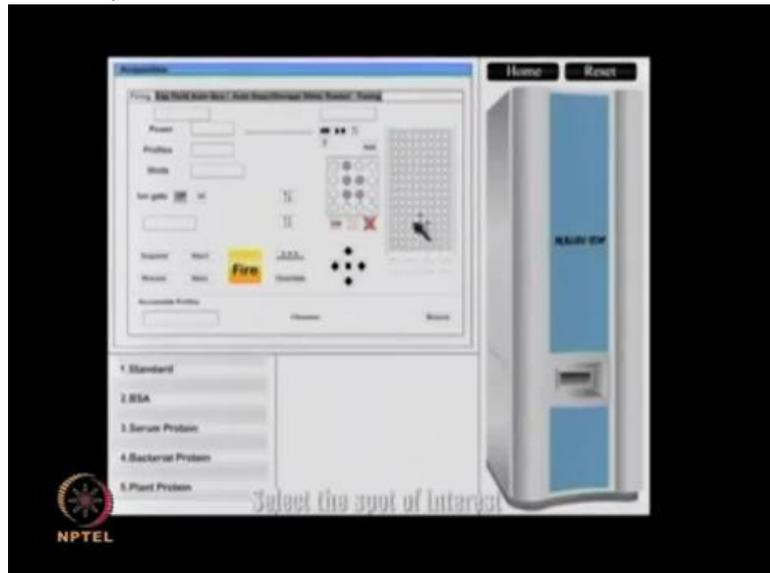
Open the acquisition window and then click on “open door”

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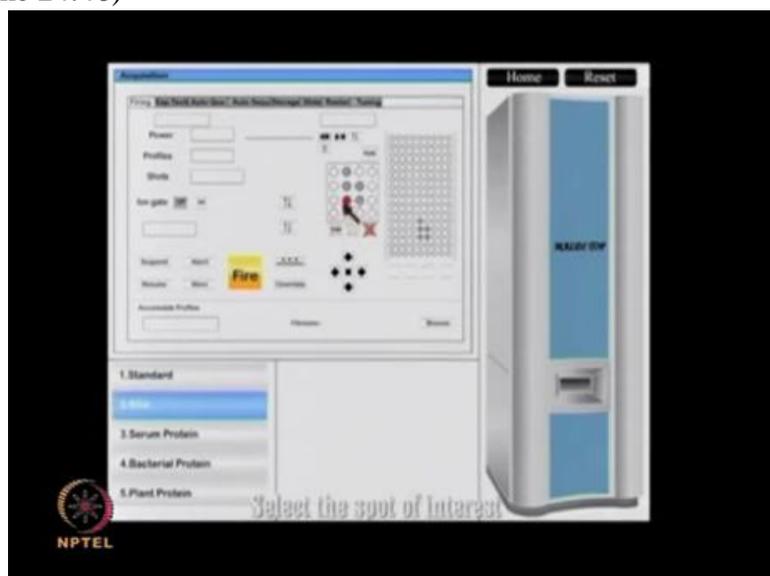
Insert MALDI target plate face up with the cut-off corner to the front.

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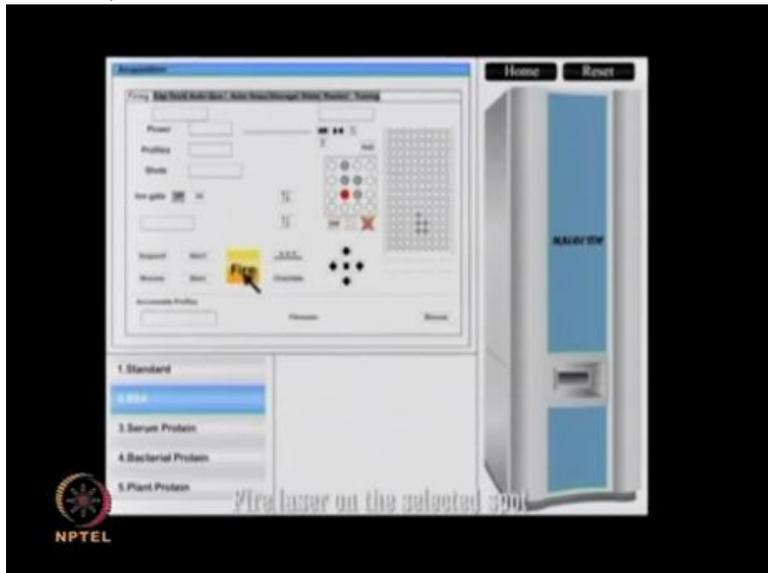
You can select the plate; you can view the overall plate on the screen and then select the spot which you want to analyze. So click on the

(Refer Slide Time 24:46)



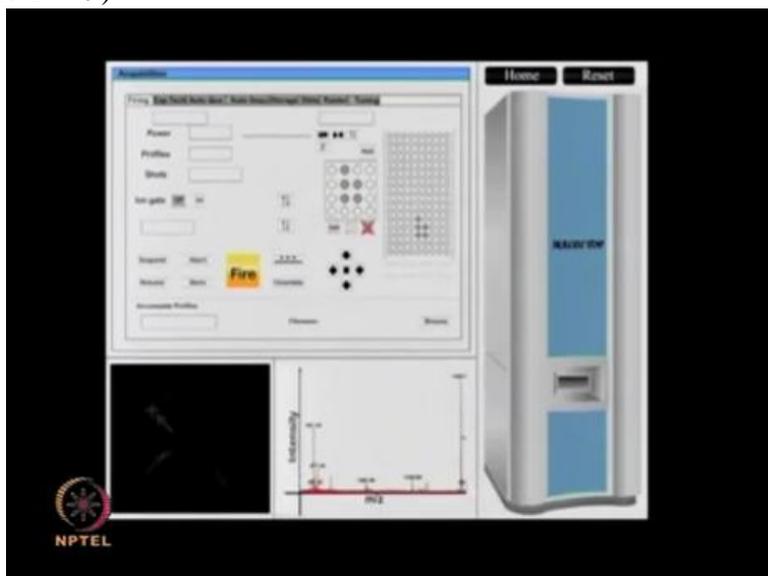
Yellow target on the acquisition window

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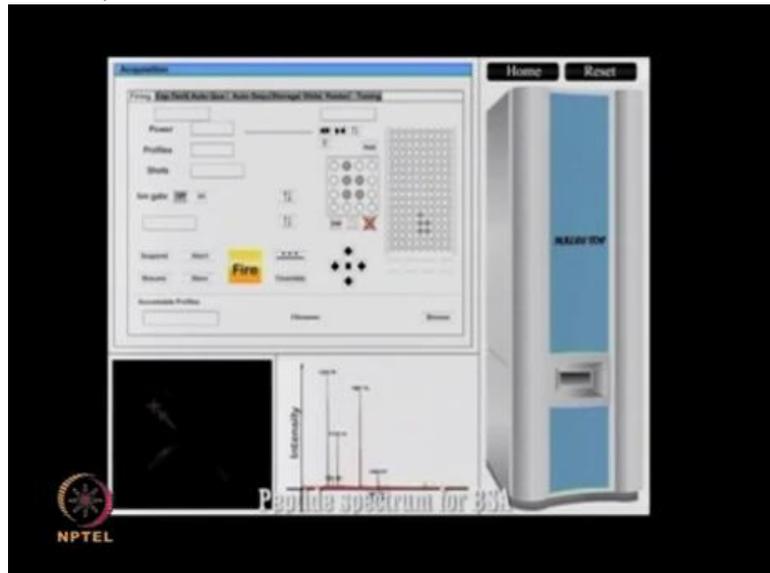


And select “go to the location”.

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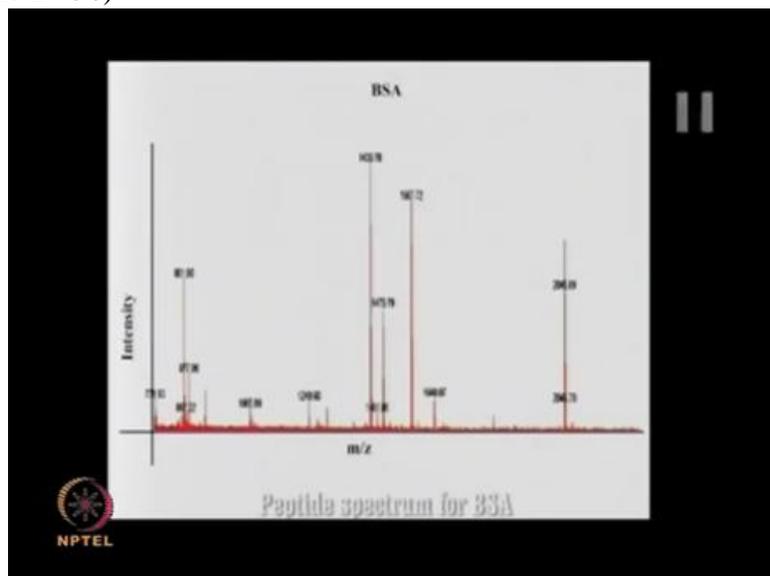


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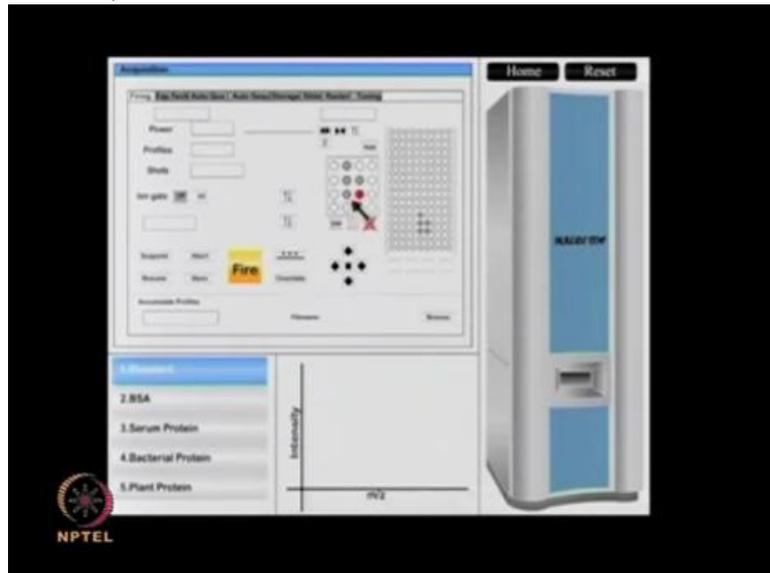
You can now do the laser bombarding and peptide spectrum is generated.

(Refer Slide Time 24:56)



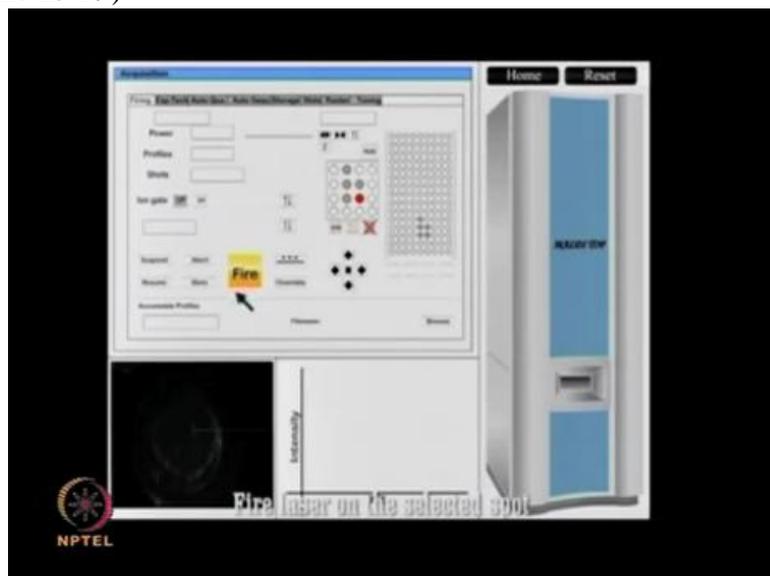
We have shown here one standard protein, bovine serum albumin. So you have to look at various locations where you can get best spectra from that spot and then you can freeze it.

(Refer Slide Time 25:16)



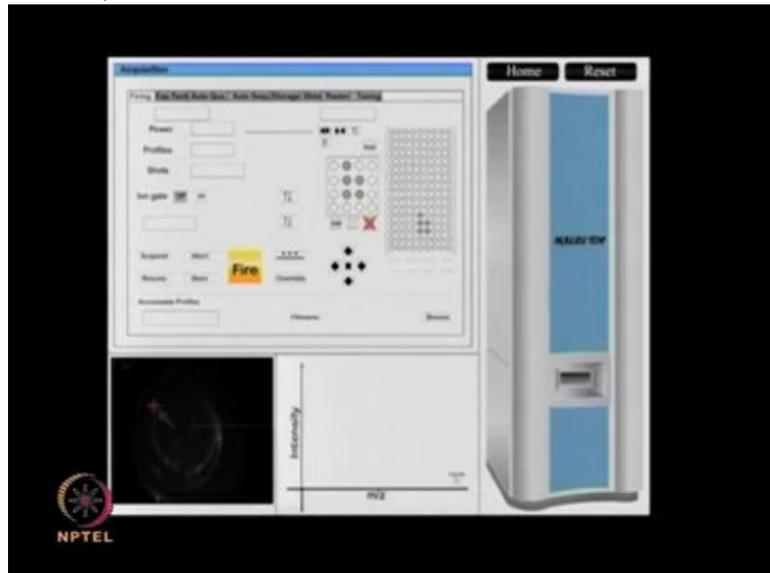
Same process

(Refer Slide Time 25:19)



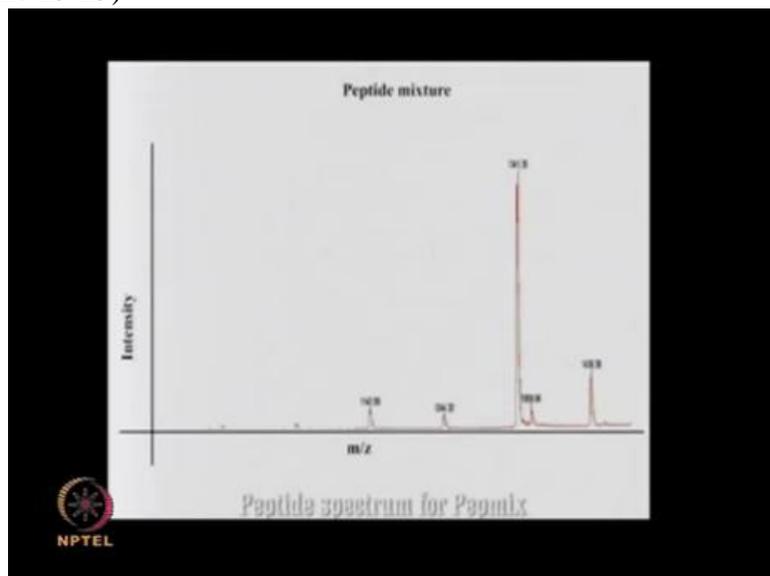
Can be performed for different spots

(Refer Slide Time 25:20)



And different regions

(Refer Slide Time 25:25)



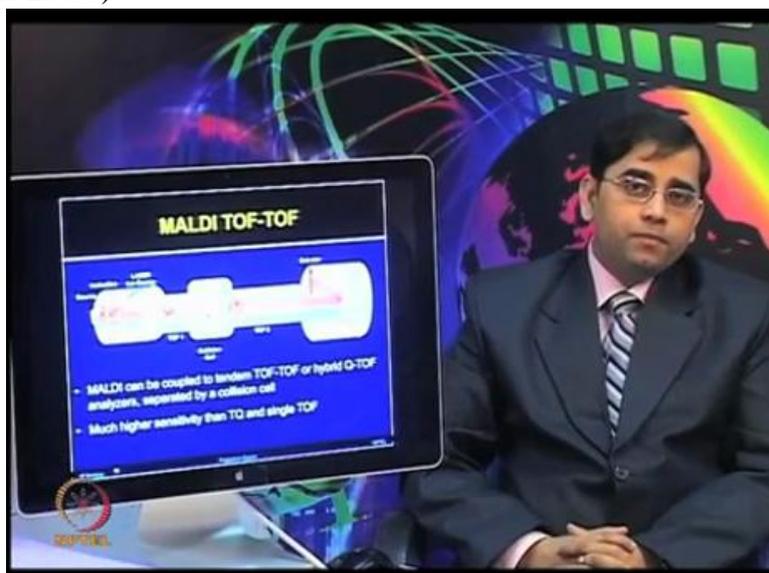
Now we have shown here a spectra for the Pepmix

(Refer Slide Time 25:29)

Section III

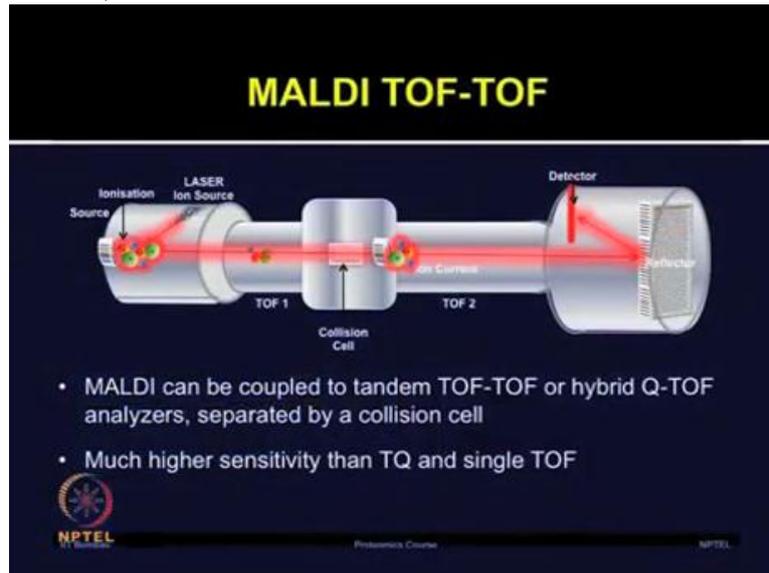
MALDI-TOF/TOF

(Refer Slide Time 25:35)



You are familiar with how to perform the MALDI TOF experiment.

(Refer Slide Time 25:35)



Now let's add one more mass analyzer. So now we have a configuration of MALDI-TOF-TOF. So MALDI can be coupled to the Tandem- Time of Flight in combination with another Time of Flight or with hybrid quadrupole-Time of Flight analyzers which are separated by collision cells.

Now for proteomic applications it is recommended to use TOF-TOF or Q-TOF. The peptide ions are accelerated through the first time of flight tube as you can see in the slide and then they are disassociated by introducing an inert gas into the collision cell. This process allows collision induced disassociation spectra from MALDI produced from the precursor ions.

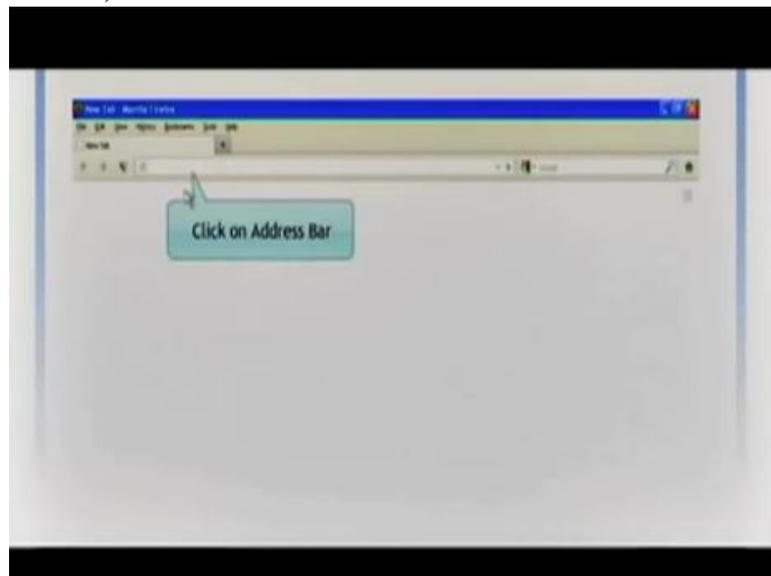
These hybrid configurations are more sensitive than triple-quad and single Time Of Flight. So the combination of TOF-TOF allows the protein identification through peptide mass fingerprinting and high-throughput analysis of proteins or proteome is possible with hybrid-TOF analyzers.

(Refer Slide Time 26:59)

Section IV

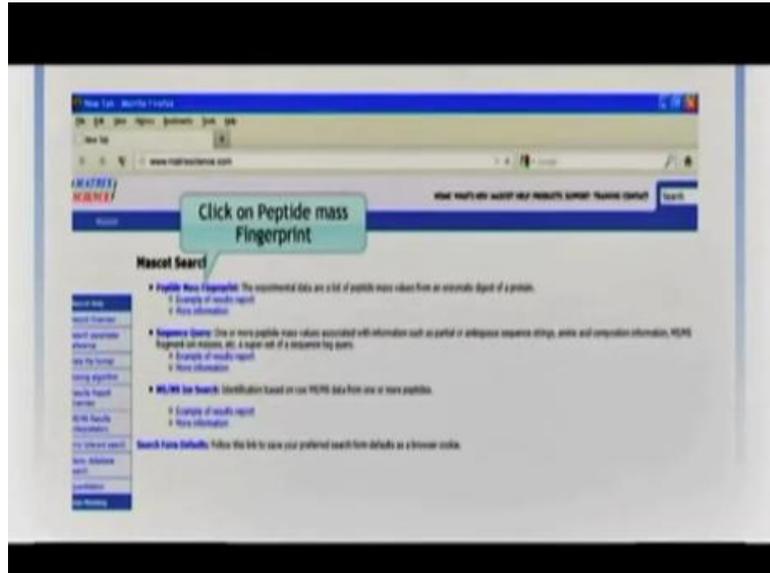
Data analysis for protein identification (PMF and MS/MS)

(Refer Slide Time 27:06)



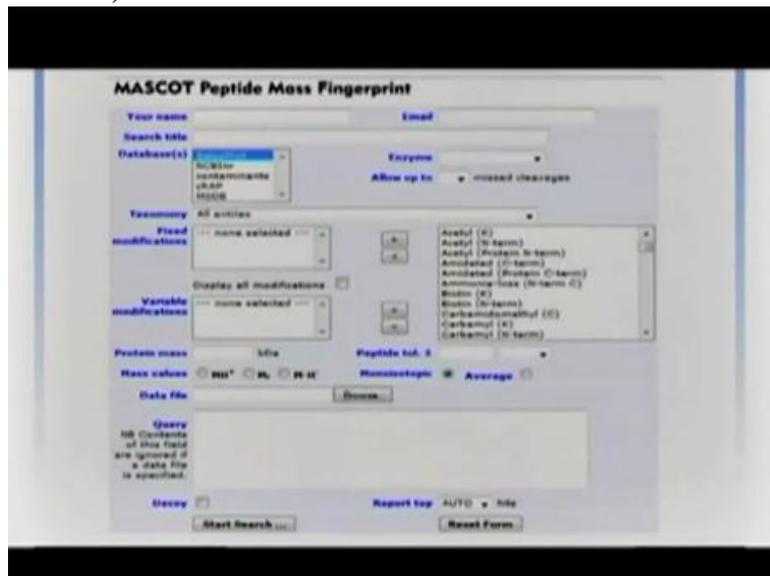
Open the Matrix Science browser window to carry out online data analysis.

(Refer Slide Time 27:17)



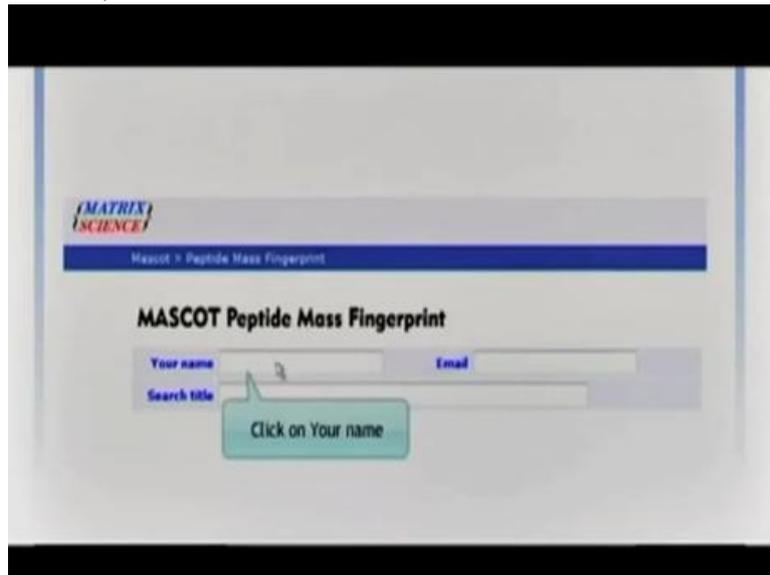
For peptide analysis click on peptide mass fingerprinting,

(Refer Slide Time 27:22)



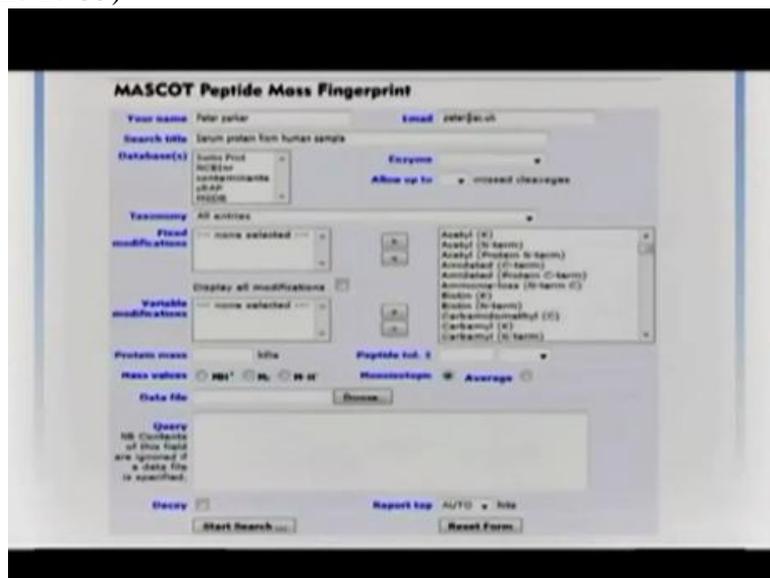
Mascot peptide mass fingerprint

(Refer Slide Time 27:29)



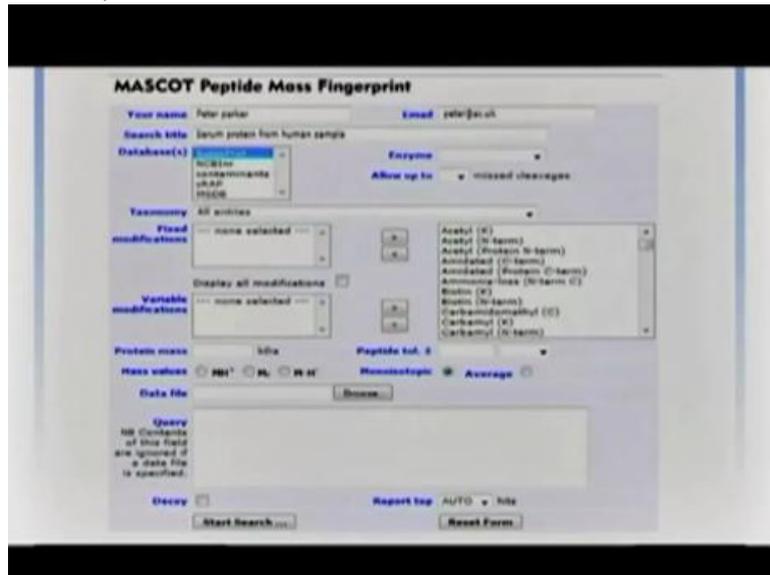
Please enter the User Id and details to acquire information in case of any network loss.

(Refer Slide Time 27:35)



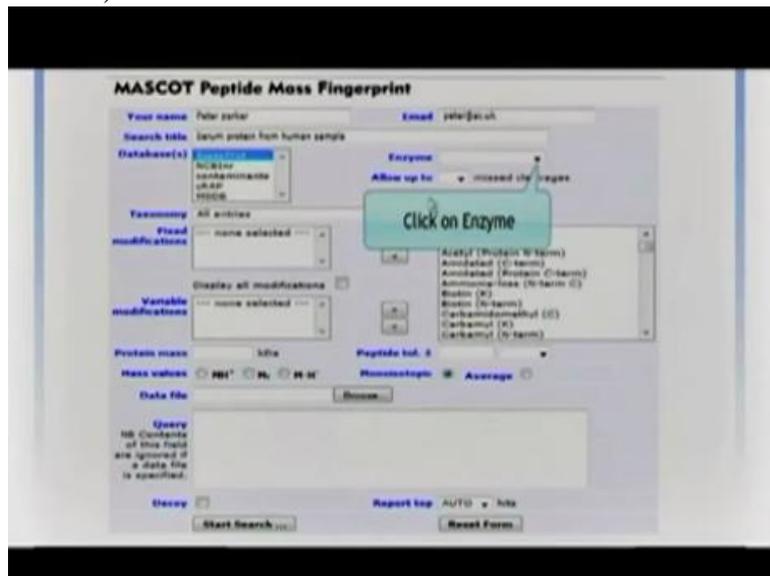
The following parameters should be selected. Database, primary databases include SwissProt and NCBI.

(Refer Slide Time 27:45)



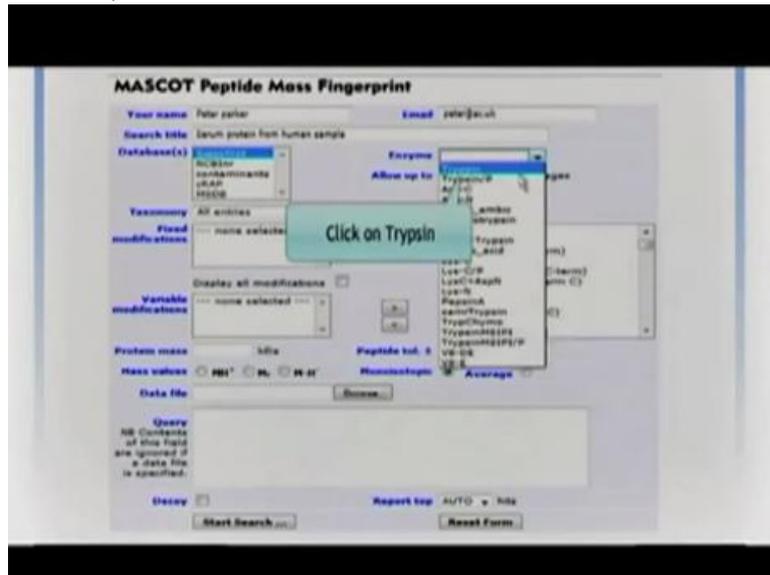
Select SwissProt database for data analysis.

(Refer Slide Time 27:51)



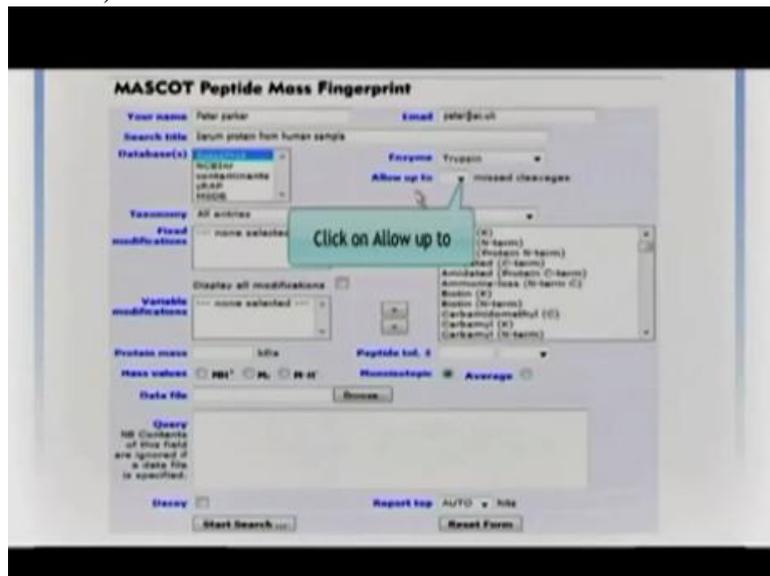
Click Enzyme

(Refer Slide Time 27:55)



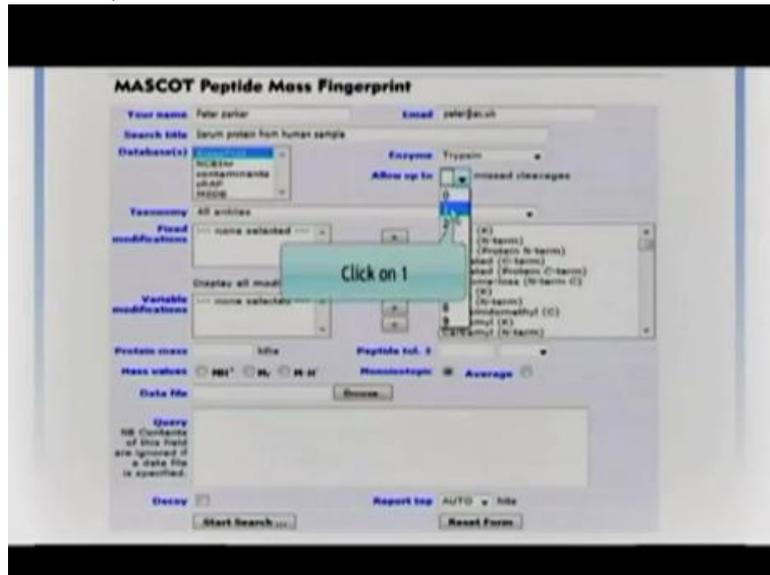
, select enzyme as Trypsin

(Refer Slide Time 27:57)



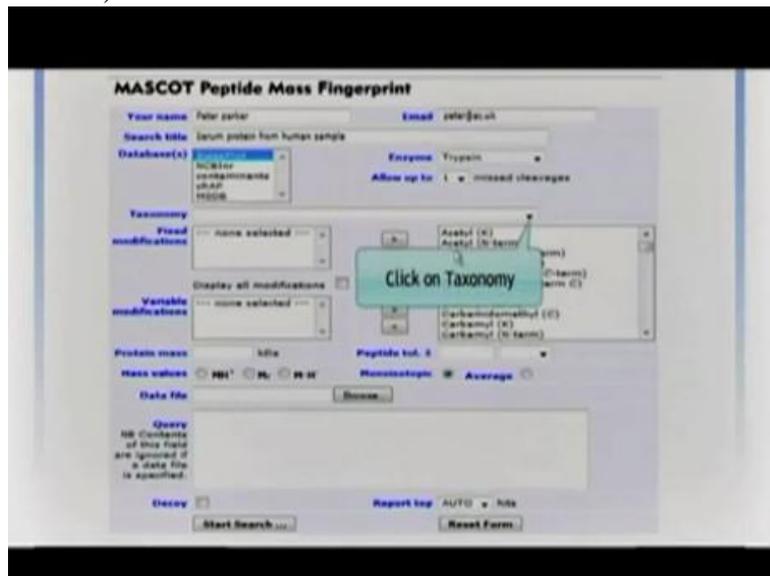
Missed Cleavages,

(Refer Slide Time 28:03)



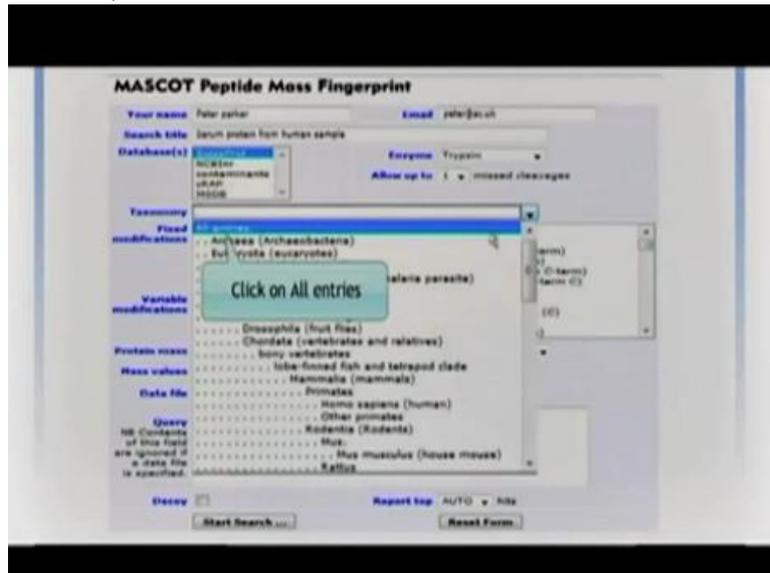
Missed cleavages are allowed up to 1

(Refer Slide Time 28:04)



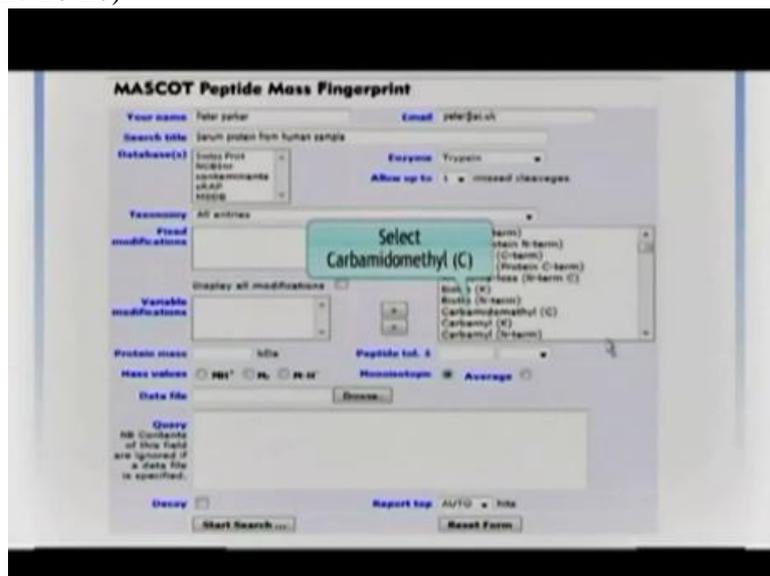
Next is taxonomy. The protein extracted from the biological specimen has to be assigned to a particular species or a group of species to which the sample belongs.

(Refer Slide Time 28:08)



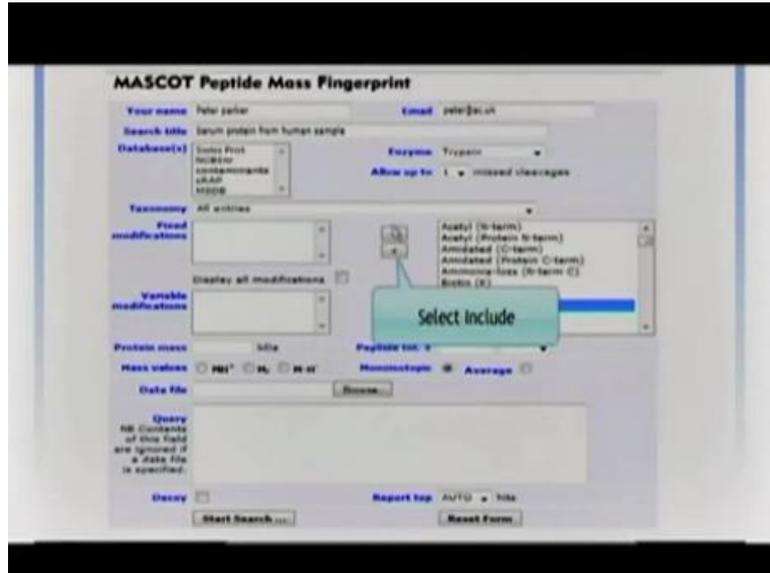
When you are not sure of organism select all entries.

(Refer Slide Time 28:20)



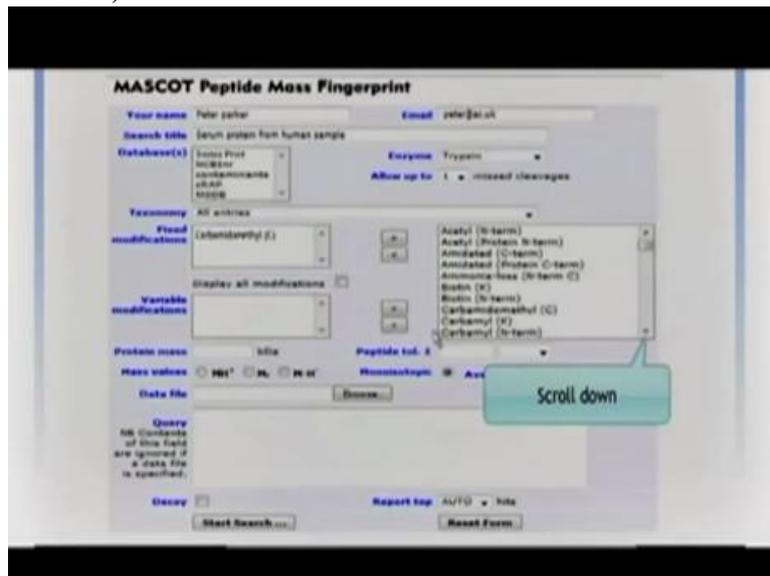
Fixed modification, select carbamidomethyl; fixed modifications are applied collectively

(Refer Slide Time 28:27)



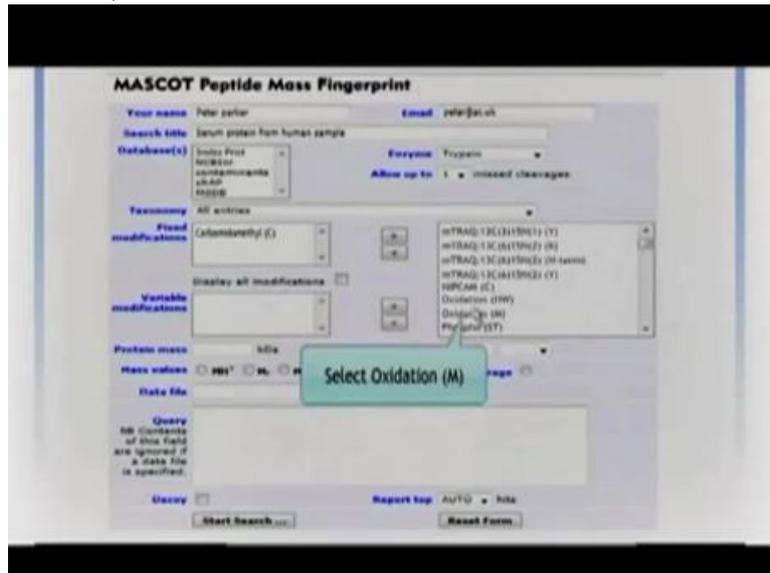
across the database to account for change in mass of specific residue

(Refer Slide Time 28:34)



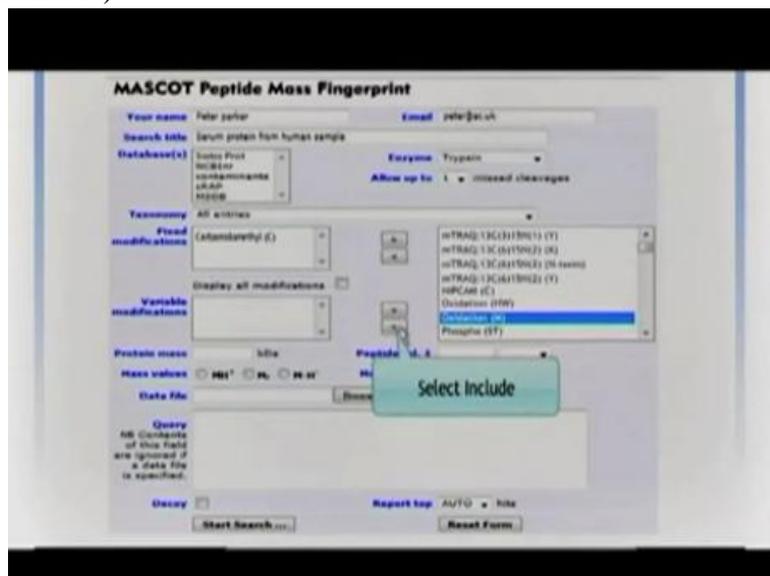
Now scroll down and select oxidation.

(Refer Slide Time 28:40)



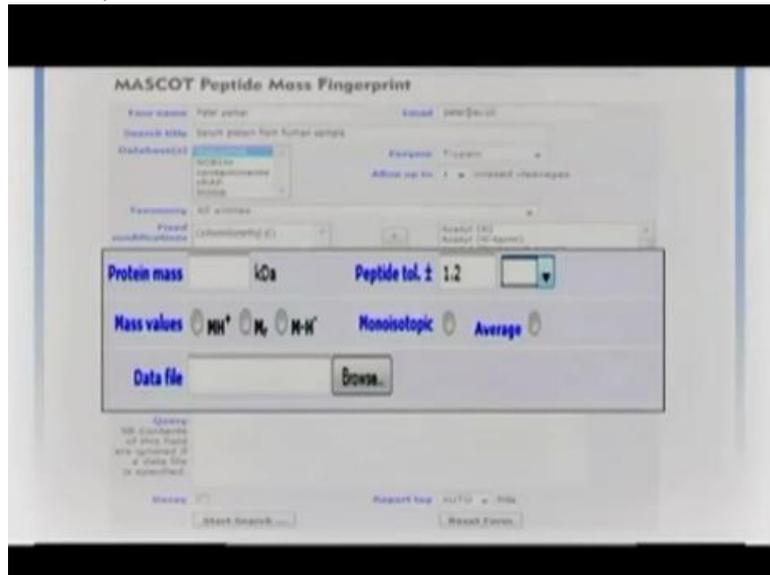
These are mass changes suspected to occur during sample handling and accounted for by increasing the number of primary sequences compared against experimental masses.

(Refer Slide Time 28:47)



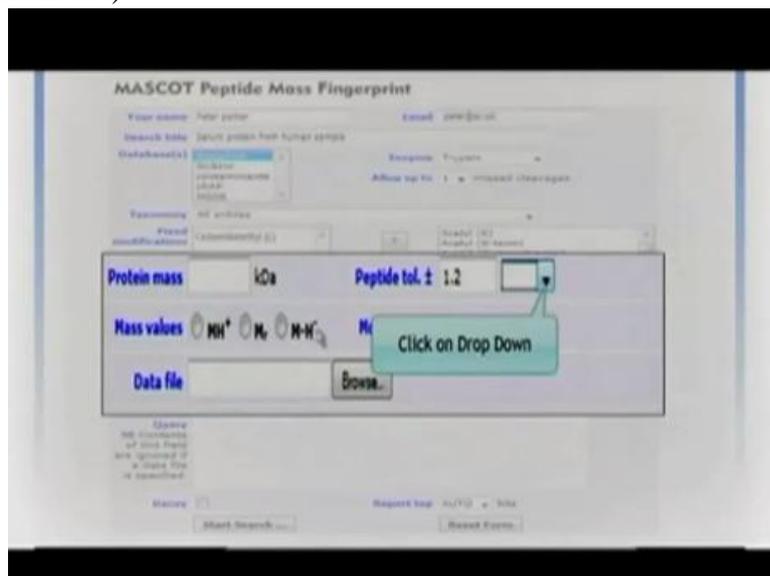
Include it as variable modification.

(Refer Slide Time 28:57)



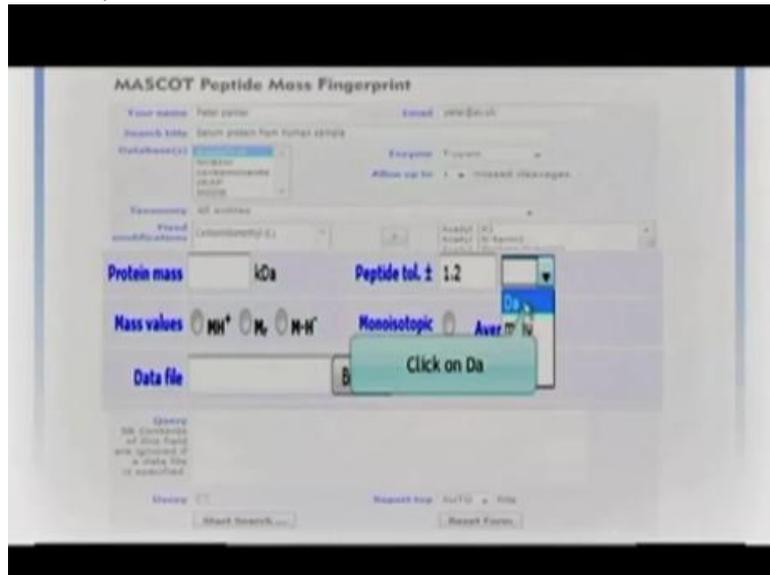
Depending upon user needs the parameters can be changed. Protein mass is the mass in that protein and is optional.

(Refer Slide Time 29:04)



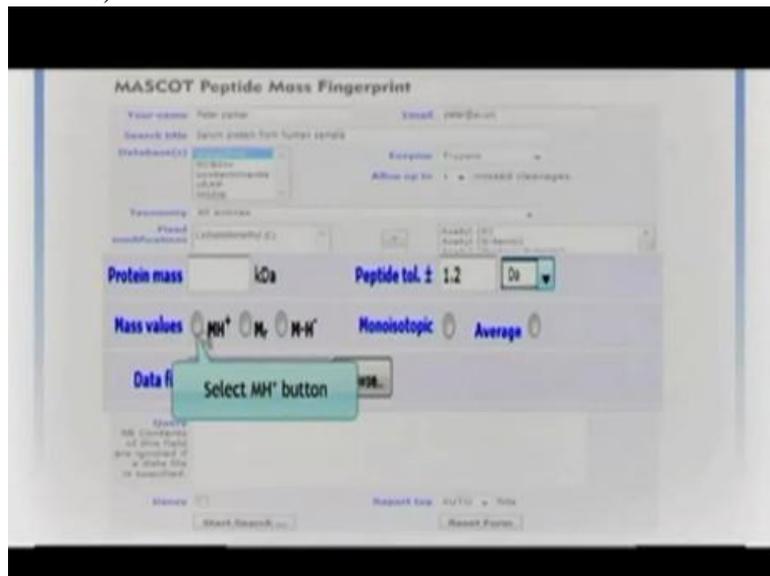
Set peptide tolerance +/- 1.2

(Refer Slide Time 29:07)



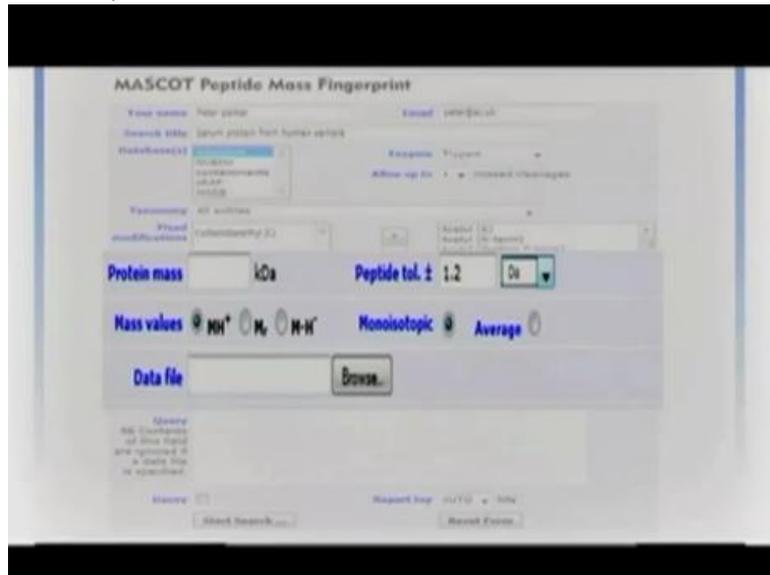
Daltons.

(Refer Slide Time 29:16)



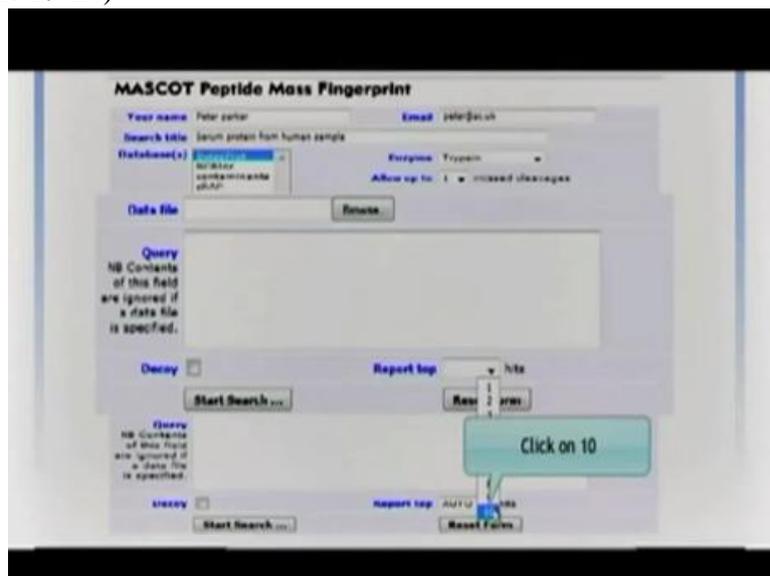
Mass values, it specifies the type of charge to be examined. Select MH positive.

(Refer Slide Time 29:21)

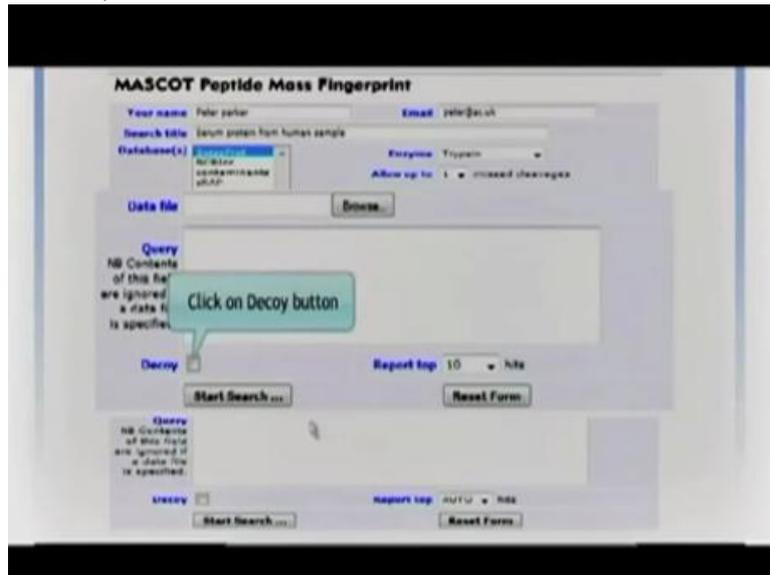


Select mono-isotopic.

(Refer Slide Time 29:24)

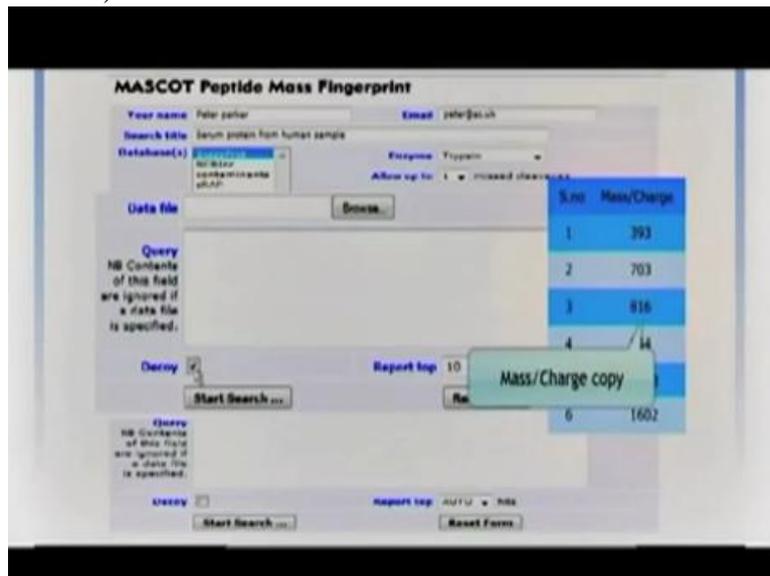


(Refer Slide Time 29:26)



Select Decoy for statistical analysis.

(Refer Slide Time 29:30)



Copy the m by z value

(Refer Slide Time 29:35)

MASCOT Peptide Mass Fingerprint

Your name: Peter Zeller Email: peter@zeller.ch

Search title: Serum protein from human sample

Database(s): UniProt Enzyme: Trypsin

Allow up to: 1 missed cleavage(s)

Data file: Browse...

Query: **paste the peptide mass data in the Query window**

Decoy: Report top: 10 hits

Start Search... Reset Form

S.no	Mass/Charge
1	393
2	703
3	816
4	944
5	1598
6	1602

And paste in the selected box for Mascot search

(Refer Slide Time 29:36)

MASCOT Peptide Mass Fingerprint

Your name: Peter Zeller Email: peter@zeller.ch

Search title: Serum protein from human sample

Database(s): UniProt Enzyme: Trypsin

Allow up to: 1 missed cleavage(s)

Data file: Browse...

Query: 393
703
816
944
1598
1602

Decoy: Report top: 10 hits

Start Search... Reset Form

S.no	Mass/Charge
1	393
2	703
3	816
4	944
5	1598
6	1602

Click on Start Search

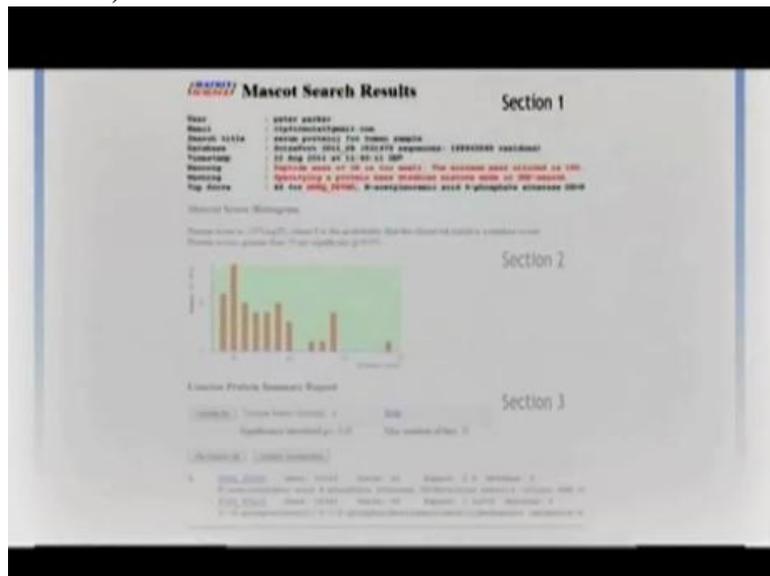
Click on search.

(Refer Slide Time 29:41)



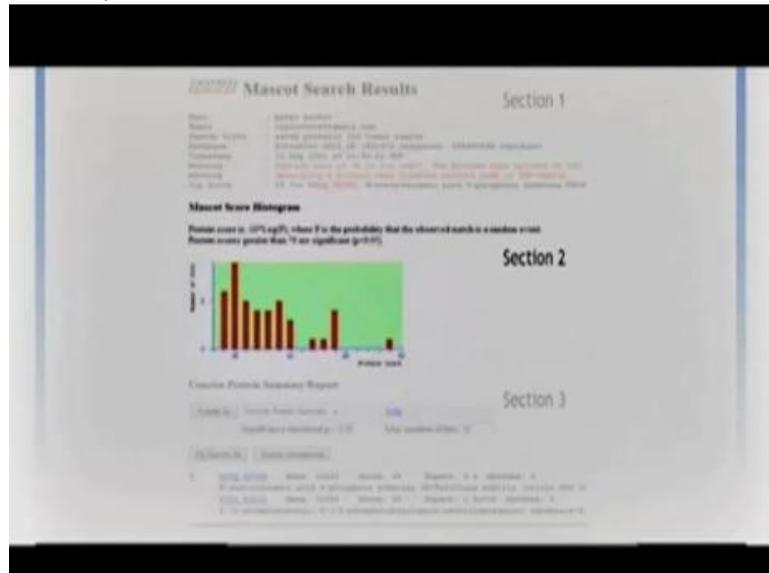
Data output

(Refer Slide Time 29:43)



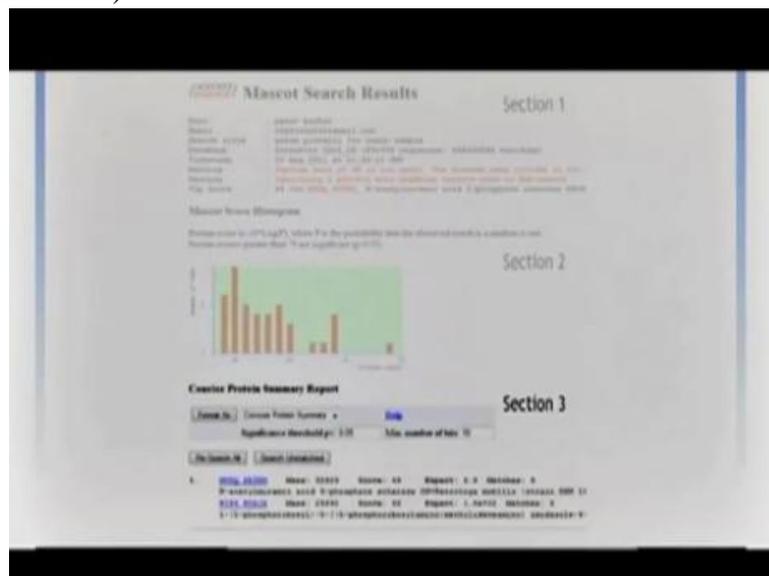
The output can be seen in 3 sections. In section 1, the summary of spec parameters defined by user.

(Refer Slide Time 29:54)



Section 2, Mascot's co-histogram, the number of proteins with score is plotted along the graph.

(Refer Slide Time 30:03)



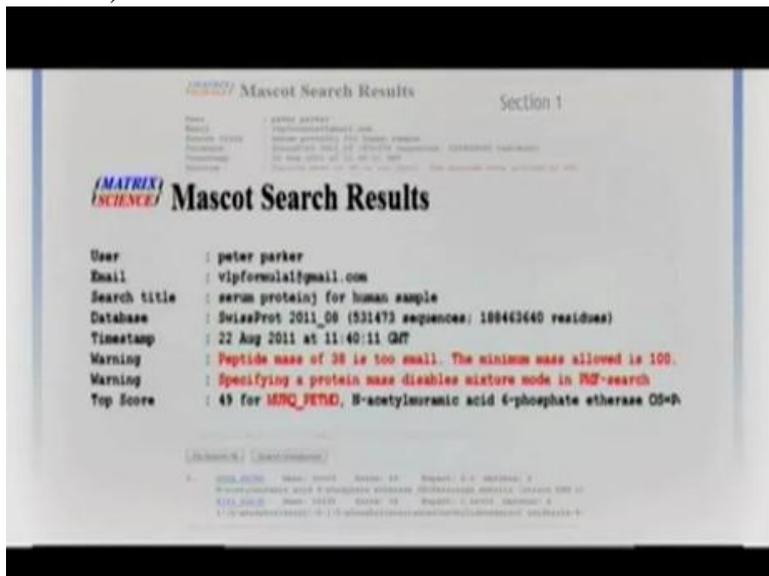
Section 3, summary report in which the matched protein from the database with the details of important parameters are displayed either in concise format, protein format and the data can be exported too.

(Refer Slide Time 30:17)



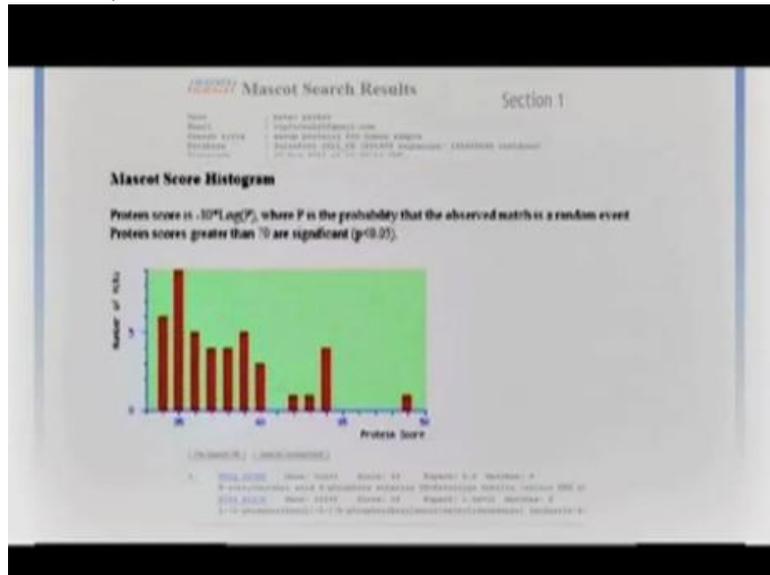
Data Analysis, If the search parameters are not the best fit,

(Refer Slide Time 30:22)



the software generates the error message Depending on the error message, the user needs to change the parameter setting and do the search again.

(Refer Slide Time 30:34)



In section 2, the Mascot score histogram, the number of protein hits and their score is displayed along the graph.

(Refer Slide Time 30:41)

Protein View

Name: [REDACTED] Score: 88 Expect: 0.0
P-antigenase acid 2-phosphate esterase (P-antigenase esterase (EC 3.1.1.1) (Pantase) (Pantase))

Unblast score (E): 0.000 (calculated at score: 0.00)
MS/MS search of [REDACTED] against the
Unformatted [REDACTED] for getting data other applications

Peptide: [REDACTED]

Peptide by Peptide: only N-term side of 88 values that matches to 0
Number of mass values searched: 18
Number of mass values searched: 3
Sequence coverage: 100

Peptide sequence shown in MS/MS And

1 MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK
44 MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK
100 MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK
156 MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK
200 MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK
256 MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK MSKSLTQK

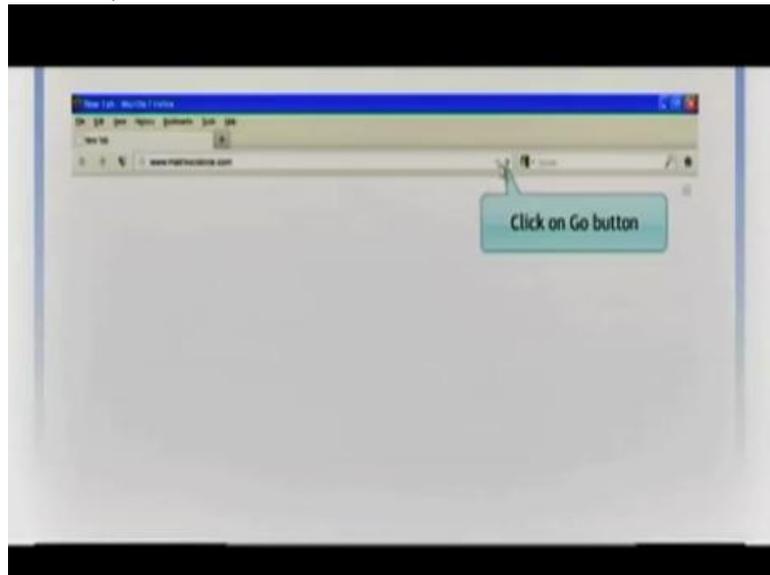
Show protein details...

Sort Options By: Score Number Increasing Mass Decreasing Mass

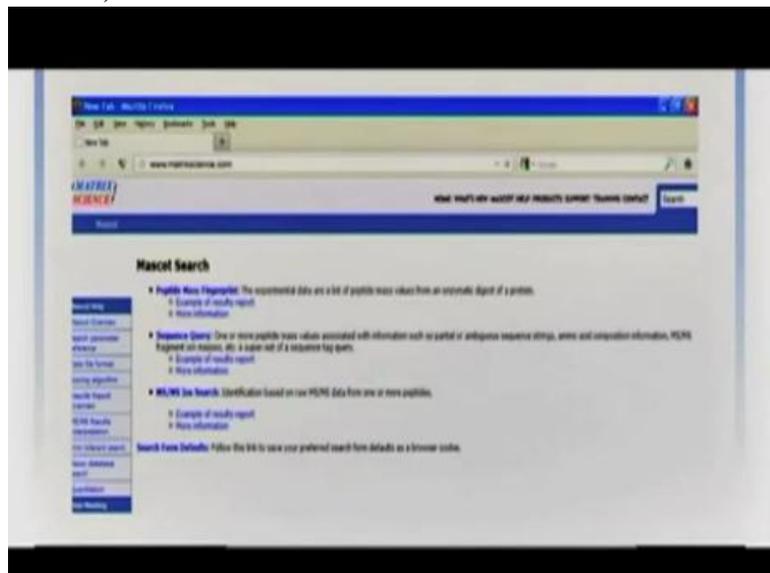
Start + End	Observed	Weighted	Weighted	Delta	Mass Sequence
10 - 15	888.2000	888.2070	888.2000	0.1130	0 N. [REDACTED]
116 - 120	888.2000	888.2170	888.2000	0.2450	0 N. [REDACTED]
120 - 123	888.2000	888.2170	888.2000	0.2450	0 N. [REDACTED]
156 - 159	888.2000	888.2170	888.2000	0.2450	0 N. [REDACTED]
200 - 203	888.2000	888.2170	888.2000	0.2450	0 N. [REDACTED]
256 - 259	888.2000	888.2170	888.2000	0.2450	0 N. [REDACTED]
257 - 261	1458.2000	1458.2170	1458.2000	0.2450	1 N. [REDACTED]

Protein view section displays matching of the query peptide to the protein sequence in the database.

(Refer Slide Time 31:02)

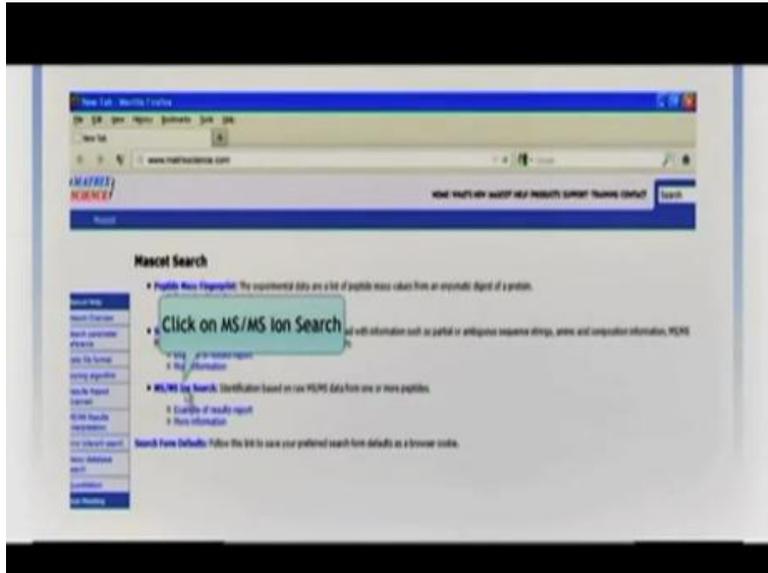


(Refer Slide Time 31:04)



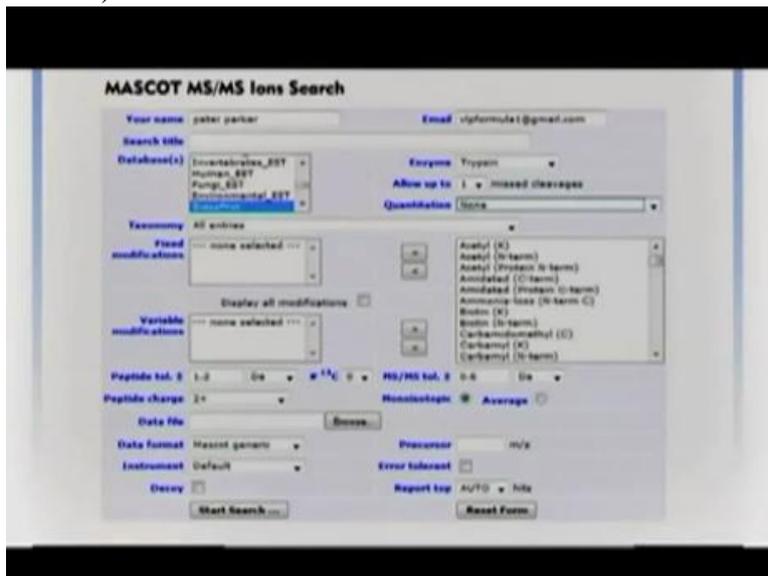
For such data analysis,

(Refer Slide Time 31:07)



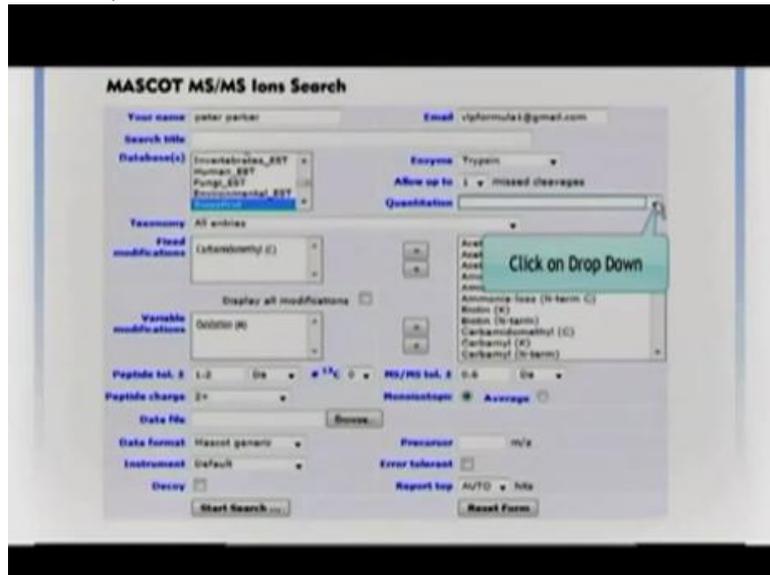
MS ion search option is selected from the Matrix Science browser window.

(Refer Slide Time 31:10)



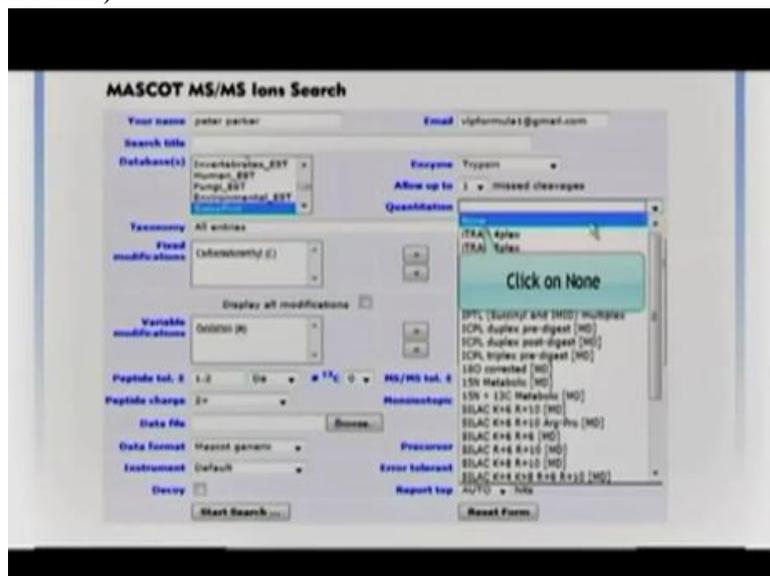
In MS/MS search tool, more input parameters like quantitation, MS/MS tolerance, peptide charge instrument etc in addition to fields for PMF and rest other parameters are similar to that of the peptide mass fingerprint.

(Refer Slide Time 31:29)



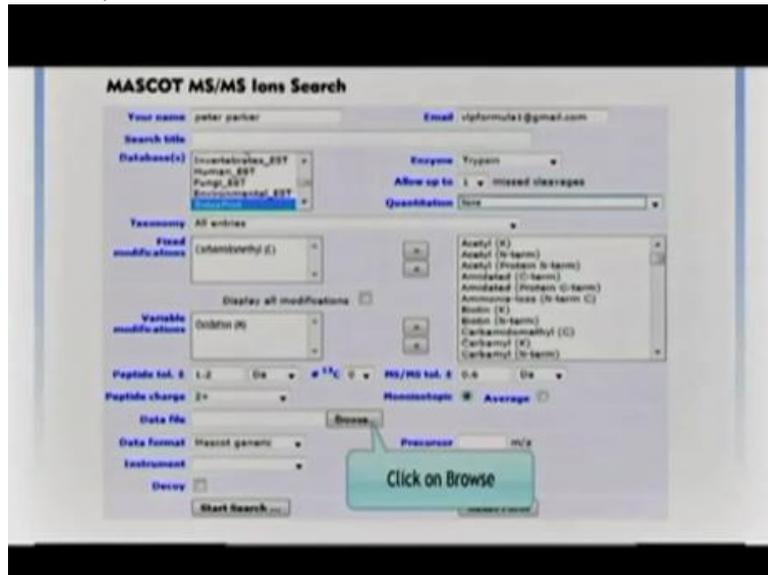
Depending upon the process carried out for data generation, a selection in the quantitation must be made.

(Refer Slide Time 31:36)



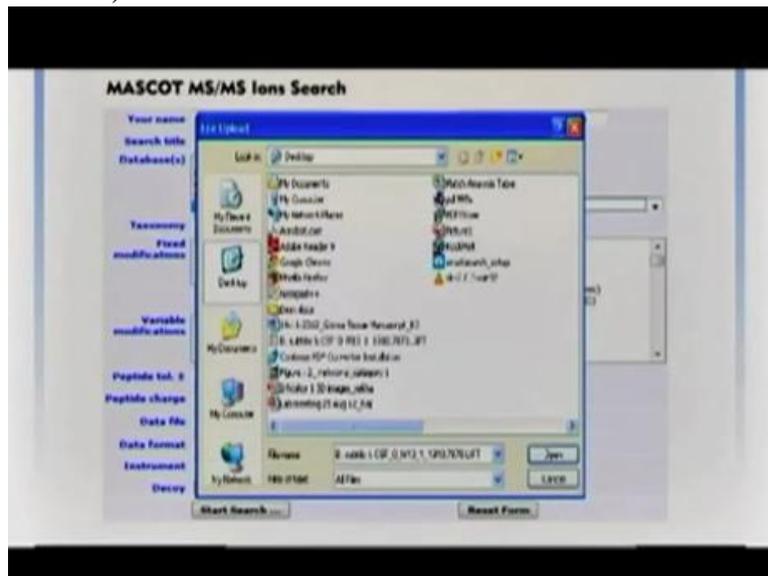
In case of label-free quantitation, select none in quantitation tab.

(Refer Slide Time 31:41)



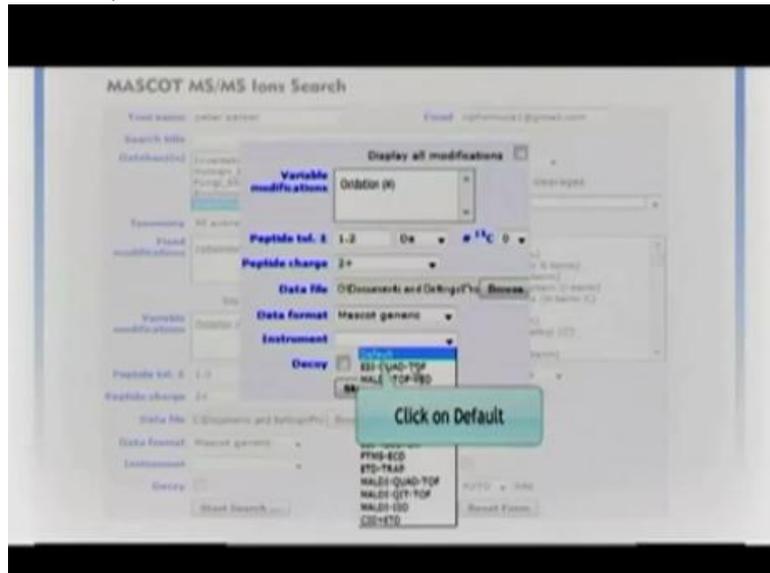
Remaining parameters remain same as PMF. Browse the MS/MS raw data file

(Refer Slide Time 31:48)



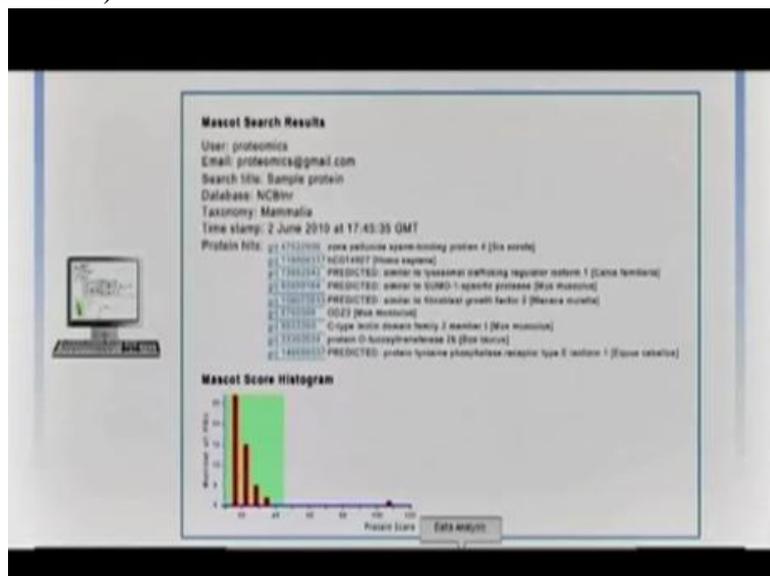
And search the results using Mascot.

(Refer Slide Time 31:56)



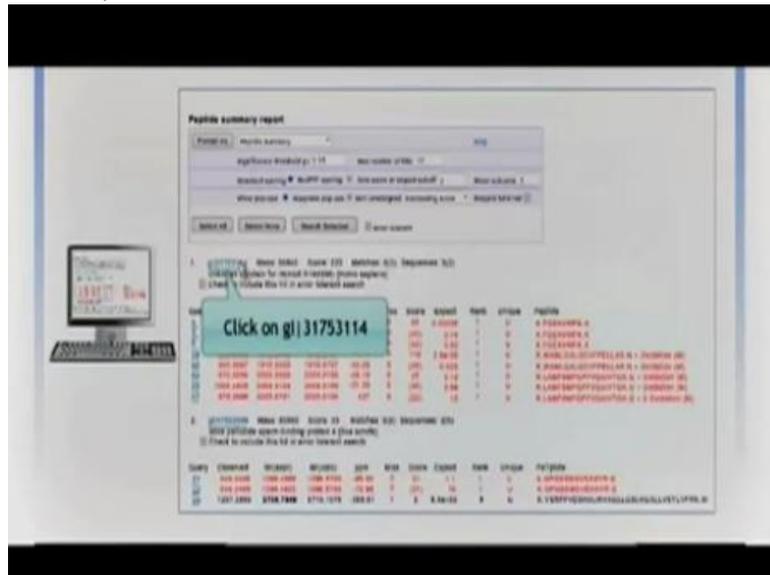
Define the instrument that has been used to generate the raw data. When we don't know the name of the instrument select default

(Refer Slide Time 32:05)



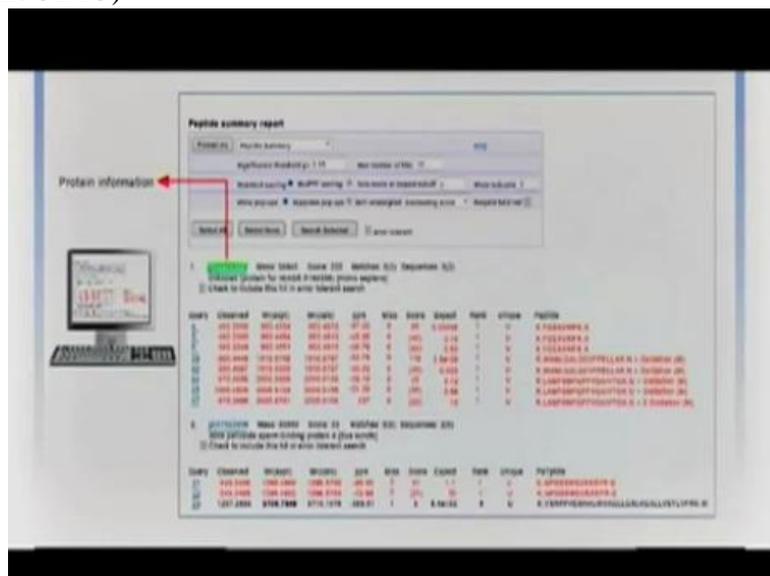
The result output generated is almost similar to PMF.

(Refer Slide Time 32:09)



The accession ID indicates the protein information

(Refer Slide Time 32:13)



Obtained from the database marked as green The protein hit outside the green box indicates the p value less than 0.05 which is statistically significant, where as if inside the green box indicates random matching.

(Refer Slide Time 32:41)

Mascot search results

Protein view

Match to: p1379214 Score: 235

Unknown protein for MS02.S14033 (30aa segment)

Found in search of: C:\Users\homer\Desktop\MS02.S14033.ms2 analysis file data file - mgf FileData File1.mgf

Nominal mass (kDa): 20.000 Calculated pI value: 4.80

NCBI BLAST search of p1379214

Other applications

Predicted mass of the protein.

Link to retrieve other details concerning this sequence from NCBI Entrez: [K122220219](#) View Data/Annotations

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Coverage by Trypsin: 100% (100% side of KR unless next residue is P)

Sequence Coverage: 100%

Matched peptide chains in Data File:

1	MSKPTFNEK	GRKTRFLLK	AAATTKAKL	YFQKAKK	ILPDAFQK
11	AKTAKETAK	AAKPTSLQK	MYGKSNLK	GLLGGPKK	LTKPTPKK
16	WETALIQK	ETFAKHEK	MYGKSNLK	ALGKPKK	AKKPKLEK
18	YKFAKSLQK	YKGLKSLK	YKIKKPKK	AKKPKLEK	GAKPKKAK
20	SPKLEPKK	YKGLKSLK	LAKPKFQK	LVKPKKAK	LAKPKPKK
23	YKATKSLQK	LVKAKSLK	YKAKKPKK	YKPKK	

Nominal mass, it is predicted mass of the protein.

(Refer Slide Time 32:48)

Mascot search results

Protein view

Match to: p1379214 Score: 235

Unknown protein for MS02.S14033 (30aa segment)

Found in search of: C:\Users\homer\Desktop\MS02.S14033.ms2 analysis file data file - mgf FileData File1.mgf

Nominal mass (kDa): 20.000 Calculated pI value: 4.80

NCBI BLAST search of p1379214

Other applications

Predicted isoelectric point of the protein.

Link to retrieve other details concerning this sequence from NCBI Entrez: [K122220219](#) View Data/Annotations

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Coverage by Trypsin: 100% (100% side of KR unless next residue is P)

Sequence Coverage: 100%

Matched peptide chains in Data File:

1	MSKPTFNEK	GRKTRFLLK	AAATTKAKL	YFQKAKK	ILPDAFQK
11	AKTAKETAK	AAKPTSLQK	MYGKSNLK	GLLGGPKK	LTKPTPKK
16	WETALIQK	ETFAKHEK	MYGKSNLK	ALGKPKK	AKKPKLEK
18	YKFAKSLQK	YKGLKSLK	YKIKKPKK	AKKPKLEK	GAKPKKAK
20	SPKLEPKK	YKGLKSLK	LAKPKFQK	LVKPKKAK	LAKPKPKK
23	YKATKSLQK	LVKAKSLK	YKAKKPKK	YKPKK	

Calculated pI value, isoelectric point of the protein.

(Refer Slide Time 32:56)

Mascot search results

Protein view

Match to: p1372314 (score: 22)

Database: Uniprot (search for UniProt: P1372314) (name: unknown)

Found in search of: C:\Users\homer\Desktop\MSI\LC-MS-MS data analysis\Raw data\Hsp\mgf\RawData\Hsp1.mgf

Normal mass (Da): 10460; Calculated pI: 4.80

MS/MS search of: p1372314 against an Uniprot database (see [Mascot help](#)) for parsing into other applications

Taxonomy: [View taxonomy](#)

Links to retrieve other entries containing this sequence from NCBI Entrez: [Accession](#) [Gene](#) [PubMed](#)

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Coverage by Trypsin: only C-term side of KR unless next residue is P

Sequence Coverage: 3%

Indicates the % of matching peptides.

126	142	960.4446	1918.8708	1918.8797	-55	0	R.WANL	GALGCVPELLAR.H	Oxidation (M)	(100% score: 112)
126	142	960.4587	1918.9029	1918.8797	-40	0	R.WANL	GALGCVPELLAR.H	Oxidation (M)	(100% score: 85)
147	154	492.2200	982.4254	982.4913	-47	0	R.FGLAVWFR.A	(100% score: 84)		
147	154	492.2305	982.4464	982.4913	-46	0	R.FGLAVWFR.A	(100% score: 79)		
147	154	492.2348	982.4551	982.4913	-37	0	R.FGLAVWFR.A	(100% score: 33)		
241	238	470.6395	2008.8964	2008.9130	-39	0	R.LAHFIMFQFFVQAVYFK.D	Oxidation (M)	(100% score: 42)	
241	238	468.4678	2008.9134	2008.9139	-31	0	R.LAHFIMFQFFVQAVYFK.D	Oxidation (M)	(100% score: 33)	
241	238	474.2964	2025.8743	2025.9104	-427	0	R.LAHFIMFQFFVQAVYFK.D	2 Oxidation (M)	(100% score: 22)	

Sequence coverage indicates the percentage of matching peptide. All the peptides are displayed with matching peptides indicated in red.

(Refer Slide Time 33:10)

Mascot search results

Protein view

Show predicted peptides also

Sort Peptides By: Residue Number Increasing Mass Decreasing Mass

Start	End	Observed	M(expt)	M(cal)	Delta	Miss	Sequence			
126	142	960.4446	1918.8708	1918.8797	-55	0	R.WANL	GALGCVPELLAR.H	Oxidation (M)	(100% score: 112)
126	142	960.4587	1918.9029	1918.8797	-40	0	R.WANL	GALGCVPELLAR.H	Oxidation (M)	(100% score: 85)
147	154	492.2200	982.4254	982.4913	-47	0	R.FGLAVWFR.A	(100% score: 84)		
147	154	492.2305	982.4464	982.4913	-46	0	R.FGLAVWFR.A	(100% score: 79)		
147	154	492.2348	982.4551	982.4913	-37	0	R.FGLAVWFR.A	(100% score: 33)		
241	238	470.6395	2008.8964	2008.9130	-39	0	R.LAHFIMFQFFVQAVYFK.D	Oxidation (M)	(100% score: 42)	
241	238	468.4678	2008.9134	2008.9139	-31	0	R.LAHFIMFQFFVQAVYFK.D	Oxidation (M)	(100% score: 33)	
241	238	474.2964	2025.8743	2025.9104	-427	0	R.LAHFIMFQFFVQAVYFK.D	2 Oxidation (M)	(100% score: 22)	

The protein view obtained on

(Refer Slide Time 33:20)

Mascot search results
Protein view

Show predicted peptides also

Indicates beginning & end of each peptide

Number Increasing Mass Decreasing Mass

Start-End	Observed	Mr(expt)	Mr(cal)	Delta	Miss	Sequence
126-142	960.4446	1918.8796	1918.8797	-55	0	R.WANLGALGCVPELLAR.N Oxidation (M) (100% SCORE 112)
126-142	960.4587	1918.9029	1918.8797	-40	0	R.WANLGALGCVPELLAR.N Oxidation (M) (100% SCORE 48)
147-154	492.2200	982.4254	982.4913	-67	0	K.FGQAVWFR.A (100% SCORE 85)
147-154	492.2305	982.4484	982.4913	-46	0	K.FGQAVWFR.A (100% SCORE 80)
147-154	492.2348	982.4551	982.4913	-37	0	K.FGQAVWFR.A (100% SCORE 73)
241-238	670.6395	2008.8966	2009.0195	-19	0	R.LAHFSPGFVQAVYTK.G Oxidation (M) (100% SCORE 42)
241-238	1008.4835	2008.9124	2009.0195	-51	0	R.LAHFSPGFVQAVYTK.G Oxidation (M) (100% SCORE 33)
241-238	676.2986	2025.8741	2025.0104	427	0	R.LAHFSPGFVQAVYTK.G 2 Oxidation (M) (100% SCORE 24)

Selecting the particular protein link is very similar to protein view obtained in PMF.

(Refer Slide Time 33:28)

Mascot search results
Protein view

Show predicted peptides also

Observed molecular weight

Number Increasing Mass Decreasing Mass

Start-End	Observed	Mr(expt)	Mr(cal)	Delta	Miss	Sequence
126-142	960.4446	1918.8796	1918.8797	-55	0	R.WANLGALGCVPELLAR.N Oxidation (M) (100% SCORE 112)
126-142	960.4587	1918.9029	1918.8797	-40	0	R.WANLGALGCVPELLAR.N Oxidation (M) (100% SCORE 48)
147-154	492.2200	982.4254	982.4913	-67	0	K.FGQAVWFR.A (100% SCORE 85)
147-154	492.2305	982.4484	982.4913	-46	0	K.FGQAVWFR.A (100% SCORE 80)
147-154	492.2348	982.4551	982.4913	-37	0	K.FGQAVWFR.A (100% SCORE 73)
241-238	670.6395	2008.8966	2009.0195	-19	0	R.LAHFSPGFVQAVYTK.G Oxidation (M) (100% SCORE 42)
241-238	1008.4835	2008.9124	2009.0195	-51	0	R.LAHFSPGFVQAVYTK.G Oxidation (M) (100% SCORE 33)
241-238	676.2986	2025.8741	2025.0104	427	0	R.LAHFSPGFVQAVYTK.G 2 Oxidation (M) (100% SCORE 24)

It provides detailed information of each of the matched peptide displayed.

(Refer Slide Time 33:31)

Mascot search results
Protein view
Show predicted peptides also
Sort Peptides: Experimental molecular weight, Missing mass, Decreasing Mass

Start	End	Observed	M(r)exptl	M(r)calc	Delta	Miss	Sequence
126	142	960.4446	1918.8746	1918.9797	-55	0	K.WANL G ALGCVPELLAR.N Oxidation (M) (IONS SCORE 118)
126	142	960.4587	1918.9029	1918.9797	-40	0	K.WANL G ALGCVPELLAR.N Oxidation (M) (IONS SCORE 85)
147	154	492.2200	982.4254	982.4913	-47	0	K.FGLAVYFK.A (IONS SCORE 85)
147	154	492.2305	982.4464	982.4913	-46	0	K.FGLAVYFK.A (IONS SCORE 79)
147	154	492.2348	982.4551	982.4913	-37	0	K.FGLAVYFK.A (IONS SCORE 73)
241	238	670.6395	2008.8946	2008.0195	-59	0	R.LANFSHPGFYVQAVYTK.G Oxidation (M) (IONS SCORE 62)
241	238	1005.4435	2008.9124	2008.0195	-51	0	R.LANFSHPGFYVQAVYTK.G Oxidation (M) (IONS SCORE 33)
241	238	676.2986	2023.8741	2023.0104	-427	0	R.LANFSHPGFYVQAVYTK.G 2 Oxidation (M) (IONS SCORE 24)

The start and end position of amino acids calculated and experimental molecular weights, number of missed tryptic cleavages, sequence of each peptide segment and their corresponding ion scores are shown.

(Refer Slide Time 33:33)

Mascot search results
Protein view
Show predicted peptides also
Sort Peptides By: Calculated molecular weight, Decreasing Mass

Start	End	Observed	M(r)exptl	M(r)calc	Delta	Miss	Sequence
126	142	960.4446	1918.8746	1918.9797	-55	0	K.WANL G ALGCVPELLAR.N Oxidation (M) (IONS SCORE 118)
126	142	960.4587	1918.9029	1918.9797	-40	0	K.WANL G ALGCVPELLAR.N Oxidation (M) (IONS SCORE 85)
147	154	492.2200	982.4254	982.4913	-47	0	K.FGLAVYFK.A (IONS SCORE 85)
147	154	492.2305	982.4464	982.4913	-46	0	K.FGLAVYFK.A (IONS SCORE 79)
147	154	492.2348	982.4551	982.4913	-37	0	K.FGLAVYFK.A (IONS SCORE 73)
241	238	670.6395	2008.8946	2008.0195	-59	0	R.LANFSHPGFYVQAVYTK.G Oxidation (M) (IONS SCORE 62)
241	238	1005.4435	2008.9124	2008.0195	-51	0	R.LANFSHPGFYVQAVYTK.G Oxidation (M) (IONS SCORE 33)
241	238	676.2986	2023.8741	2023.0104	-427	0	R.LANFSHPGFYVQAVYTK.G 2 Oxidation (M) (IONS SCORE 24)

The highest ion scores are used for computing the final protein score.

(Refer Slide Time 33:40)

Mascot search results

Protein view

Show predicted peptides also

Sort Peptides By

- Residue Number
- Increasing Mass
- Decreasing Mass

Indicates score of each ion fragment. Used for calculation of the protein score.

Start	End	Observed	M(expt)	M(calc)	Delta	Miss	Sequence
126	142	960.4446	1918.8798	1918.8797	-55	0	K.WANLGLGCYPELLAR.K Oxidation (M) (Score: 11.11)
126	142	960.4587	1918.9029	1918.8797	-40	0	K.WANLGLGCYPELLAR.K Oxidation (M) (Score: 10.41)
147	154	492.2200	982.4254	982.4913	-47	0	K.FGLAVYK.A (Score: 9.59)
147	154	492.2305	982.4464	982.4913	-46	0	K.FGLAVYK.A (Score: 9.59)
147	154	492.2348	982.4551	982.4913	-37	0	K.FGLAVYK.A (Score: 7.7)
241	238	470.6395	2008.8946	2009.0135	-39	0	K.LANFIMPFYVQIVYTK.G Oxidation (M) (Score: 6.42)
241	238	1005.4635	2008.9124	2009.0135	-51	0	K.LANFIMPFYVQIVYTK.G Oxidation (M) (Score: 3.3)
241	238	476.2989	2025.8741	2025.0194	427	0	K.LANFIMPFYVQIVYTK.G Oxidation (M) (Score: 2.4)

(Refer Slide Time 33:47)

Mascot search results

Peptide view

MS/MS Fragmentation of **FGLAVYK**

Found in [gi|21753114](#), Uniprot protein for IMAGE:5194336a [Homo sapiens]

Match to Query #: 982 Peptide sequence whose fragmentation pattern is shown. (9920.0000) to 1173 (r+1732.47, f+2, l+184) [i]qstqfqlqf

Title: Sum of 11 scans

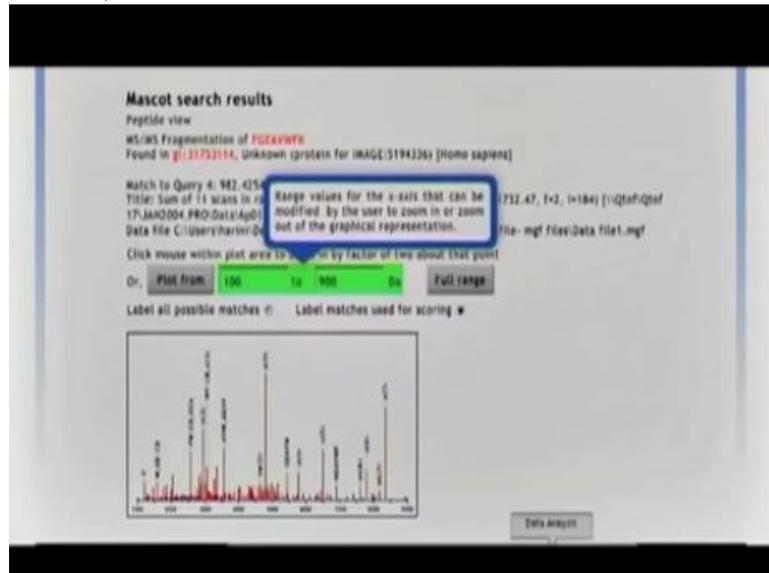
Data File C:\Users\sharni\Desktop\MS-MS data analysis Raw data file- mgf files\Data file1.mgf

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 100 to 900 Da Full range

Label all possible matches Label matches used for scoring

(Refer Slide Time 33:55)



This provides significantly larger amount of information regarding each peptide segment which can be viewed by clicking on the peptide link provided in the summary report. The fragmentation pattern is displayed graphically

(Refer Slide Time 34:13)

Mascot search results
 Peptide view
 Monoisotopic mass of neutral peptide M(zcalc): 982.4913
 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
 Ions Score: 46. Expect: 0.00016
 Matches: 23/78 fragment ions using 16 most intense peaks [View](#)

#	Intron	a	a'	b	b'	Seq	y	y'	y''	#	
1		120.0808	120.0808		148.0757					8	
2		30.0338	177.1022		205.0972	G	816.4301	819.4036	818.4196	7	
3		162.0550	306.1448	288.1343	334.1397	316.1292	L	779.4087	762.3821	761.3981	6
4		44.0495	377.1819	359.1714	405.1769	387.1663	A	650.3661	633.3395	5	
5		72.0808	476.2504	458.2398	504.2453	486.2347	V	579.3289	562.3024	4	
6		159.0917	662.3297	644.3191	690.3246	672.3140	W	680.2407	663.2140	3	
7		120.0808	809.3981	791.3875	837.3930	819.3824	F	294.1812	277.1547	2	
8		101.1073					K	147.1128	130.0863	1	

which can be zoomed into as per the requirement

(Refer Slide Time 34:30)

Points to ponder

- Higher the score, better the hit
- The hit should have at least two peptides
- Higher the sequence coverage, better the hit
- y and b ions should be matched with MS/MS spectrum

(Refer Slide Time 34:35)

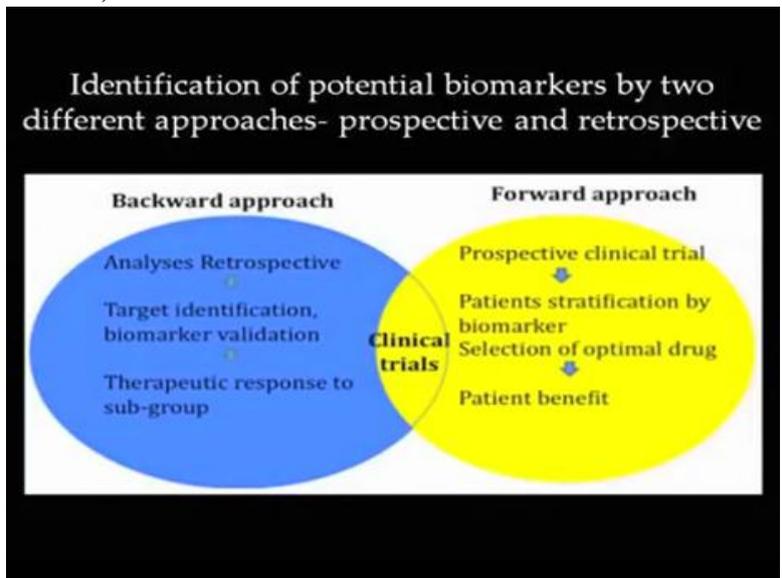
Section V

Challenges associated with translational
research

(Refer Slide Time 34:48)

- Translational proteomics research is a broad field that aims to identify novel diagnostic and prognostic marker proteins for cancer and other human diseases
- Also focuses on identification of novel therapeutic targets for drug and vaccine development
- Long term objective is to provide personalized medication therapy in health care sector
- Translation of knowledge obtained from basic proteomics research is a challenging task
- There is huge gap between the “bench-side” research and “bed-side” implications, often making it difficult to translate findings obtained under lab environments in actual applications

(Refer Slide Time 34:57)



(Refer Slide Time 35:18)

Challenges associated with translational research

- Lack of standard protocols
- Cannot amplify proteins (like PCR used for gene amplification)
- Presence of protein isoforms
- Huge variation in proteome with time within a same individual
- Broad dynamic range of protein concentrations and complexity of biological fluids
- Variations among individuals of same or different populations; difficult to establish standard biomarkers applicable for all populations

(Refer Slide Time 35:42)

Summary

- Sample preparation was discussed
- MALDI-TOF instrumentation demonstrated
- MS hybrid configuration: MALDI-TOF/TOF was discussed
- PMF and MS/MS analysis were discussed
- An overview of challenges associated with translational research provided