Proteins and Gel-Based Proteomics
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Mod 05 Lecture Number 21

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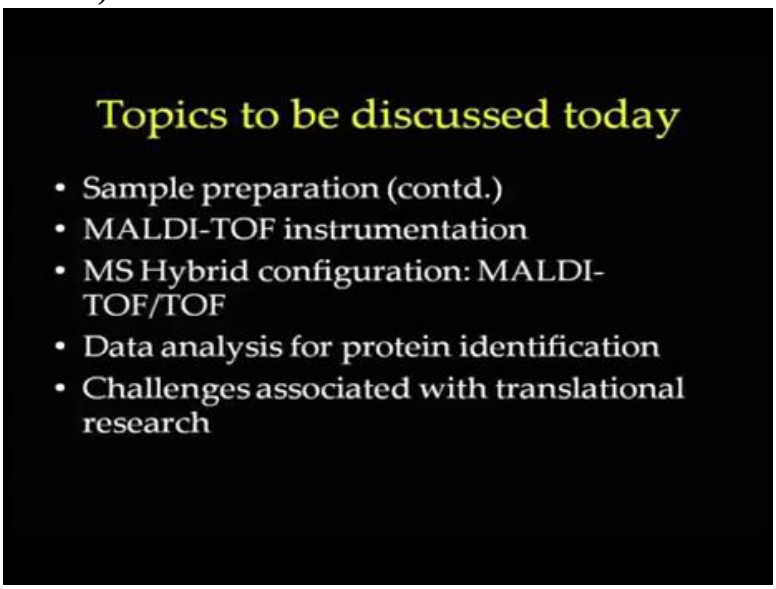

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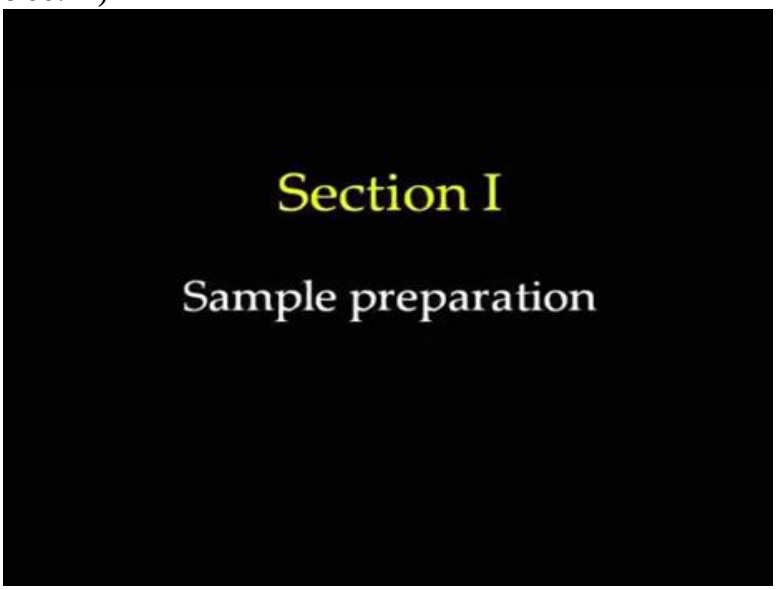

So the in-gel digestion of the proteins isolated by the gel electrophoresis remains a core area in the mass spectrometry or in any of the proteomics applications.

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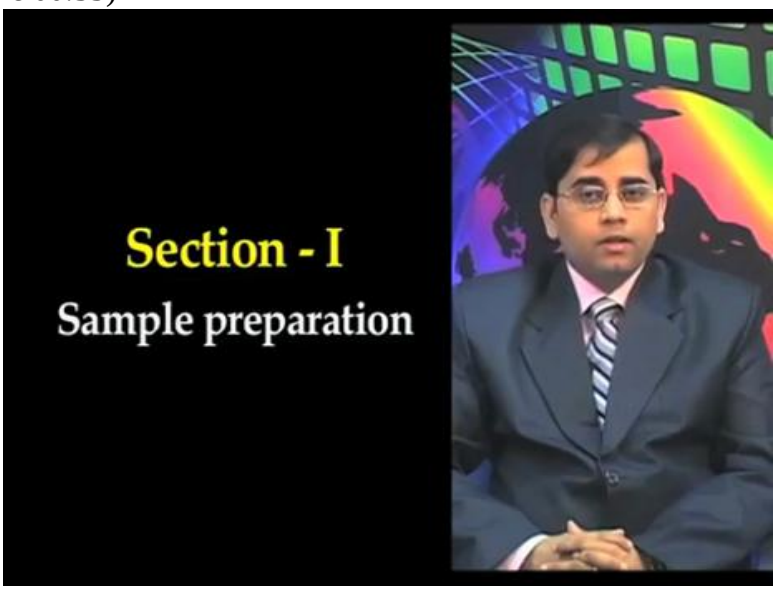

The following video which we are going to watch is going to provide the broad guideline for the in-gel digestion. However the recipe is very flexible and it varies from lab to lab to meet the specific requirements of particular proteomic experiments.

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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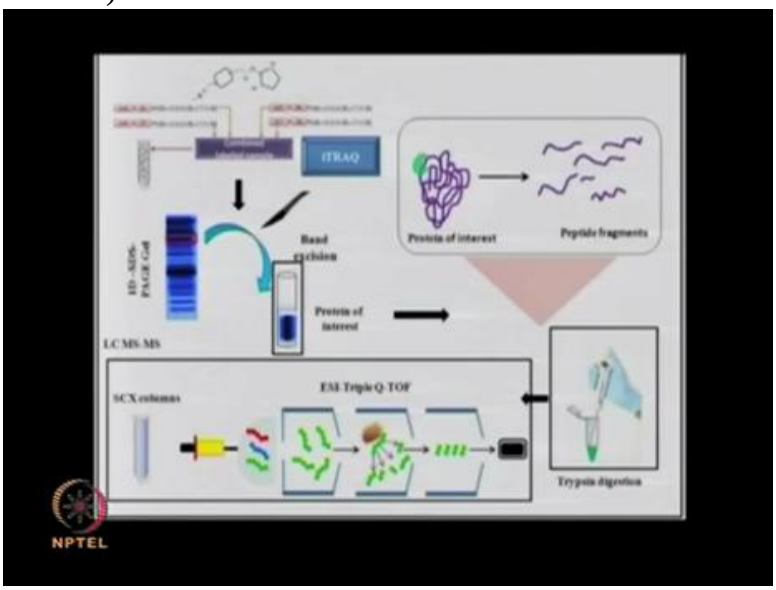

In-gel digested protein for MS Analysis Video

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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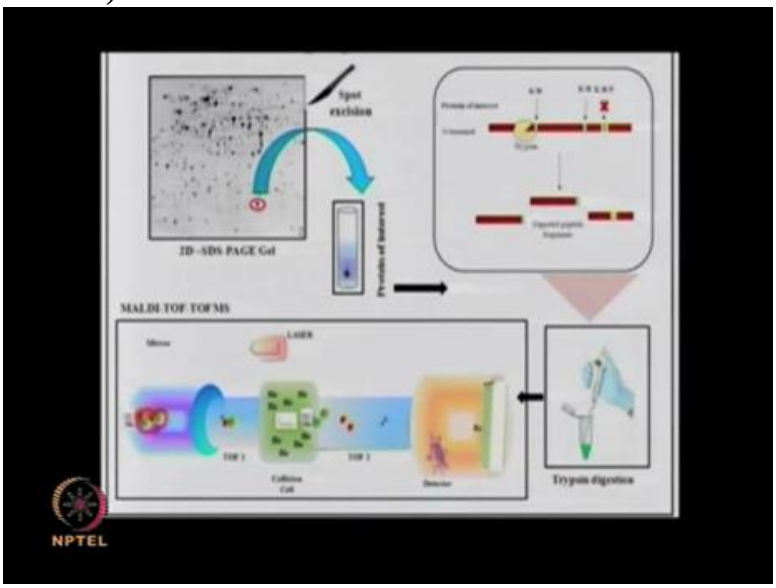

That generates mixture of peptides from the target protein through proteolytic digestion.

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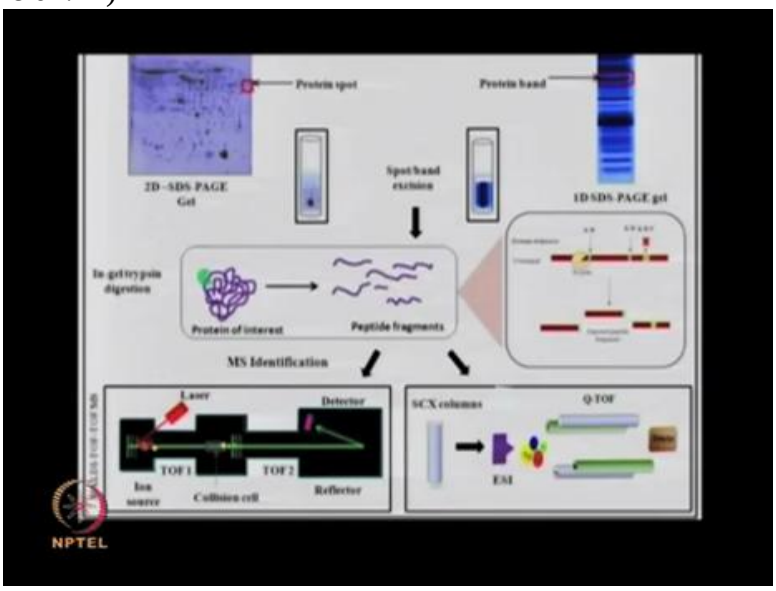

This slide gives you an overview of various steps involved in the in-gel digestion process and also how various of samples can be analyzed by using this method, whether you have 1D SDS PAGE gel, 2D SDS PAGE gel.

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.

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

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

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

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Then cut the slice into cubes

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

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

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

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

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For large gel slices,

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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,

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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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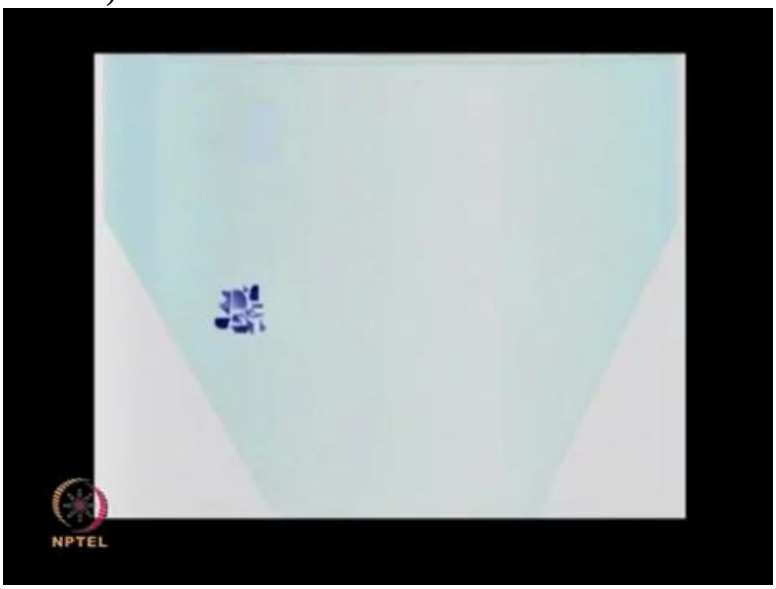

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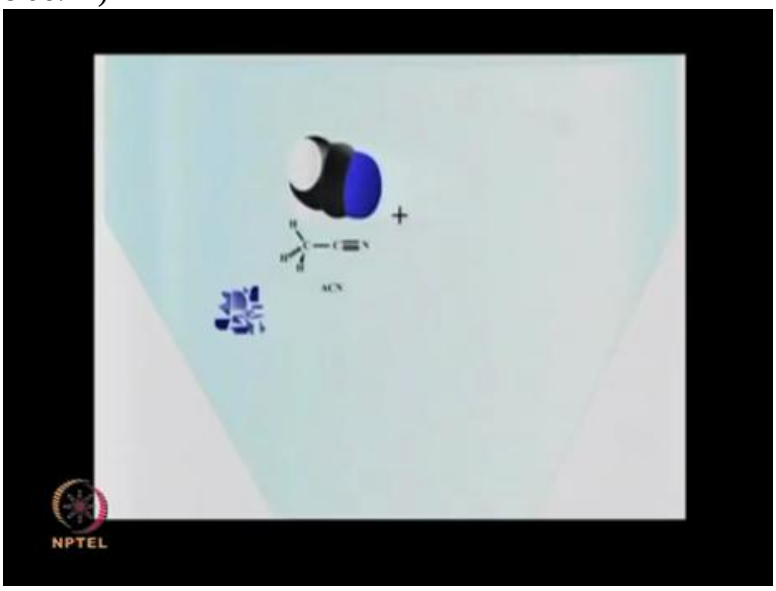

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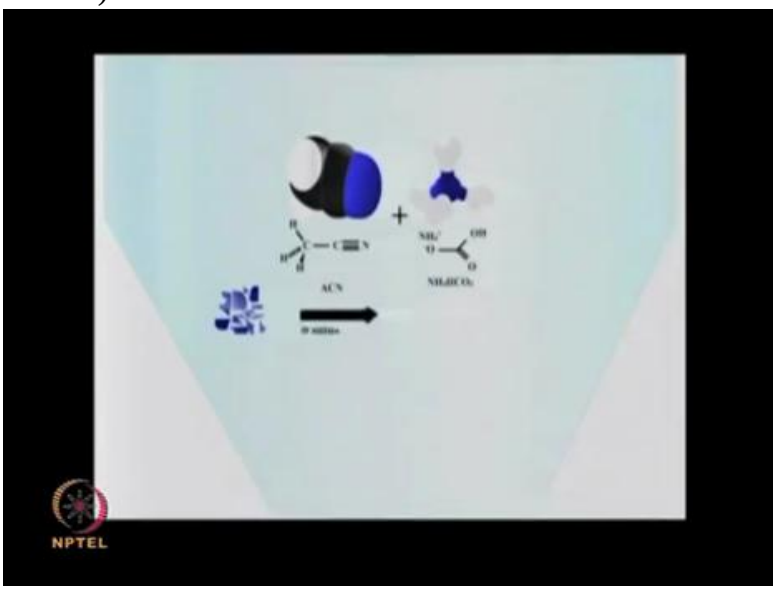

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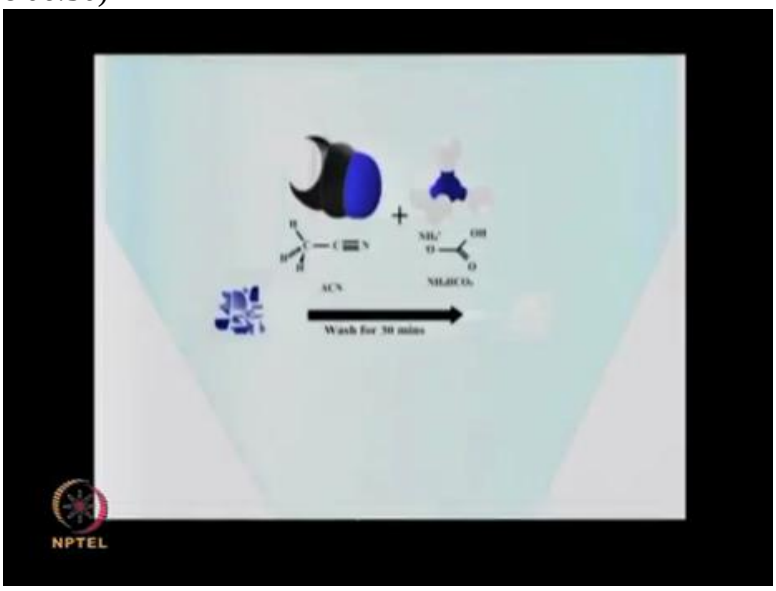

By changing the solution and removing it after every 10 minutes, the Coomassie Brilliant Blue gel pieces, they become colorless. You have seen the animation. Now once the staining is removed,

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

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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.

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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.

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After removing the solution completely,

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

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To completely cover gel pieces

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Incubate it for 30 minutes at 56 degrees Centigrade.

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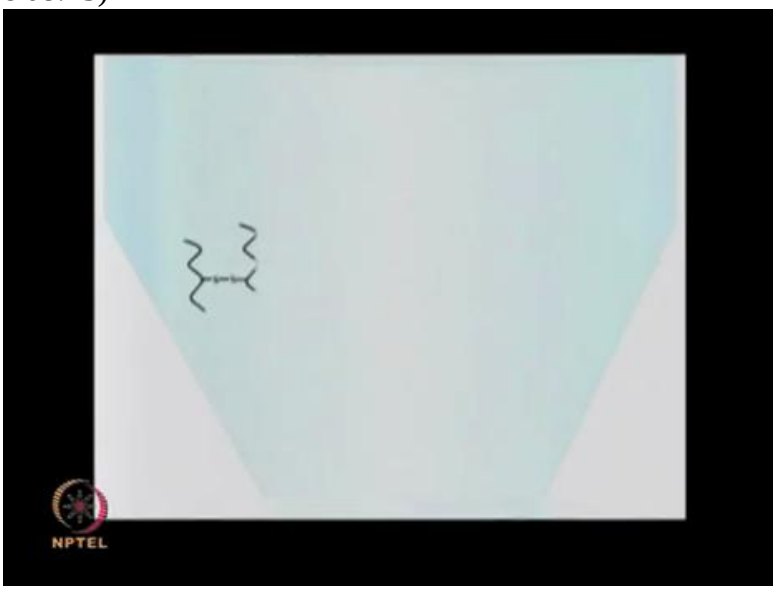

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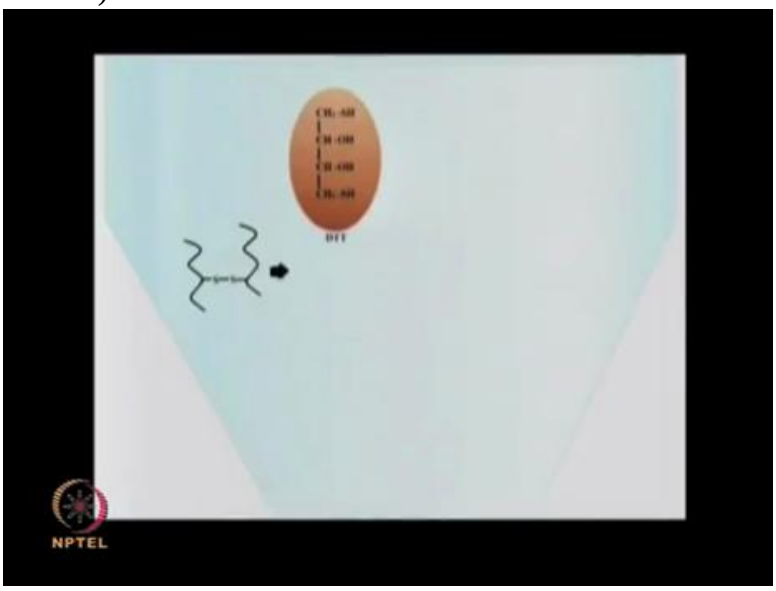

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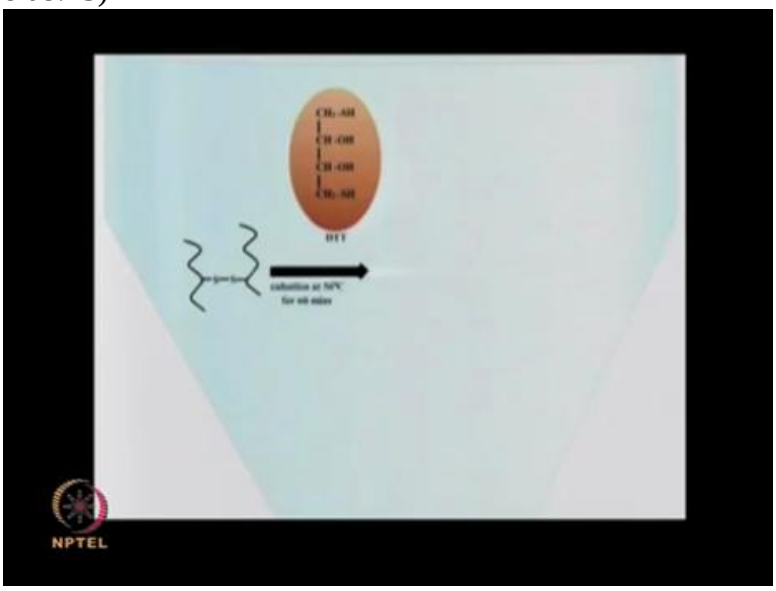

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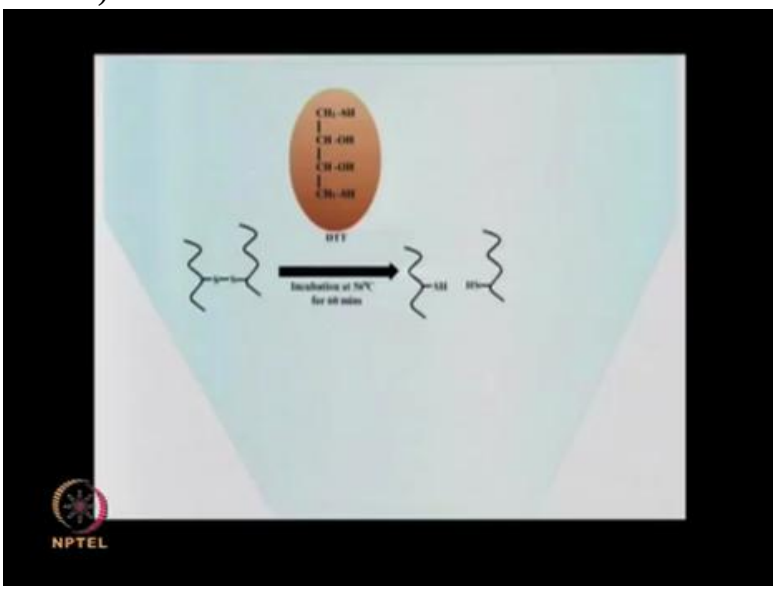

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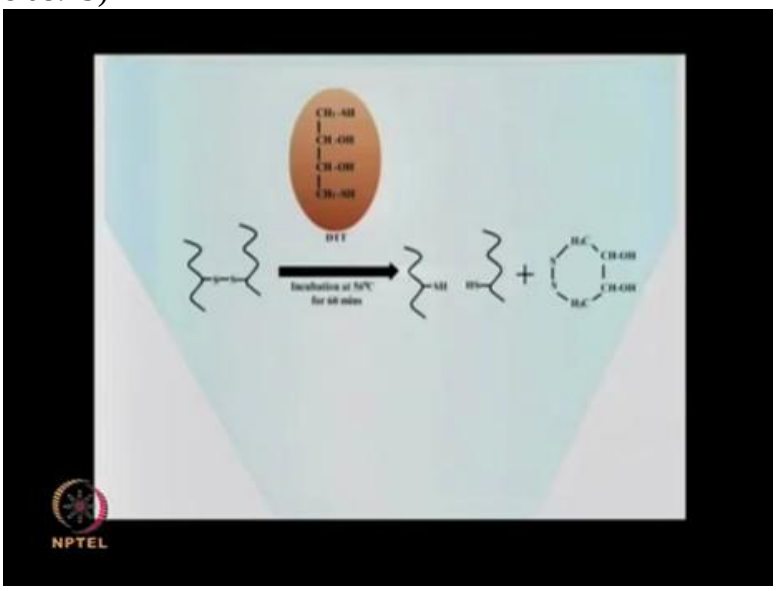

For treatment of the protein residues with Dithiothreitol breaks the disulfide bonds.

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

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And incubate for 10 minutes

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Now remove all the liquids

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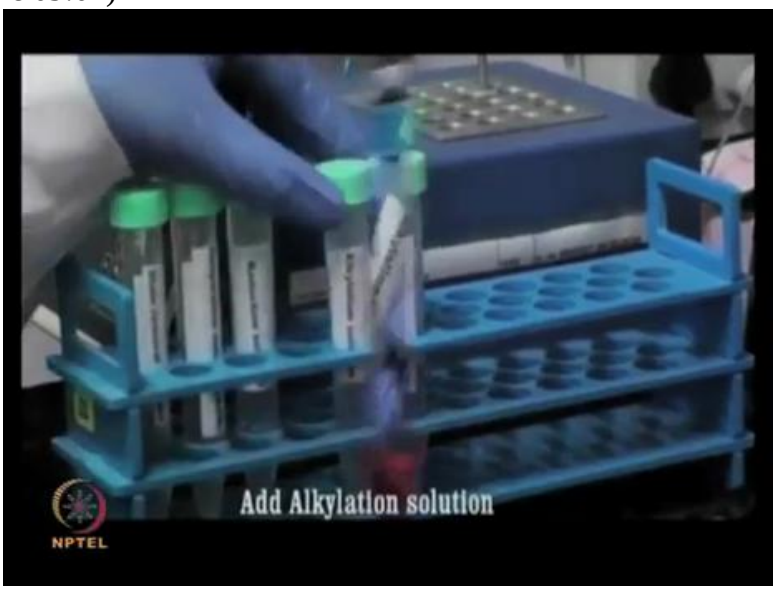

Then add the alkylation solution.

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Add 30 to 50 micro liters of the alkylation solution

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

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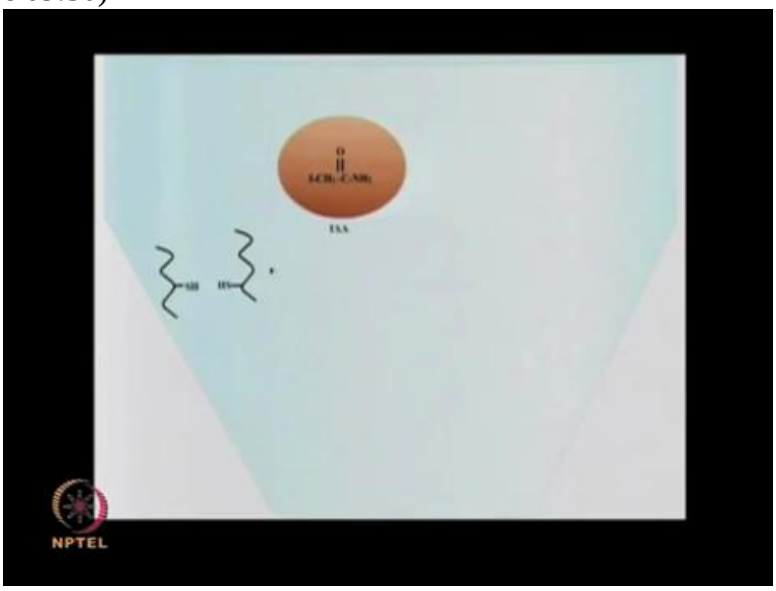

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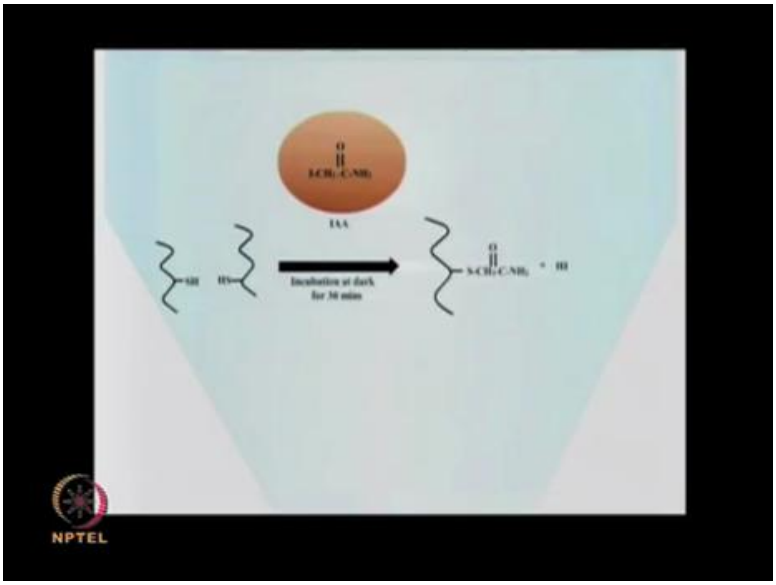

Iodoacetamide prevents the reformation of disulfide bonds. It is an alkylating agent.

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

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And now remove the solution.

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Now

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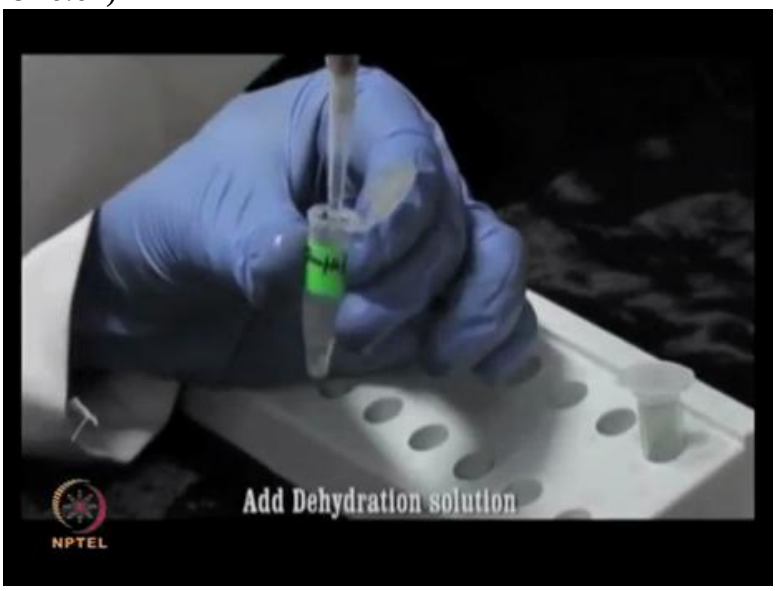

Add 50 micro liters of dehydration solution,

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Mix it properly by vortexing and incubate for it 10 minutes

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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

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

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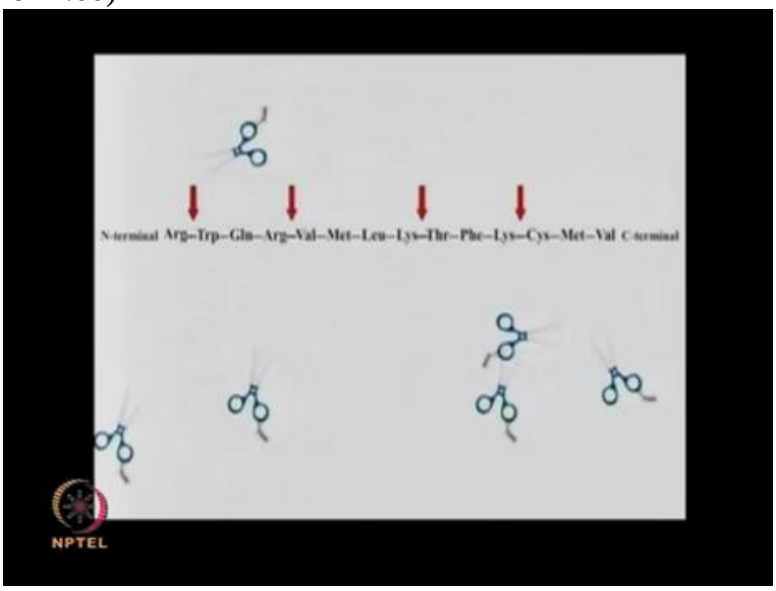

It is same buffer in which Trypsin is prepared; and incubate at 37 degrees C for overnight, 12-16 hours

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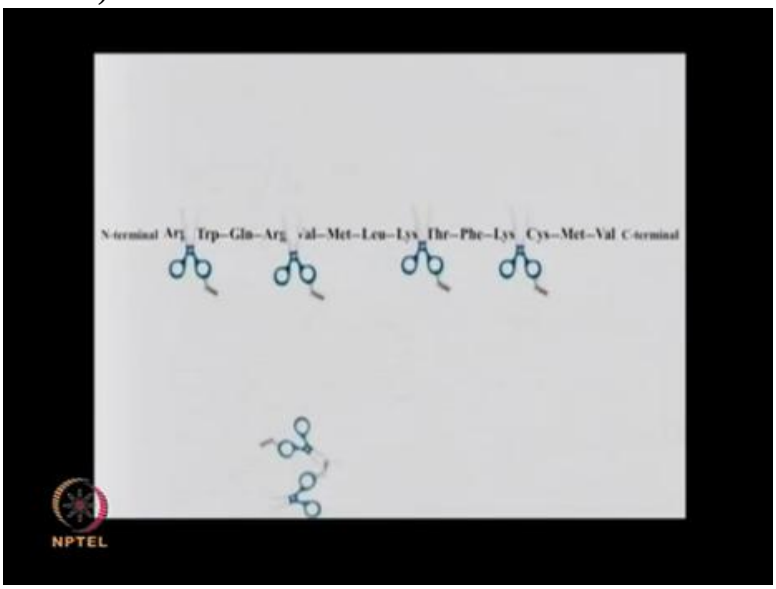

For proper proteolytic cleavage so prior to MS identification, proteins are digested to generate peptides. Several proteolytic enzymes are available. Chymotrypsin, trypsin, pepsin are some of the enzymes commonly used for proteolysis.

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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.

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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

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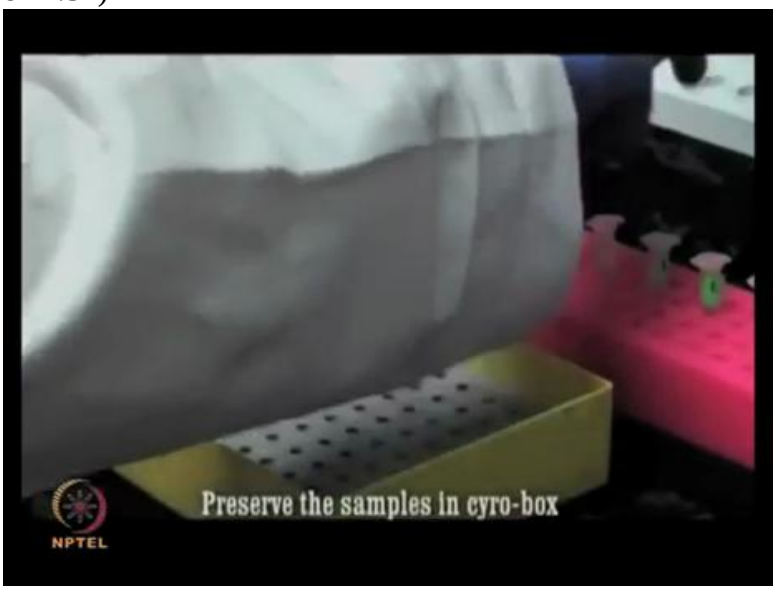

All the solution volumes described in the protocol are recommended volumes but depending upon the experimental requirement, you can optimize and change these volumes and the incubation time according to your experimental requirements.

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

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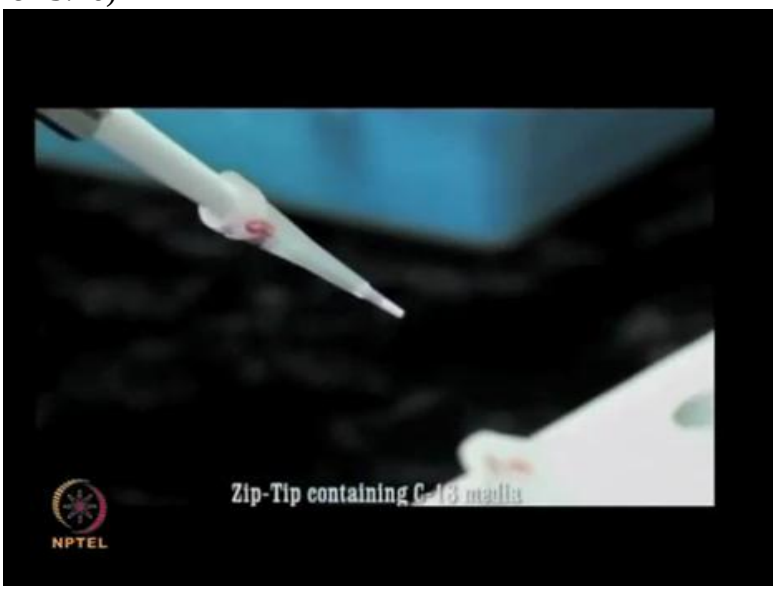

In-gel digested protein samples are further processed by using ZipTip pipette tips

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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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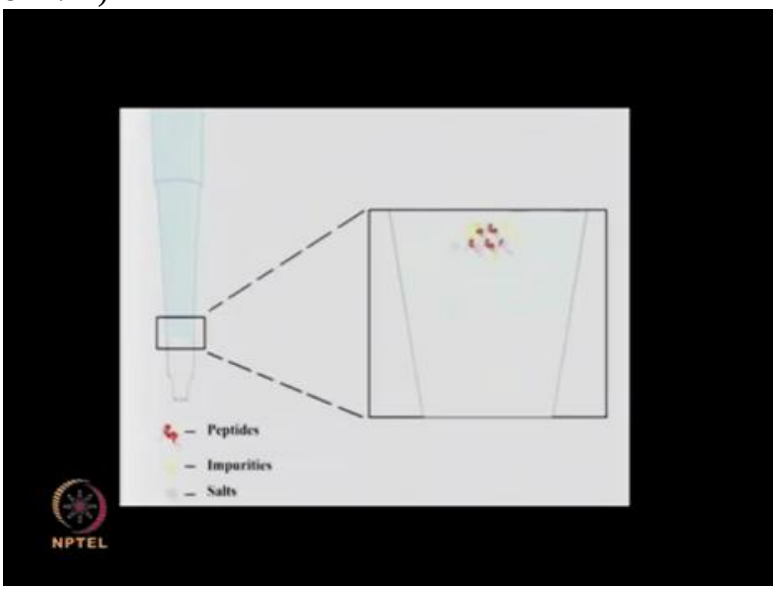

So first, you need to attach the ZipTip pipette tip on the top of a suitable micropipette

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.

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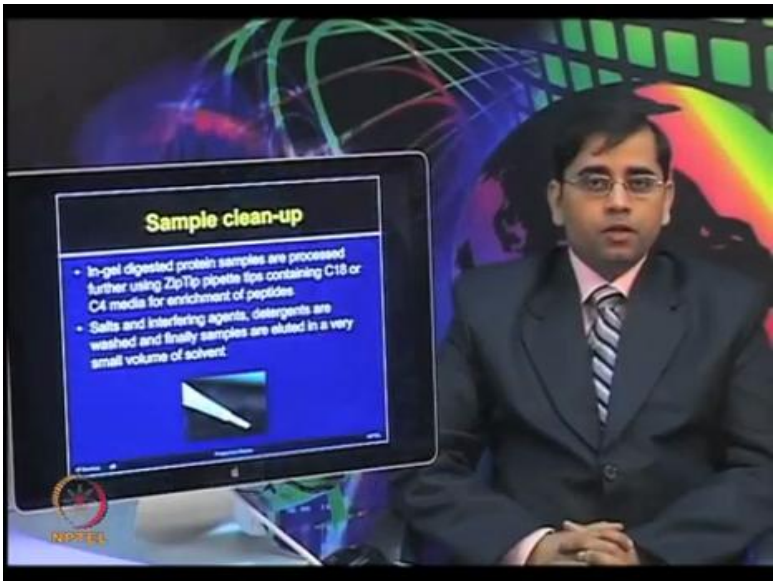

After watching this video, now you are very clear about the in-gel digestion process how various steps are important to perform these experiments.

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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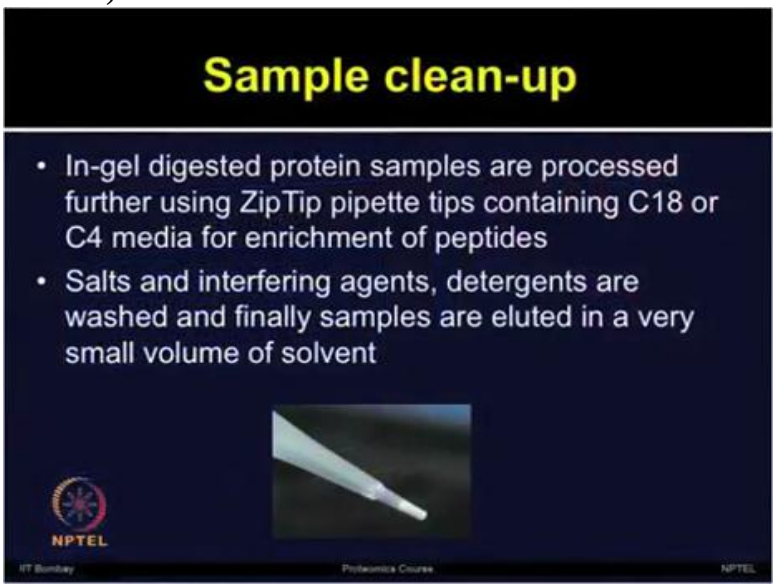

So it is recommended that one should use the cleanup step in between. In-gel digested protein samples can be cleaned up by processing further ZipTip pipette tips which contain C18 or C4 media for enrichment of the peptides

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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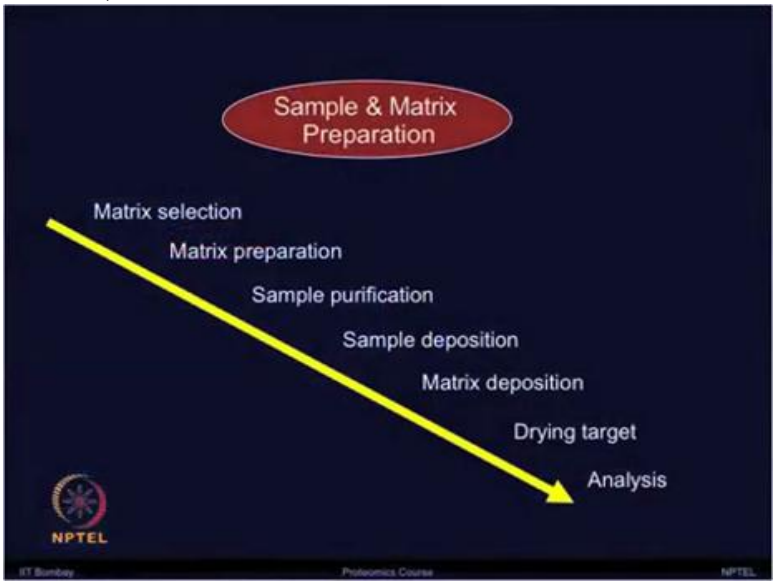

You need to select the matrix. You need to prepare the matrix. You have already done the sample purification. Now sample need to be deposited on the MALDI plate. Either you can mix it with matrix or you can do this separately.

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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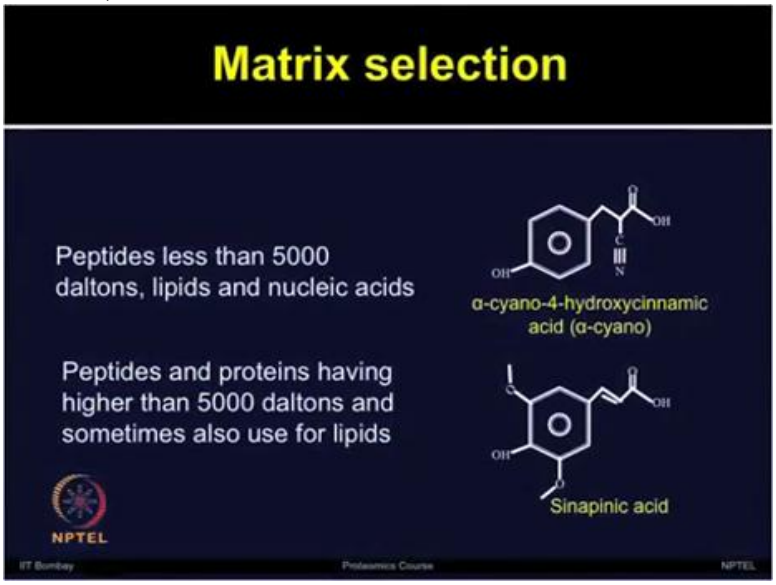

One is alpha-cyano-4- hydroycinnamic acid. When you have peptides less than 5000 Daltons or lipids and nucleic acids one can use this matrix.

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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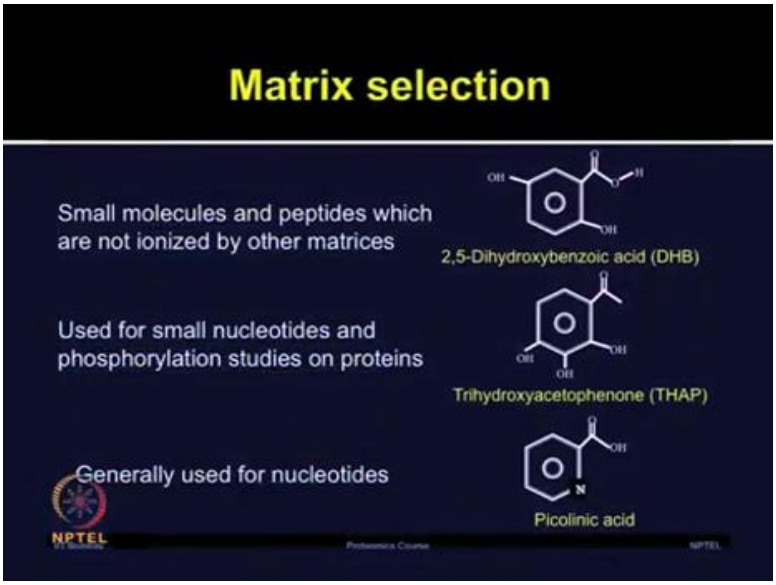

Then you have options such as 2-5-dihydroxybenzoic acid also known as DHB. Small molecules and peptides which are not ionized by other molecules can be analyzed by using this matrix.

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 converts 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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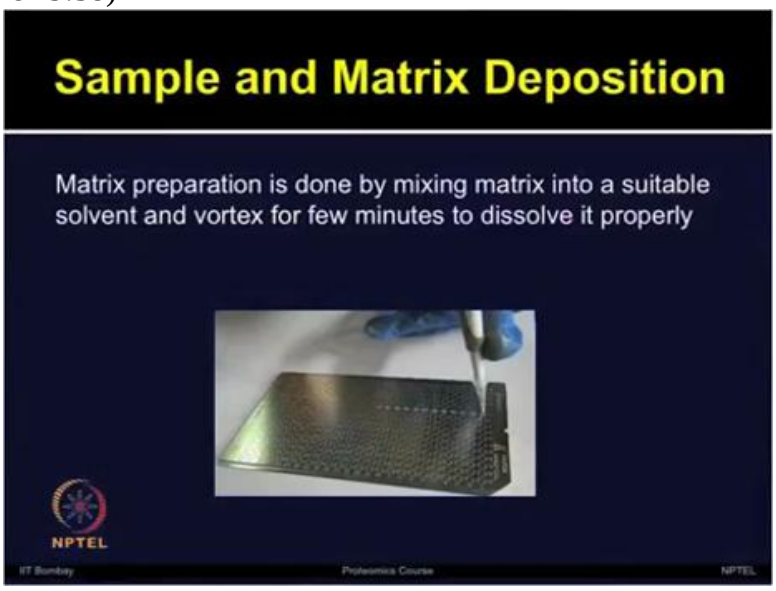

Now once you have selected the matrix, matrix can be prepared by mixing it in a suitable solvent and vortex it for few minutes so that it can dissolve properly. Now you are ready with both analyte the protein which you want to analyze as well as the matrix which we have selected for your application.

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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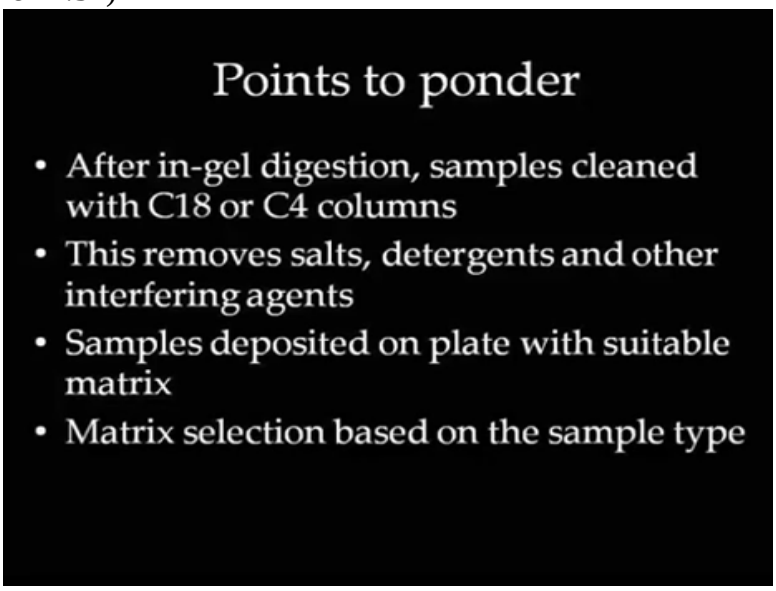

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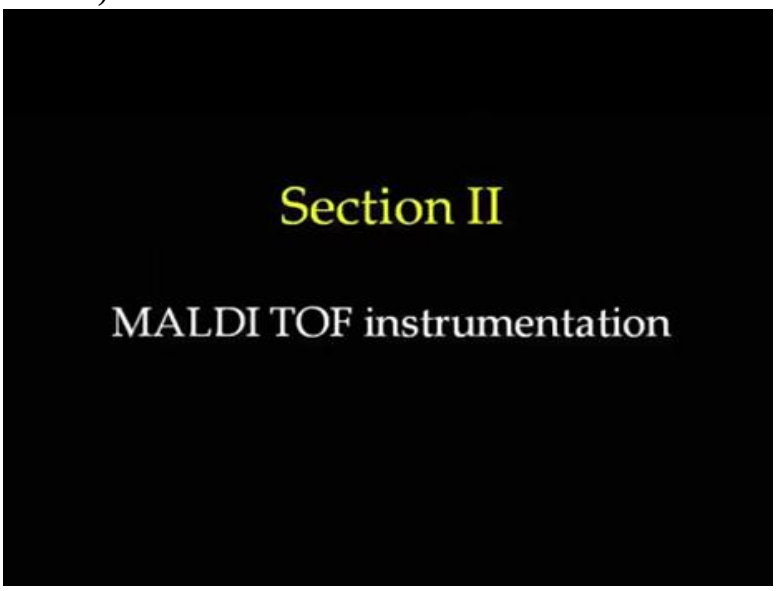

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After spotting is done and the MALDI plate is dried almost 30 minutes, then the instrument can be turned on and MS analysis can be performed. Now there are various types of configuration of these instruments available as well as there are various types 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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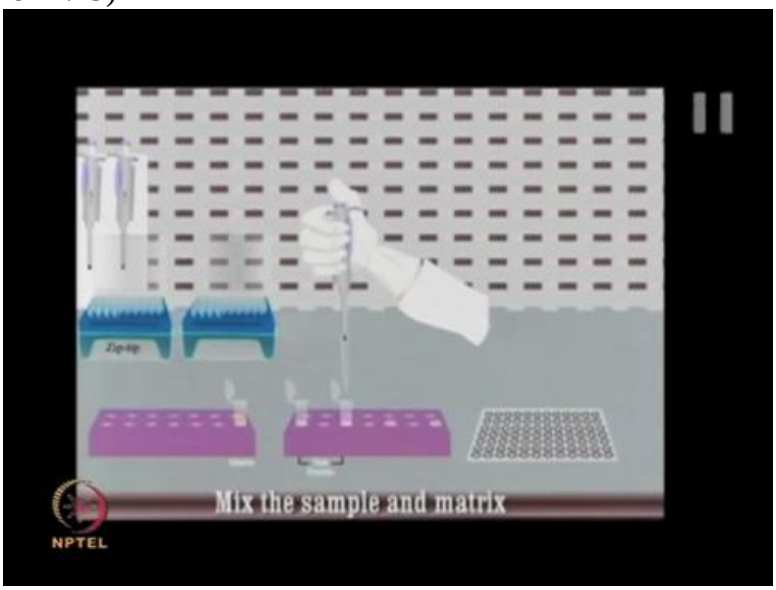

MALDI is performed in two steps. In first step, the compound for the analysis should be dissolved in a solvent containing small organic molecules, known as matrix.

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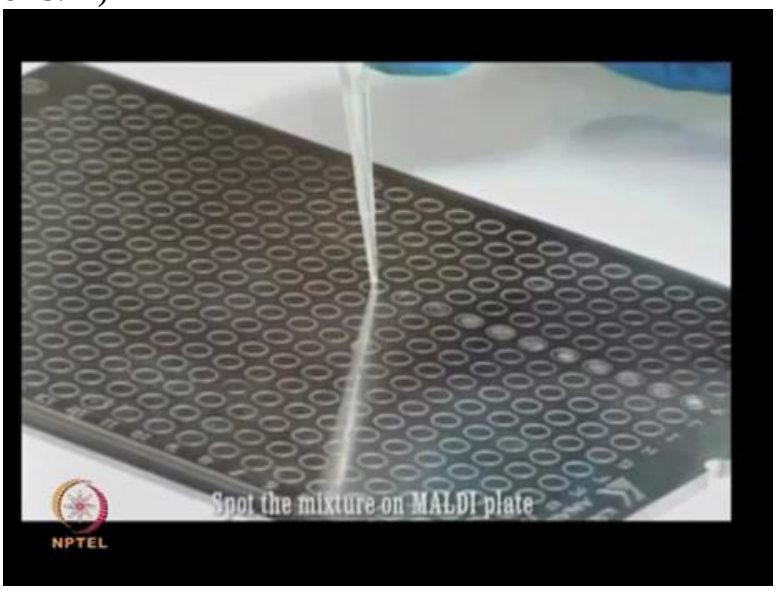
This mixture is dried before analysis and liquid solvent used in the preparation of the solution is removed.

(Refer Slide Time 22:46)

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.

(Refer Slide Time 23:12)



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.

(Refer Slide Time 23:30)

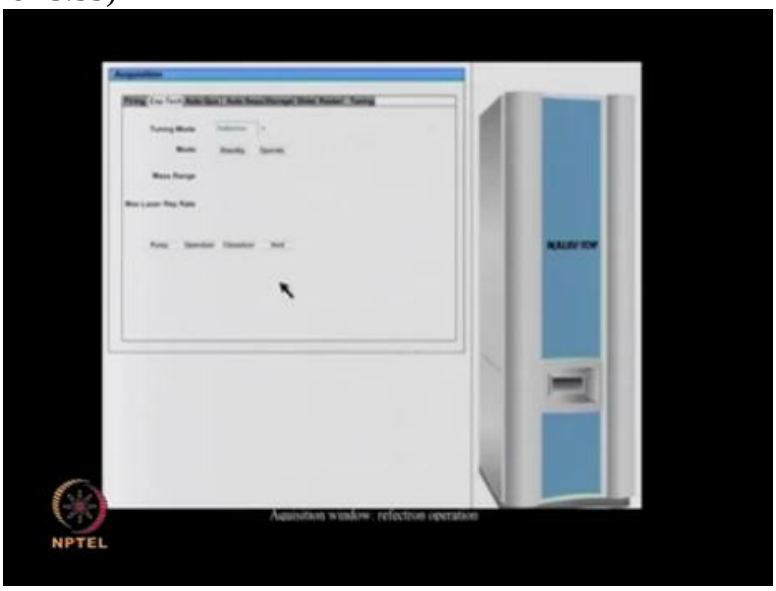
(Refer Slide Time 23:31)

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

(Refer Slide Time 23:47)

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

(Refer Slide Time 23:55)



You need to ensure that the instrument is on.

(Refer Slide Time 23:59)

So click on the software and

(Refer Slide Time 24:02)

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

(Refer Slide Time 24:06)

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

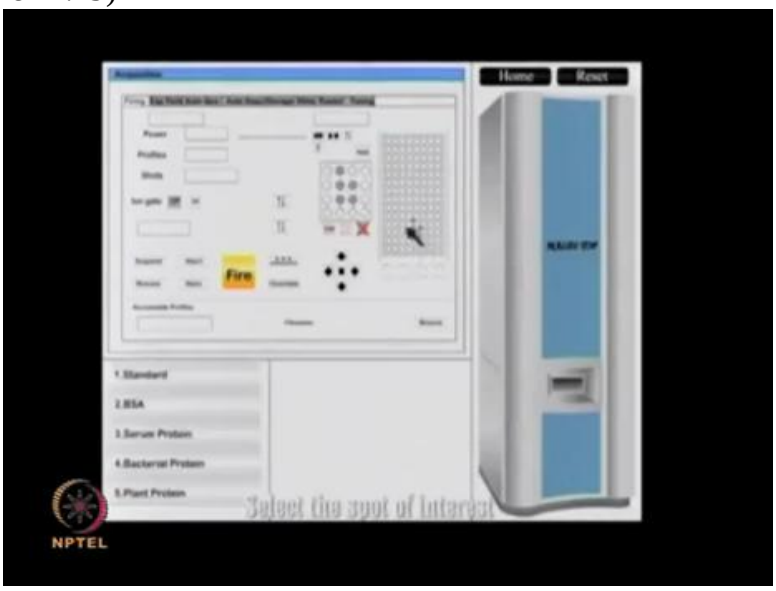
(Refer Slide Time 24:19)

And now by using software, close the door.

(Refer Slide Time 24:23)

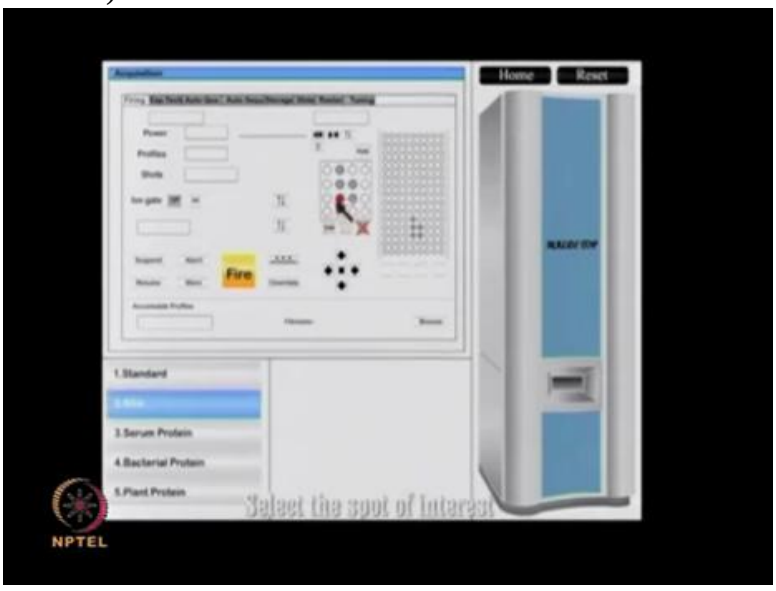
The door of the insertion chamber is now closed.

(Refer Slide Time 24:29)



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)



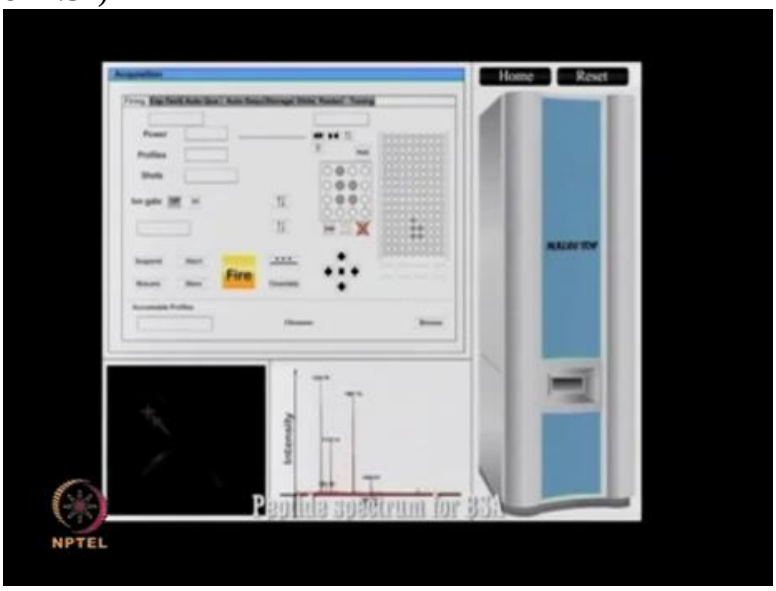
Yellow target on the acquisition window

(Refer Slide Time 24:47)

And select “go to the location”.

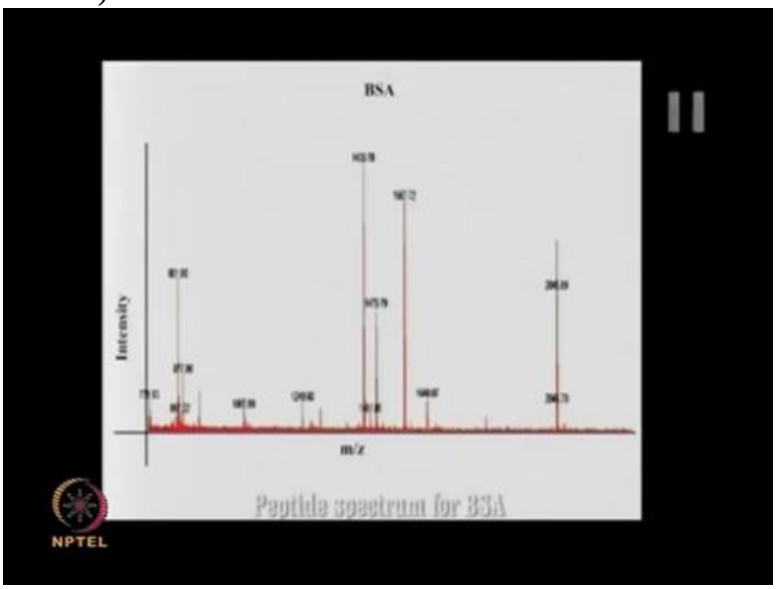
(Refer Slide Time 24:49)

(Refer Slide Time 24:52)



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

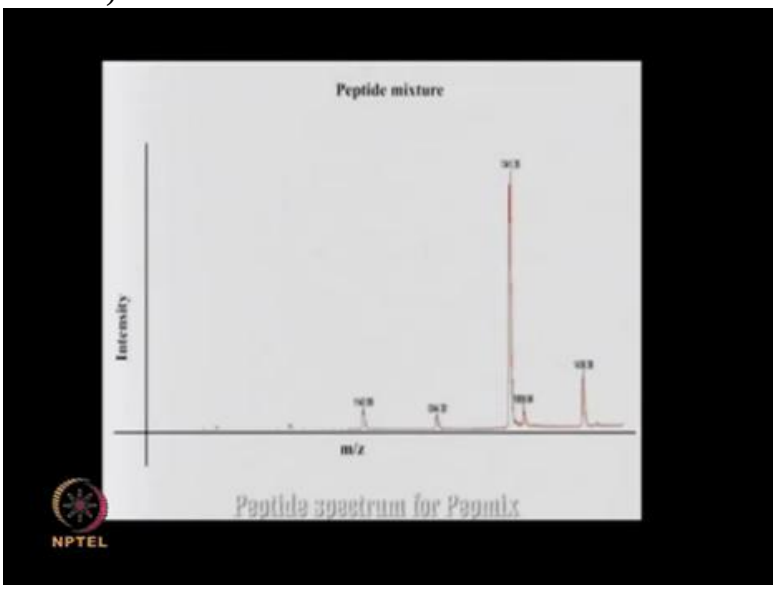
(Refer Slide Time 25:19)

Can be performed for different spots

(Refer Slide Time 25:20)

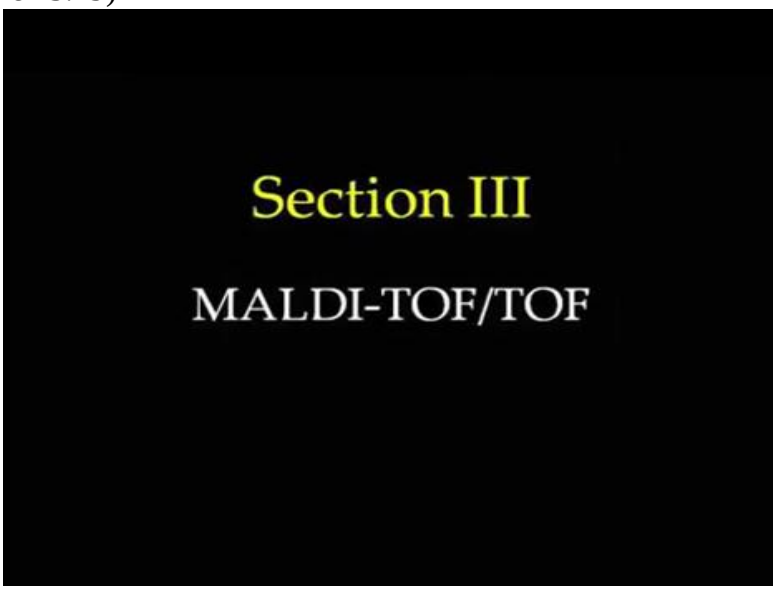
And different regions

(Refer Slide Time 25:25)

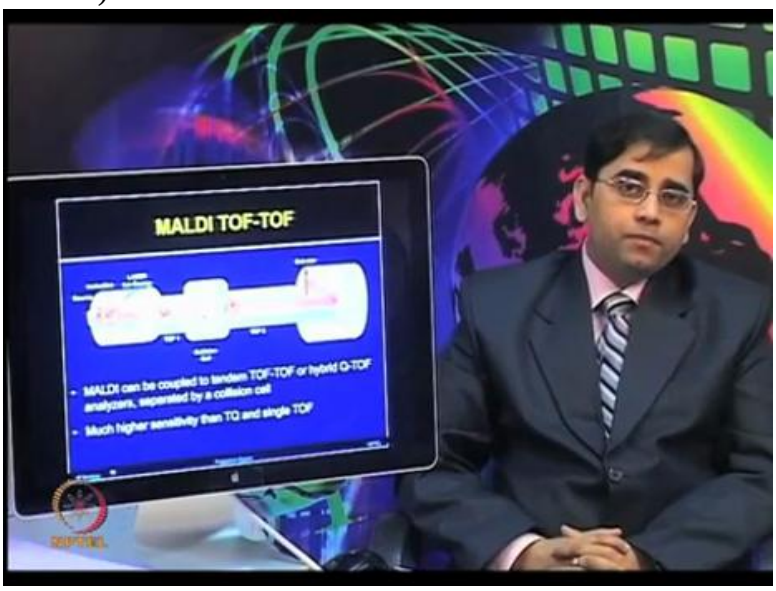


Now we have shown here a spectra for the Pepmix

(Refer Slide Time 25:29)

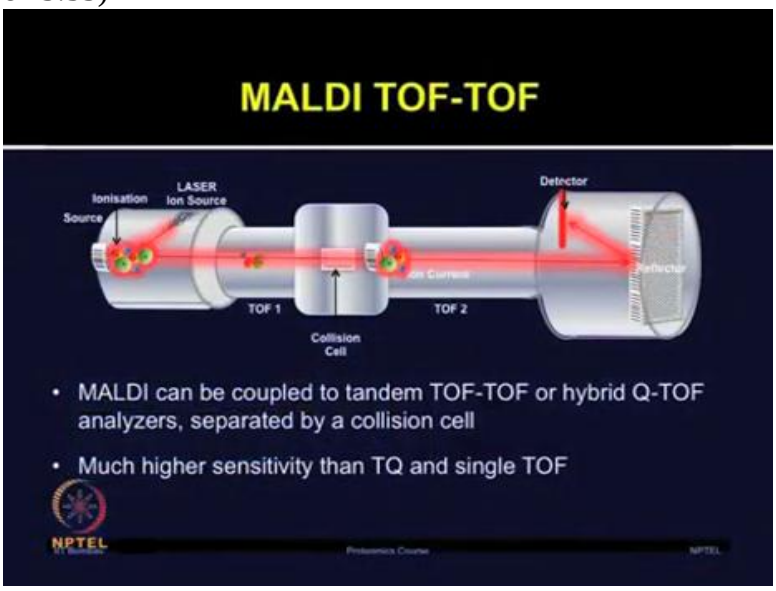


(Refer Slide Time 25:35)



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

(Refer Slide Time 25:35)

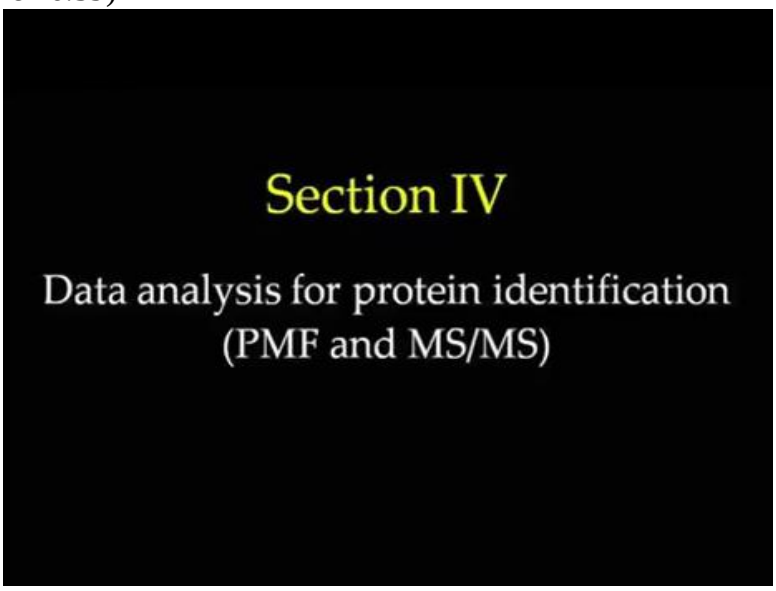


Now let's add one more mass analyzer. So now we have a configuration of MALDI-TOF-TOF. So MALDI can be coupled to the Tandem- Time of Flight in combination with another Time of Flight or with hybrid quadrupole-Time of Flight analyzers which are separated by collision cells.

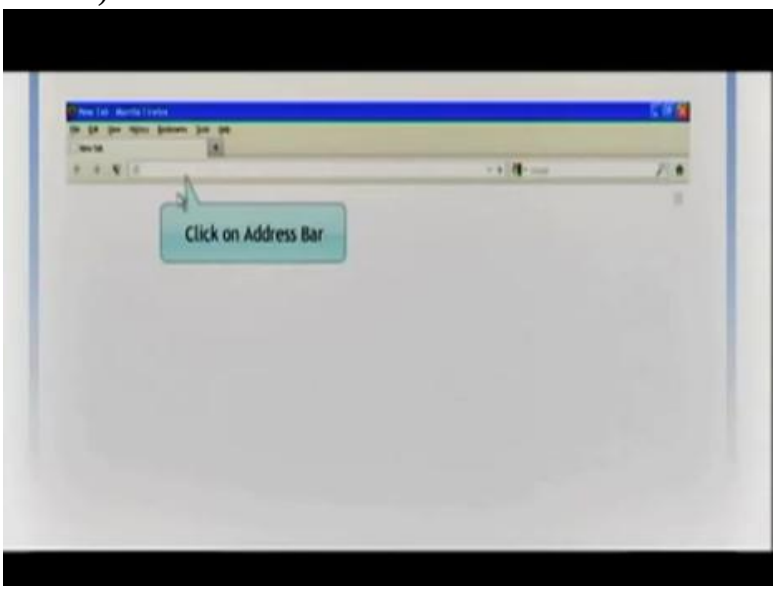
Now for proteomic applications it is recommended to use TOF-TOF or Q-TOF. The peptide ions are accelerated through the first time of flight tube as you can see in the slide and then they are disassociated by introducing an inert gas into the collision cell. This process allows collision induced disassociation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

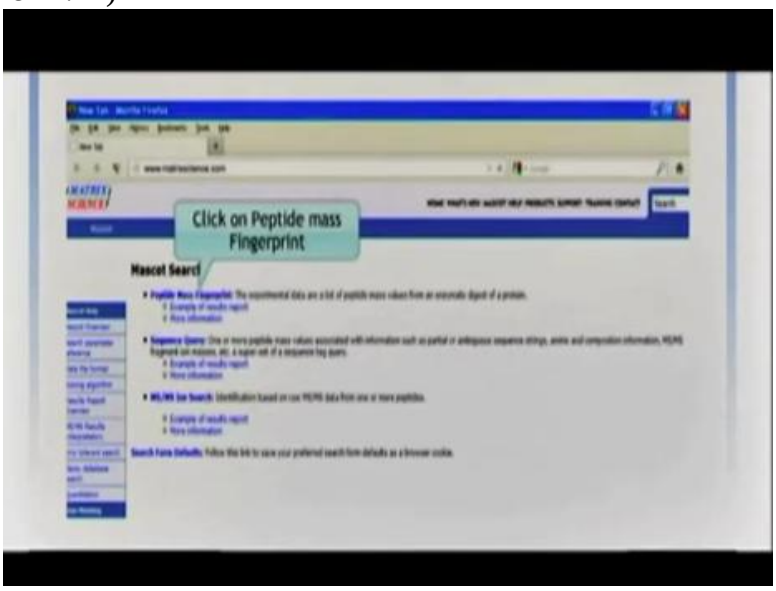


(Refer Slide Time 27:06)



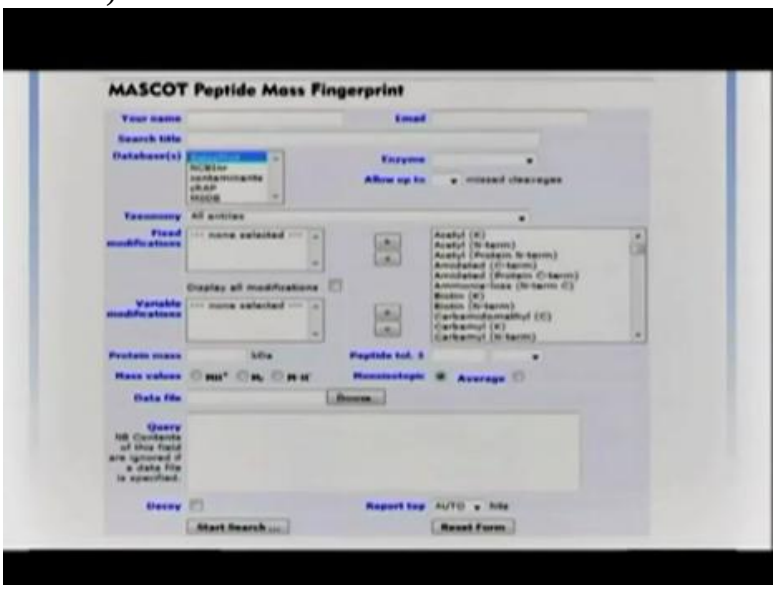
Open the Matrix Science browser window to carry out online data analysis.

(Refer Slide Time 27:17)



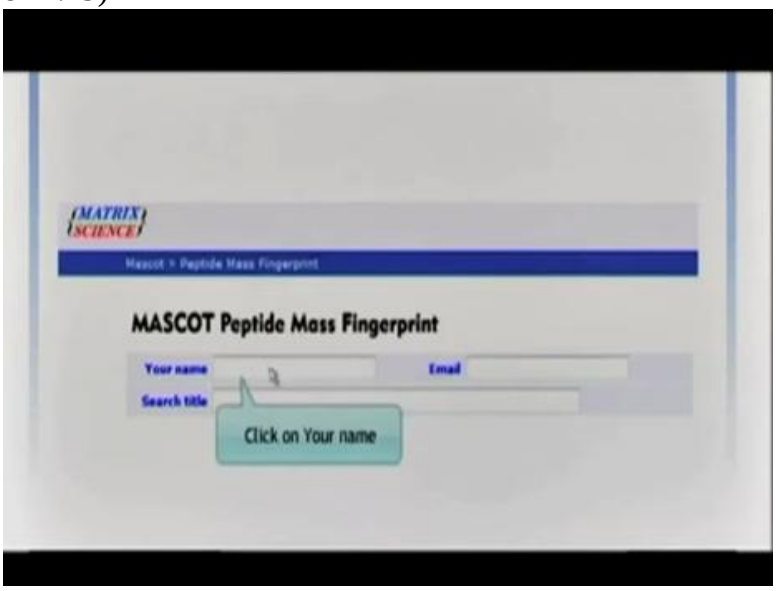
For peptide analysis click on peptide mass fingerprinting,

(Refer Slide Time 27:22)



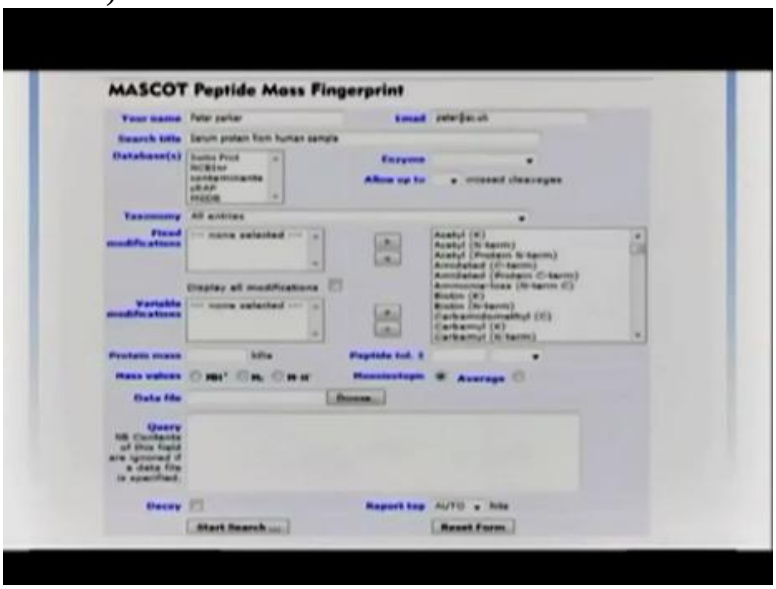
Mascot peptide mass fingerprint

(Refer Slide Time 27:29)



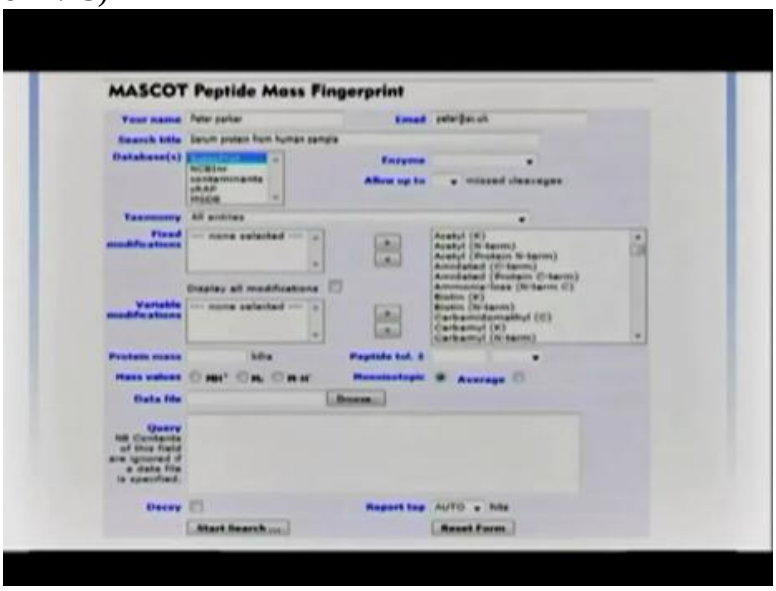
Please enter the User Id and details to acquire information in case of any network loss.

(Refer Slide Time 27:35)



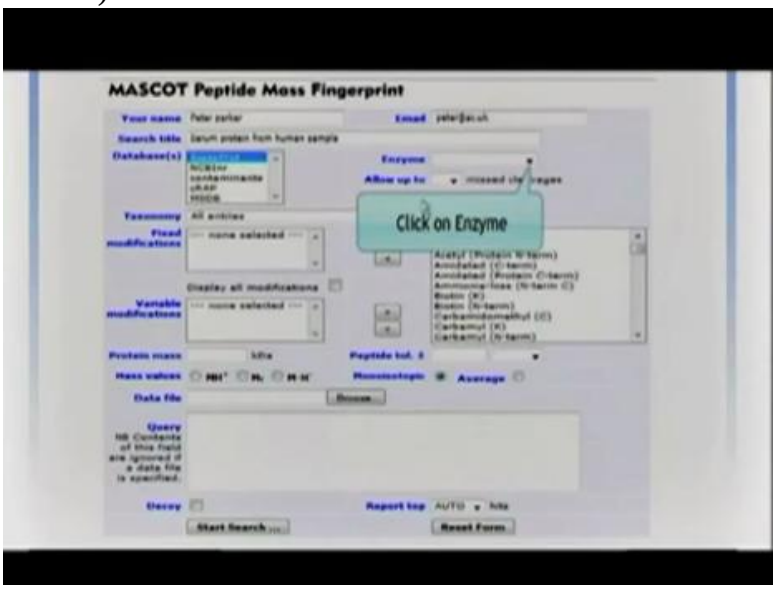
The following parameters should be selected. Database, primary databases include SwissProt and NCBI.

(Refer Slide Time 27:45)



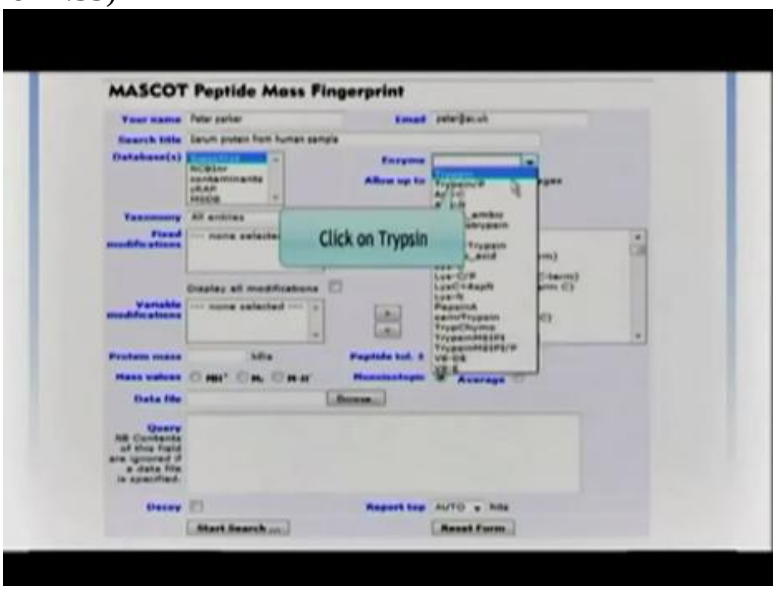
Select SwissProt database for data analysis.

(Refer Slide Time 27:51)



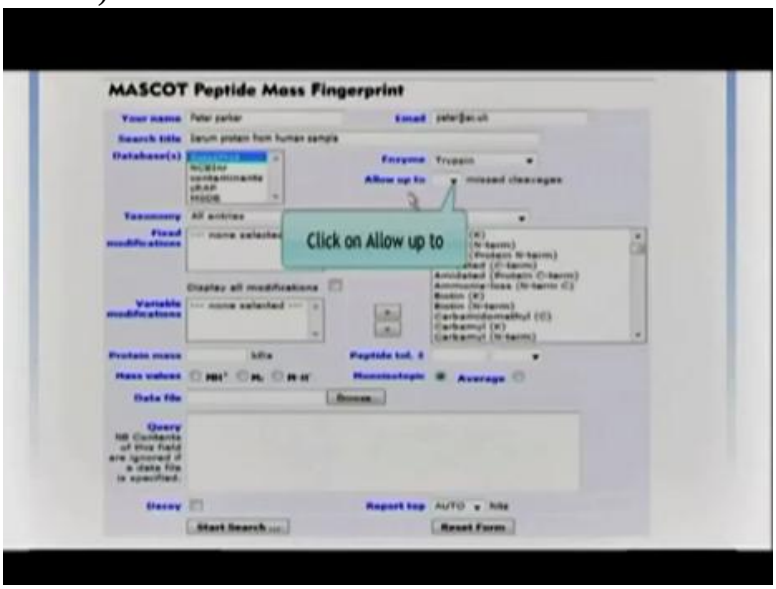
Click Enzyme

(Refer Slide Time 27:55)



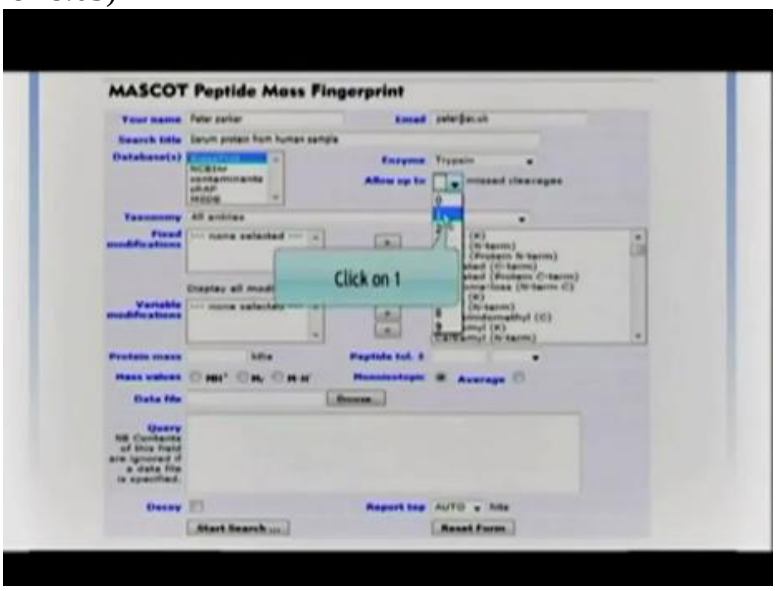
, select enzyme as Trypsin

(Refer Slide Time 27:57)



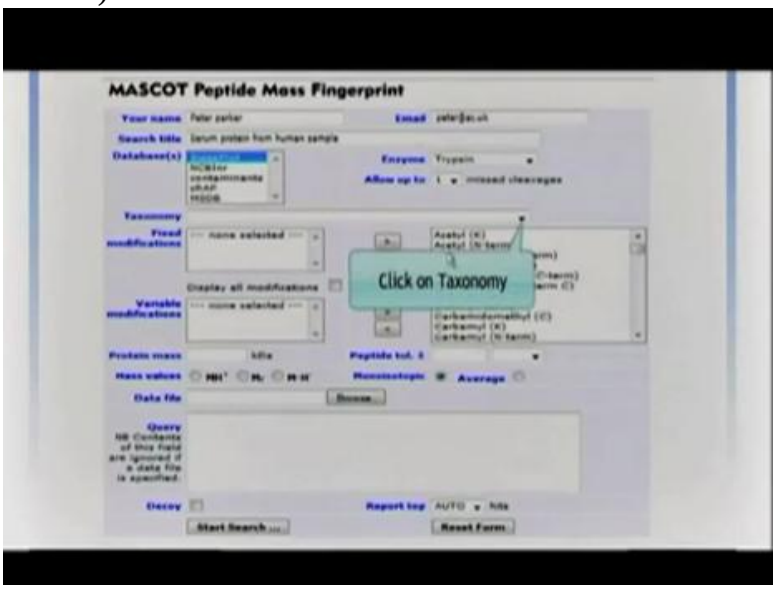
Missed Cleavages,

(Refer Slide Time 28:03)



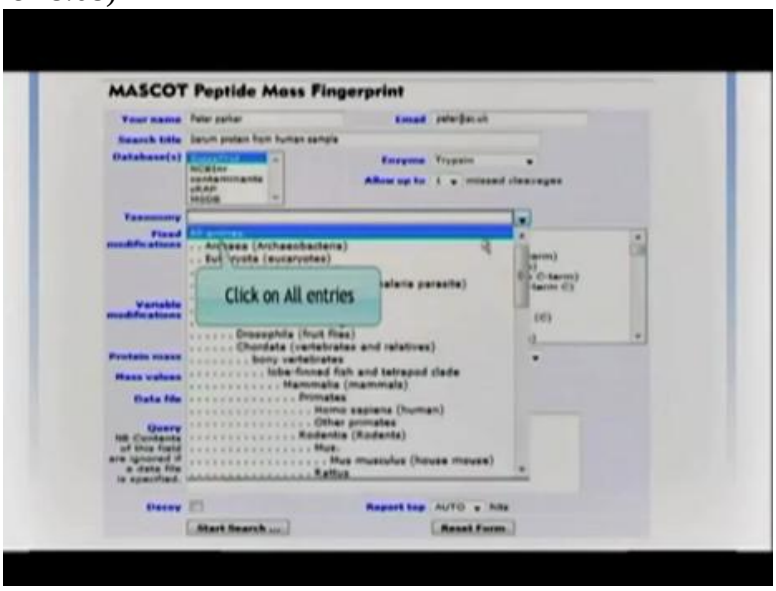
Missed cleavages are allowed up to 1

(Refer Slide Time 28:04)



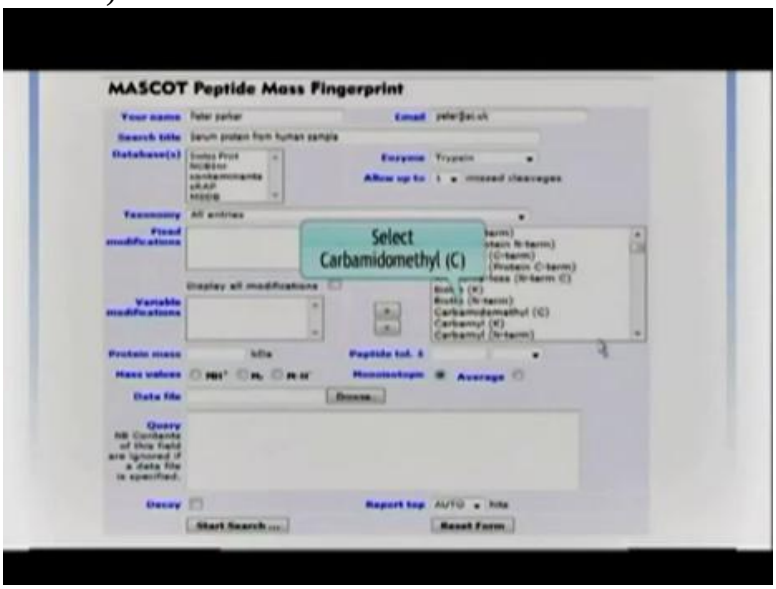
Next is taxonomy. The protein extracted from the biological specimen has to be assigned to a particular species or a group of species to which the sample belongs.

(Refer Slide Time 28:08)



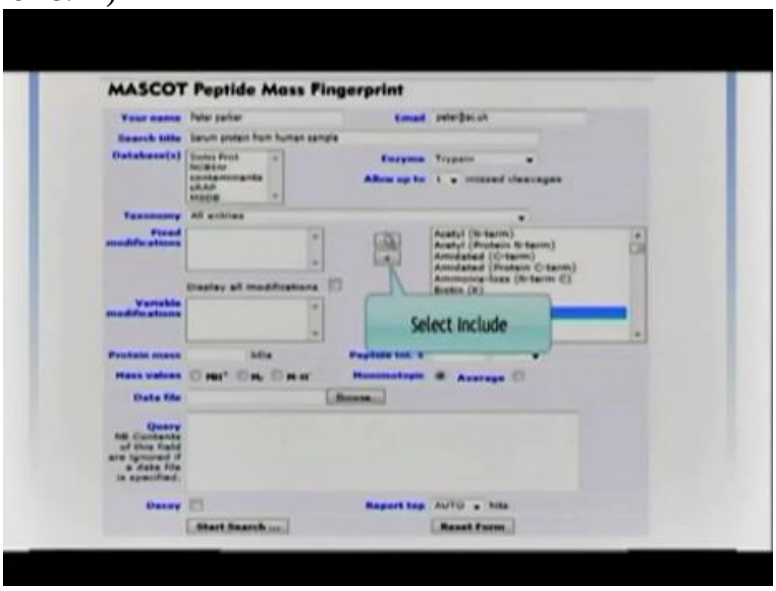
When you are not sure of organism select all entries.

(Refer Slide Time 28:20)



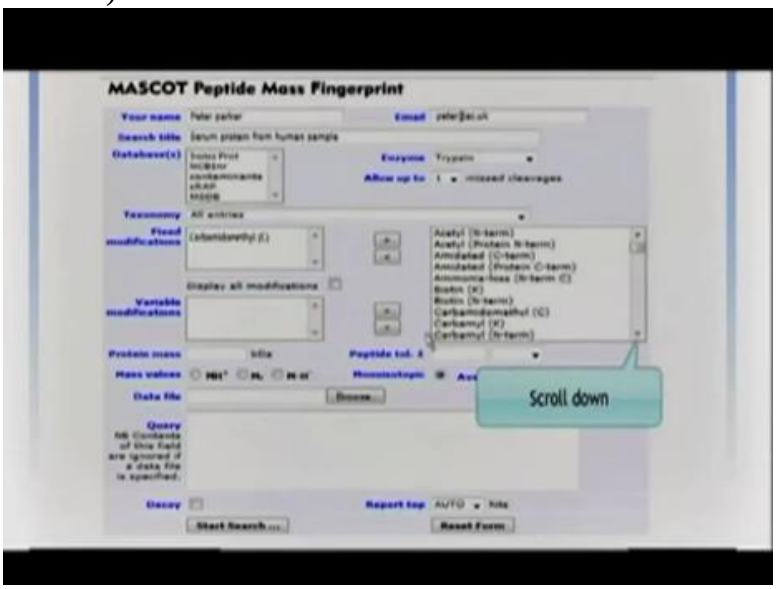
Fixed modification, select carbamidomethyl; fixed modifications are applied collectively

(Refer Slide Time 28:27)



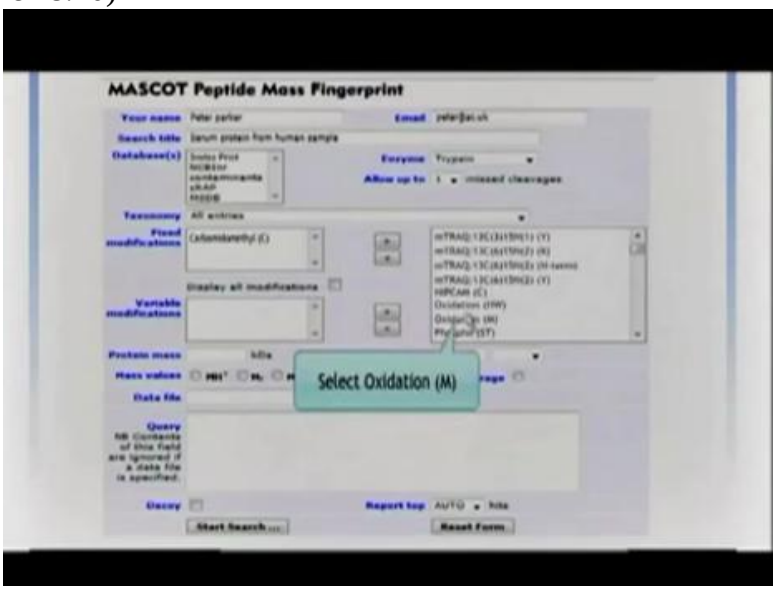
across the database to account for change in mass of specific residue

(Refer Slide Time 28:34)



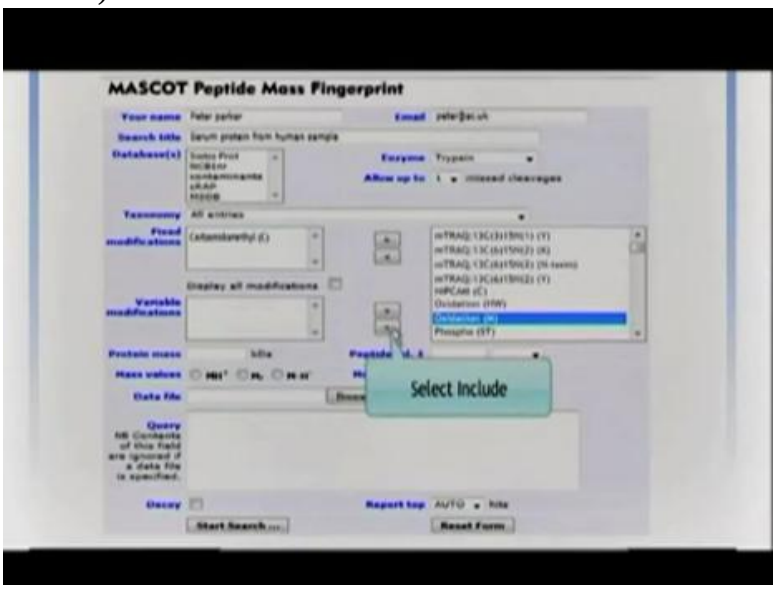
Now scroll down and select oxidation.

(Refer Slide Time 28:40)



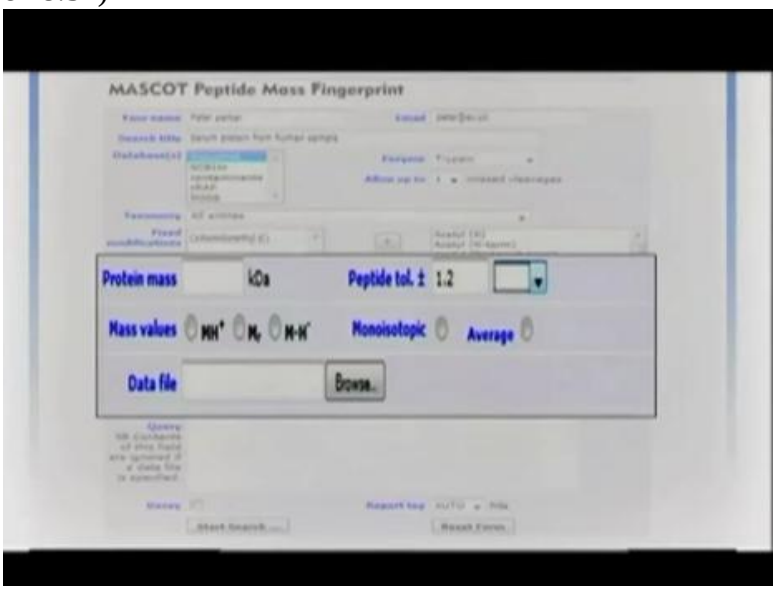
These are mass changes suspected to occur during sample handling and accounted for by increasing the number of primary sequences compared against experimental masses.

(Refer Slide Time 28:47)



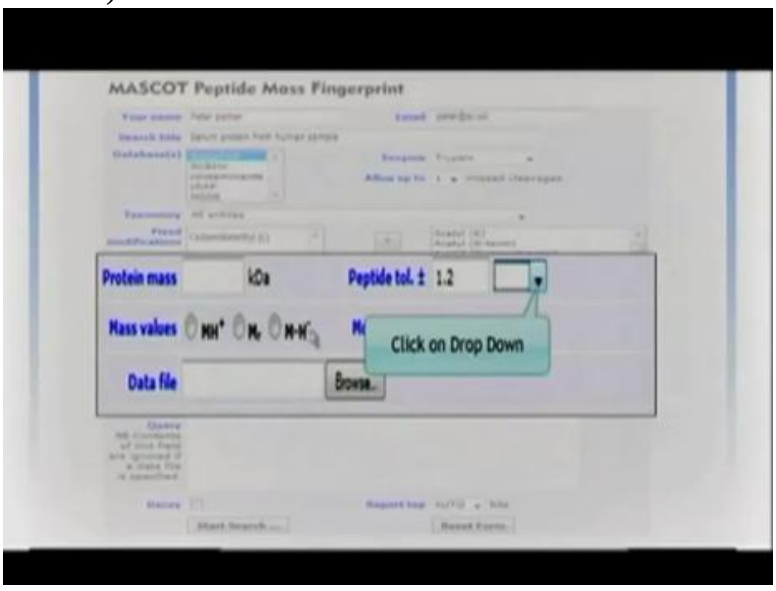
Include it as variable modification.

(Refer Slide Time 28:57)



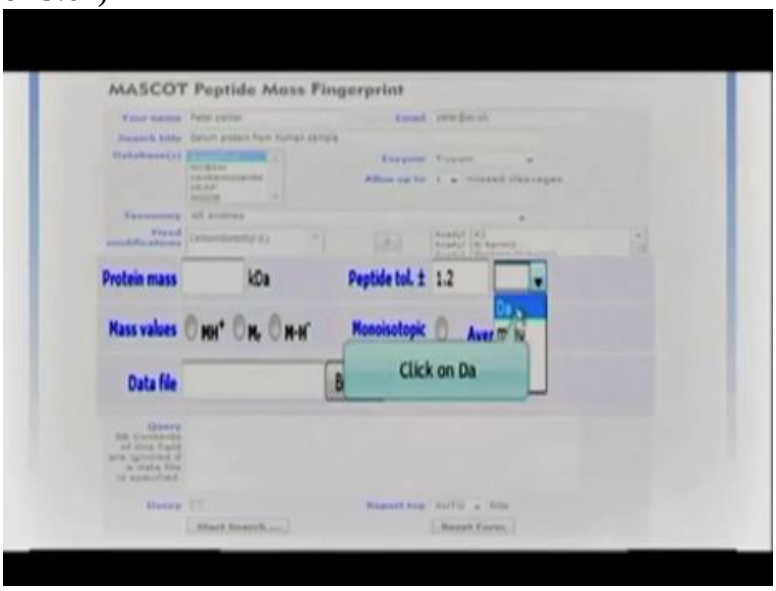
Depending upon user needs the parameters can be changed. Protein mass is the mass in that protein and is optional.

(Refer Slide Time 29:04)



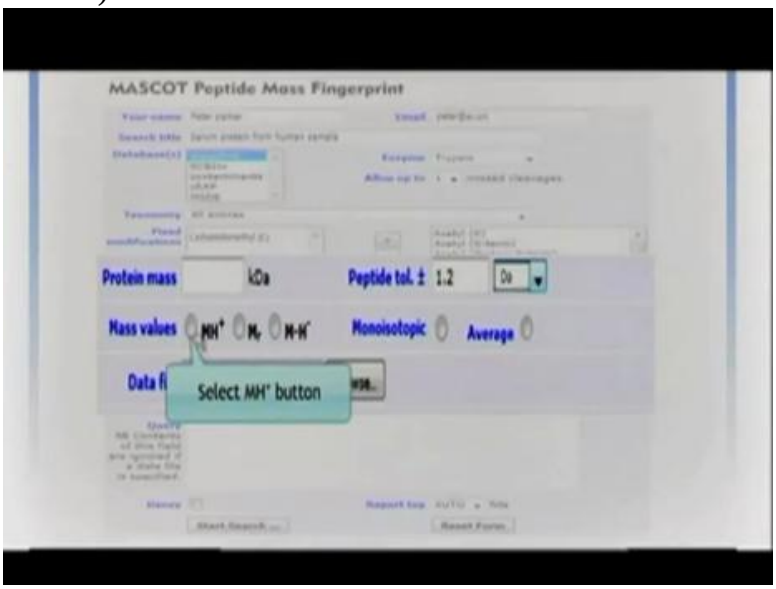
Set peptide tolerance +/- 1.2

(Refer Slide Time 29:07)



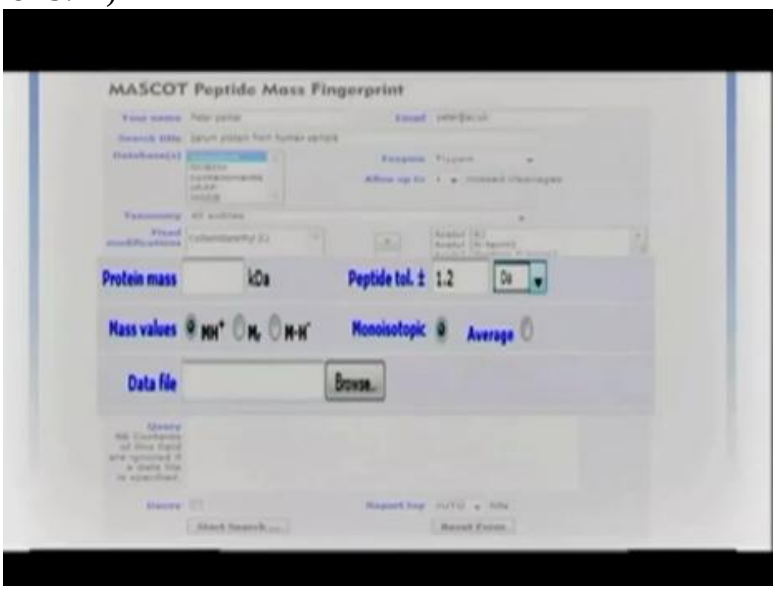
Daltons.

(Refer Slide Time 29:16)



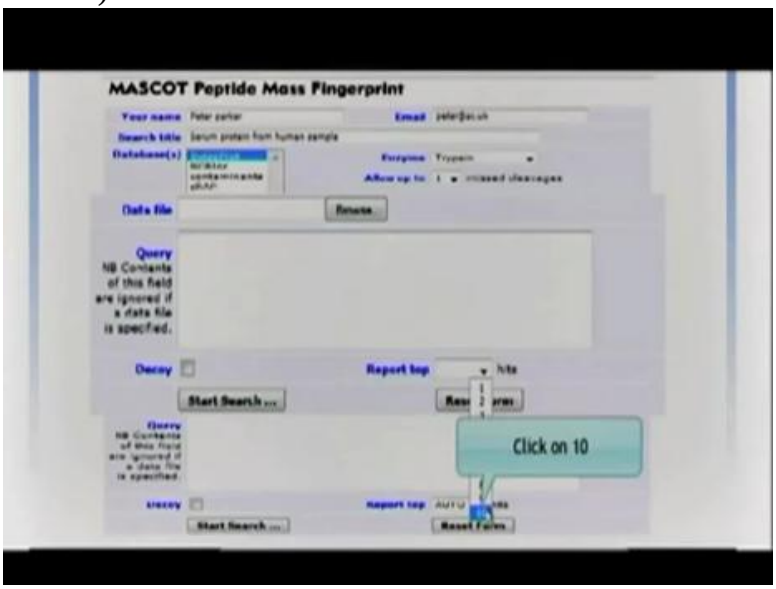
Mass values, it specifies the type of charge to be examined. Select MH positive.

(Refer Slide Time 29:21)

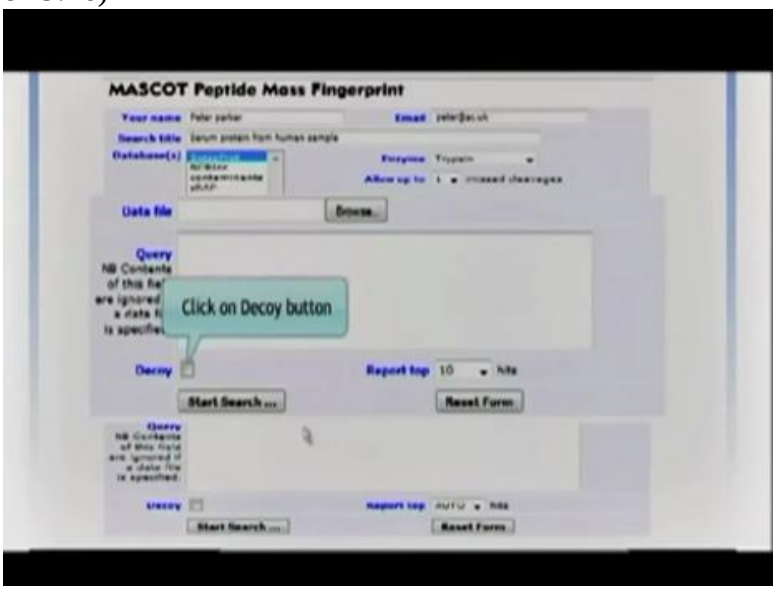


Select mono-isotopic.

(Refer Slide Time 29:24)

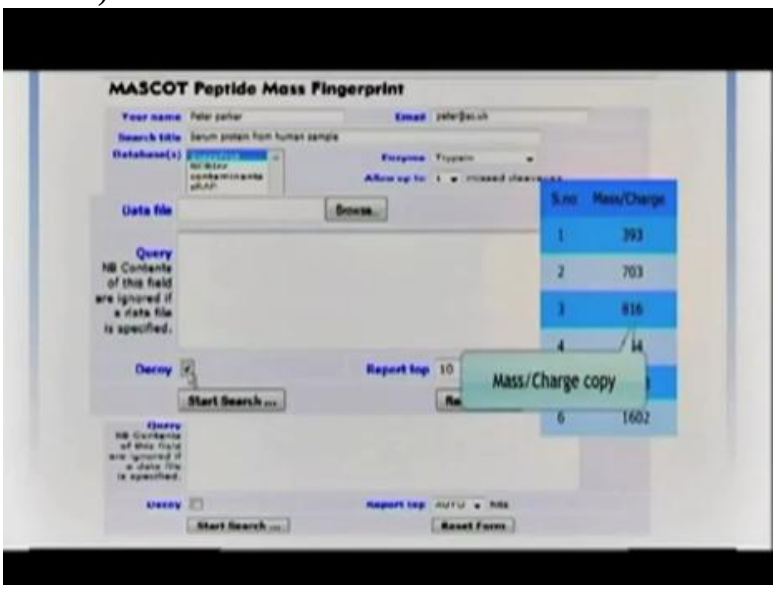


(Refer Slide Time 29:26)



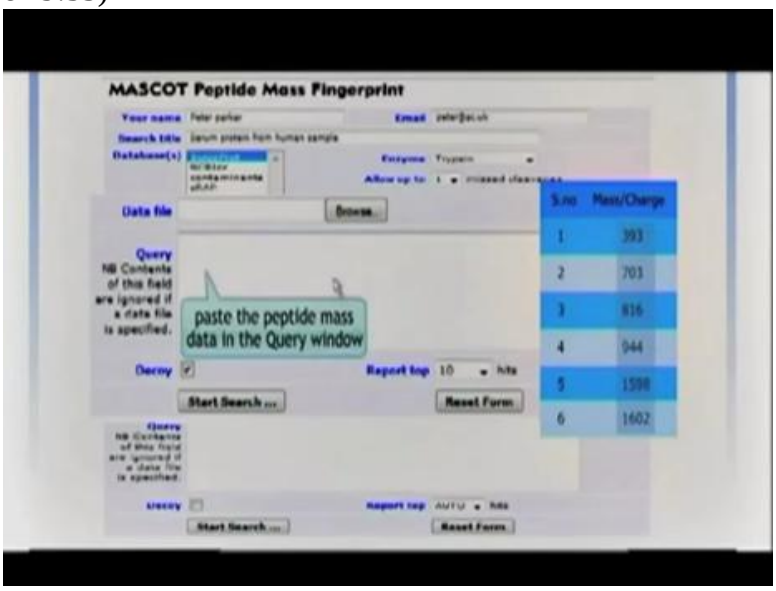
Select Decoy for statistical analysis.

(Refer Slide Time 29:30)



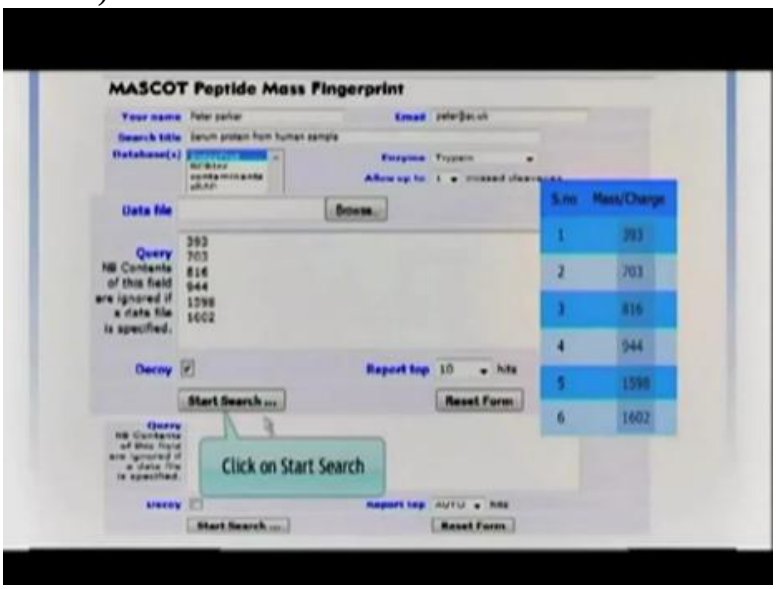
Copy the m by z value

(Refer Slide Time 29:35)



And paste in the selected box for Mascot search

(Refer Slide Time 29:36)

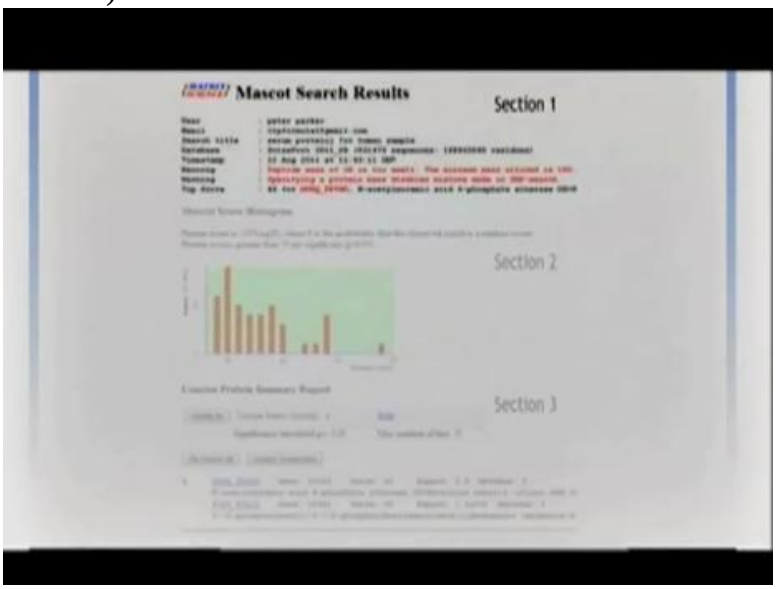


Click on search.

(Refer Slide Time 29:41)

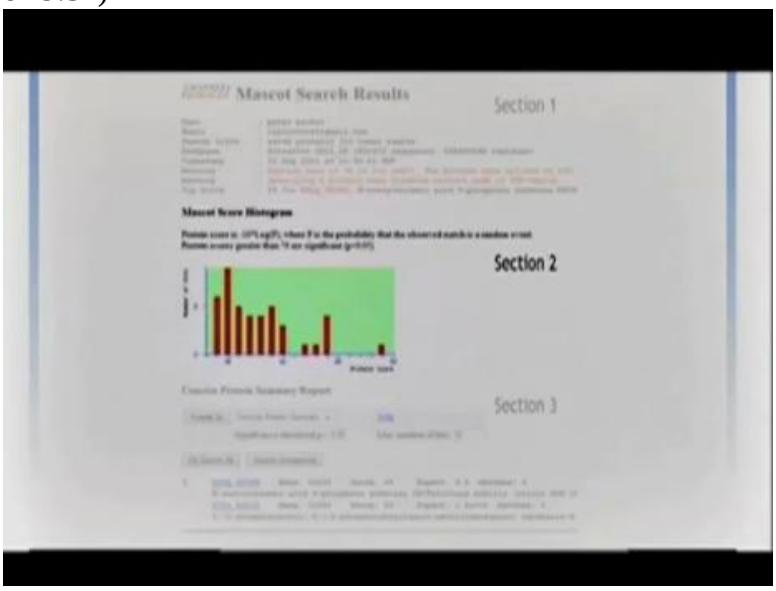
Data output

(Refer Slide Time 29:43)



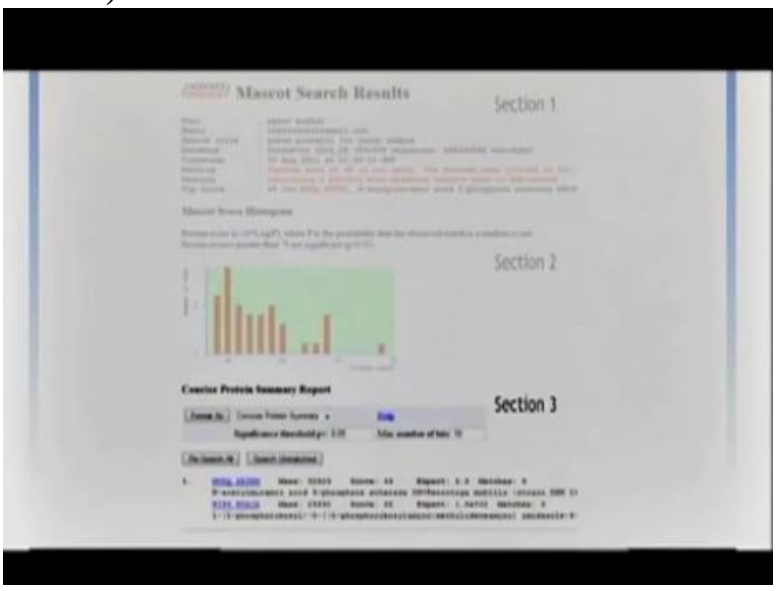
The output can be seen in 3 sections. In section 1, the summary of spec parameters defined by user.

(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)

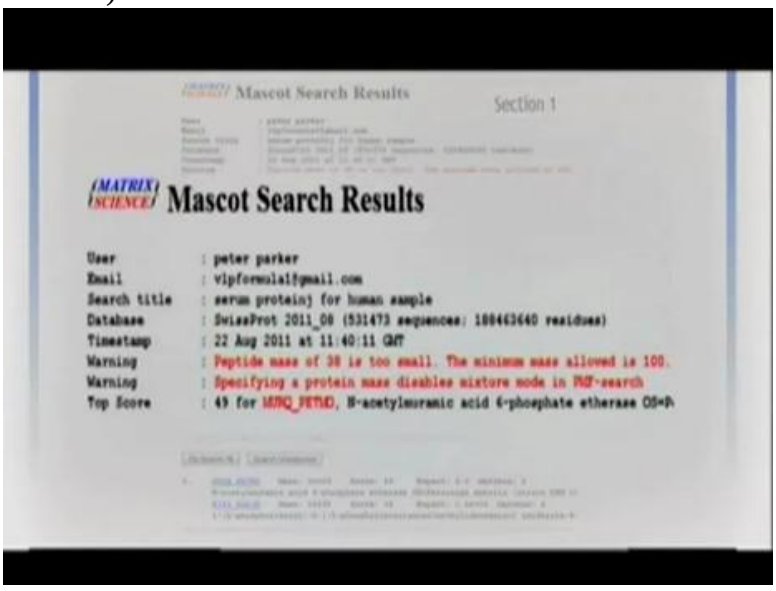


Section 3, summary report in which the matched protein from the database with the details of important parameters are displayed either in concise format, protein format and the data can be exported too.

(Refer Slide Time 30:17)

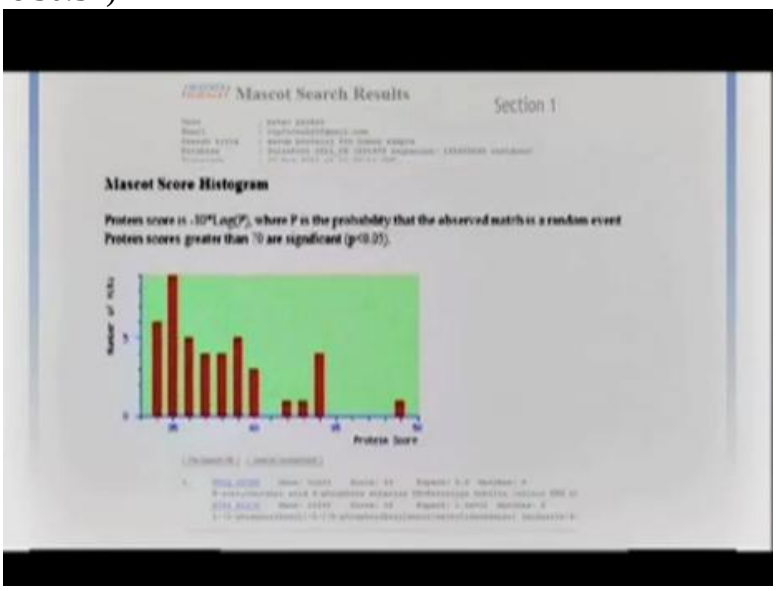
Data Analysis, If the search parameters are not the best fit,

(Refer Slide Time 30:22)



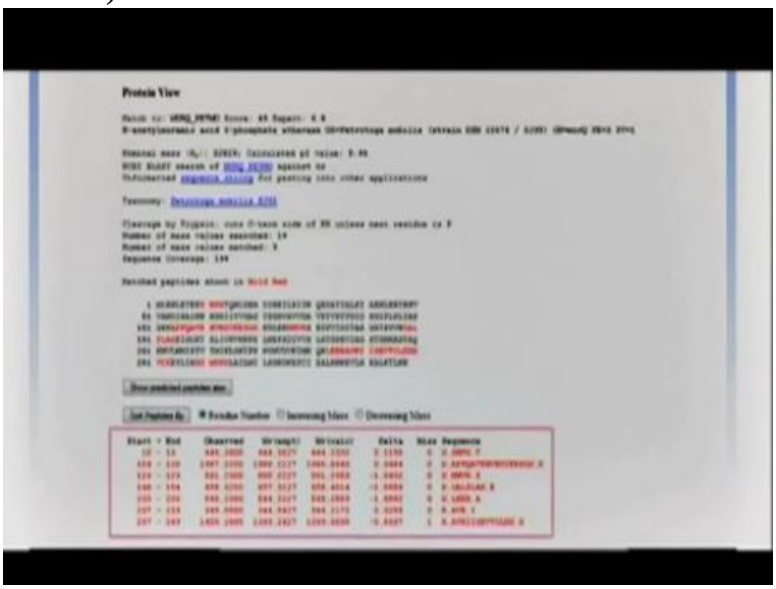
the software generates the error message Depending on the error message, the user needs to change the parameter setting and do the search again.

(Refer Slide Time 30:34)



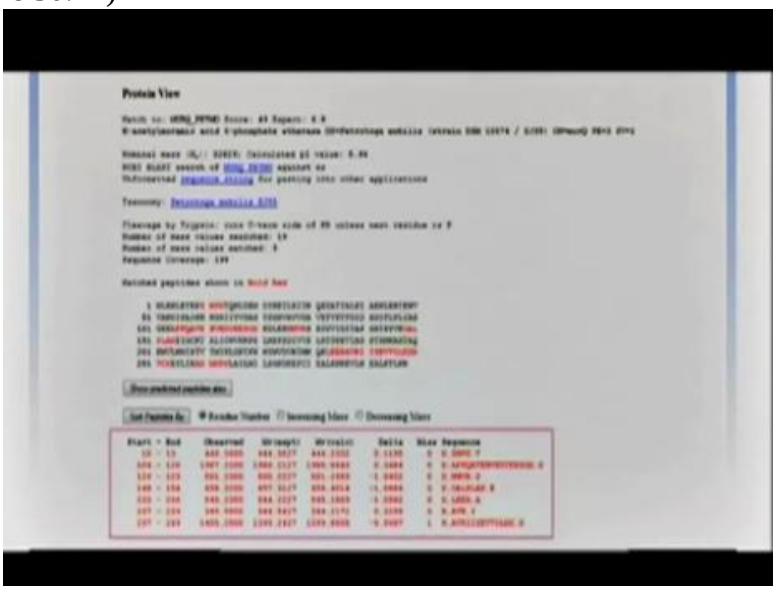
In section 2, the Mascot score histogram, the number of protein hits and their score is displayed along the graph.

(Refer Slide Time 30:41)



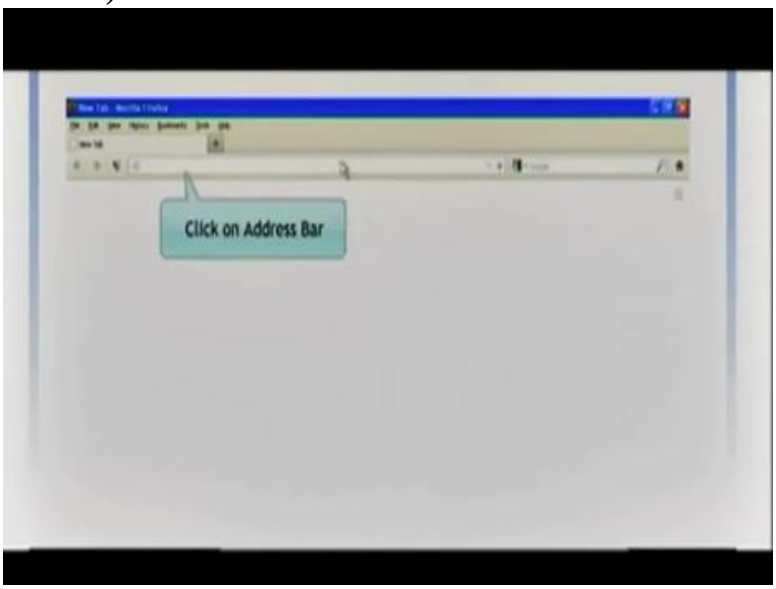
Protein view section displays matching of the query peptide to the protein sequence in the database.

(Refer Slide Time 30:47)



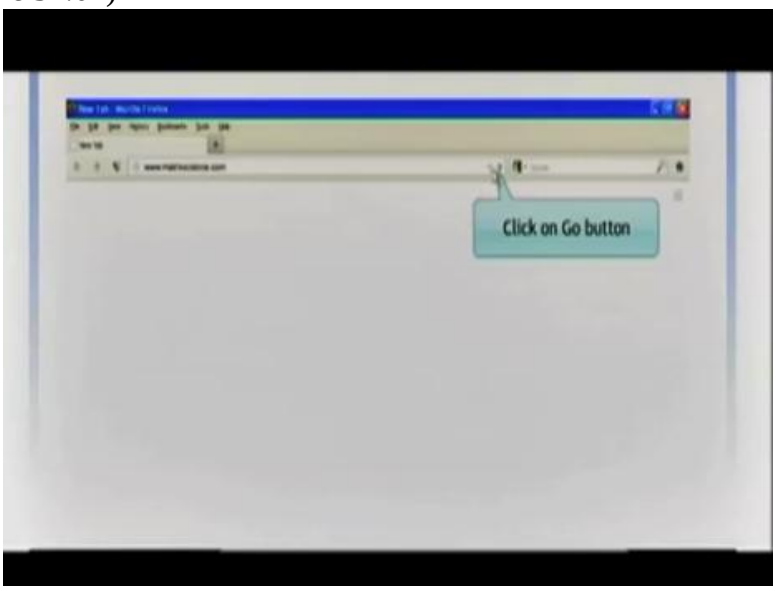
The sequence type, the matched region, what is the expected and the calculated value of the query peptide and the sequence details

(Refer Slide Time 30:58)

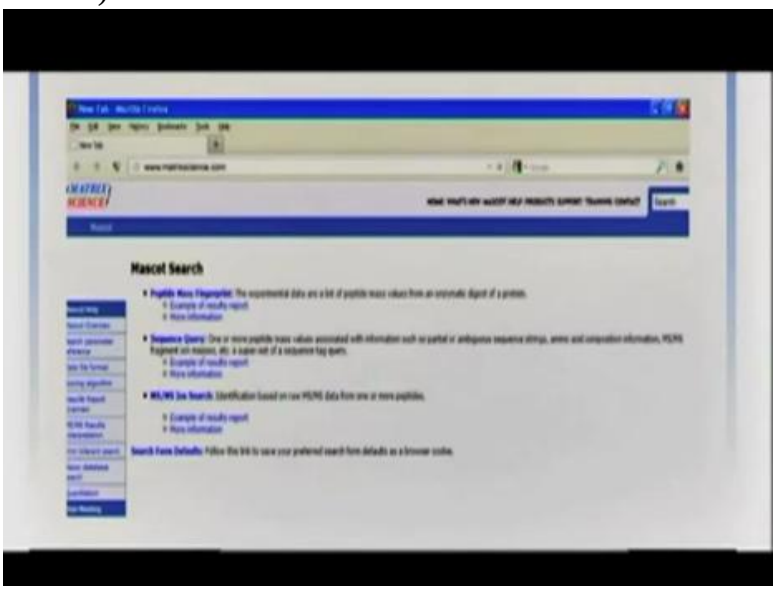


For better protein identification and to increase protein score, CID of each peak generated is carried out to generate MS/MS data.

(Refer Slide Time 31:02)

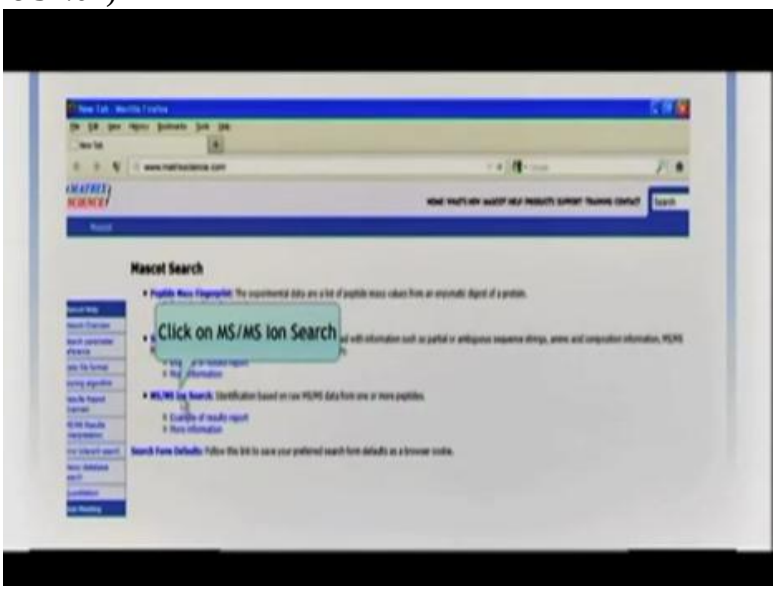


(Refer Slide Time 31:04)



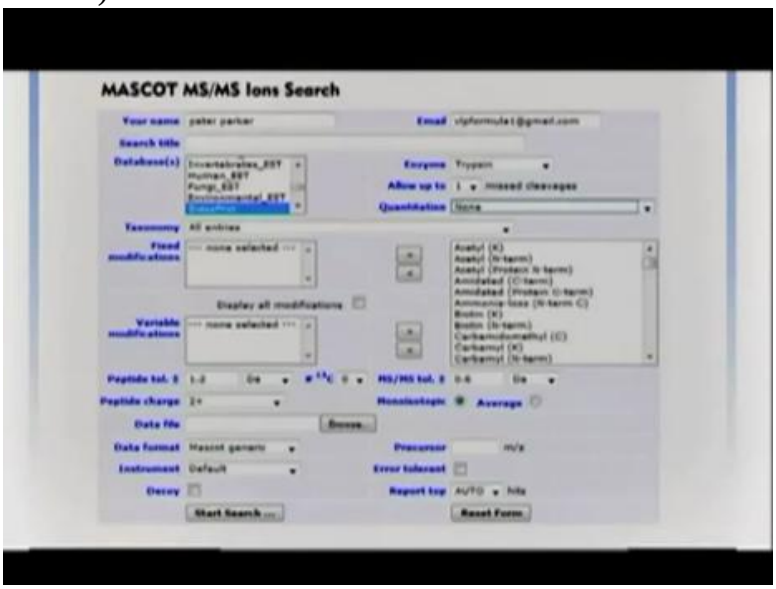
For such data analysis,

(Refer Slide Time 31:07)



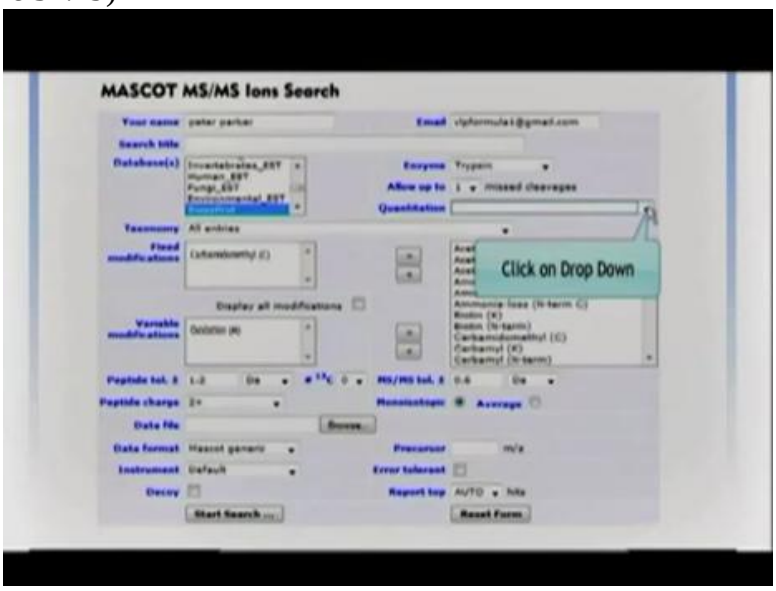
MS ion search option is selected from the Matrix Science browser window.

(Refer Slide Time 31:10)



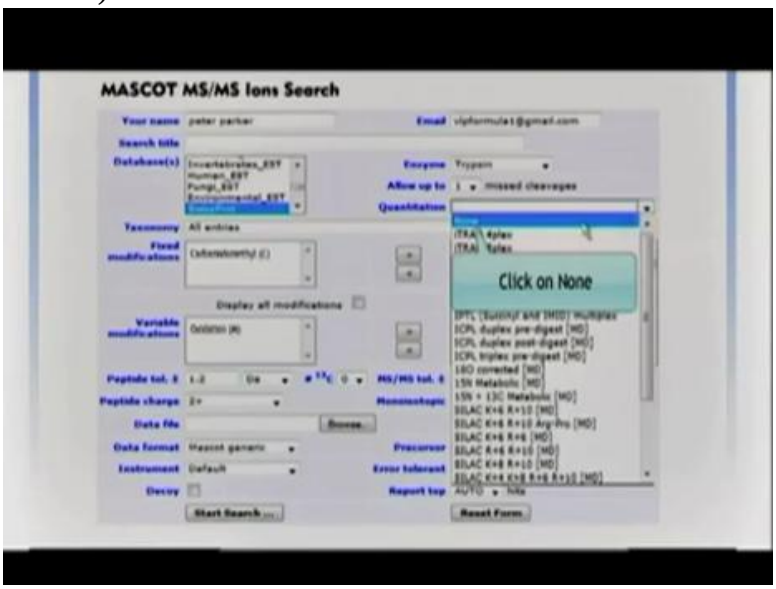
In MS/MS search tool, more input parameters like quantitation, MS/MS tolerance, peptide charge instrument etc in addition to fields for PMF and rest other parameters are similar to that of the peptide mass fingerprint.

(Refer Slide Time 31:29)



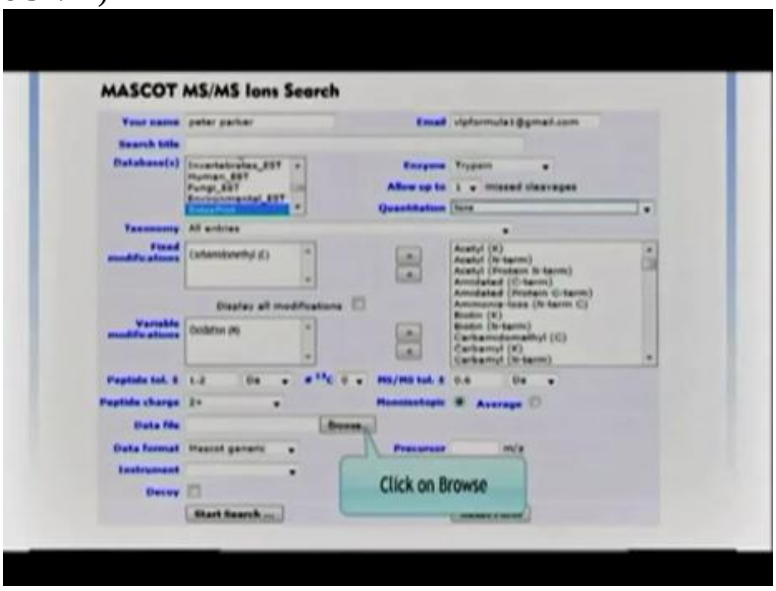
Depending upon the process carried out for data generation, a selection in the quantitation must be made.

(Refer Slide Time 31:36)



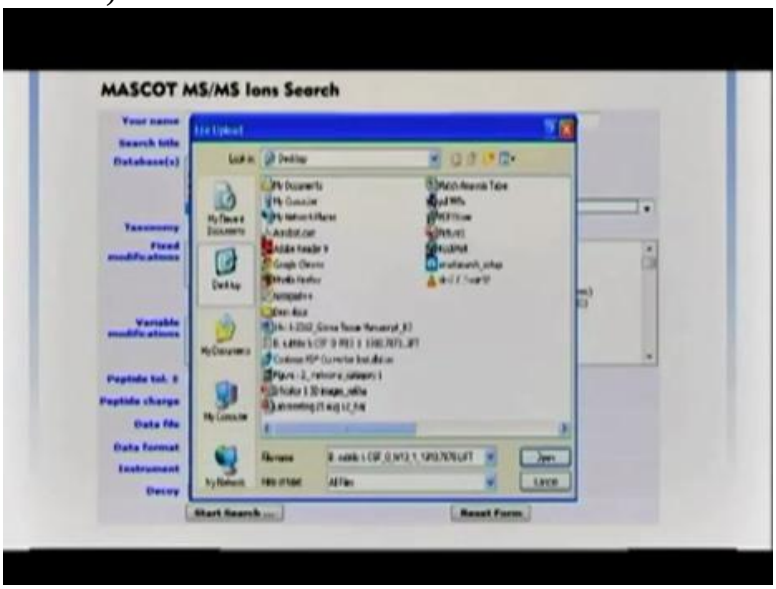
In case of label--free quantitation, select none in quantitation tab.

(Refer Slide Time 31:41)



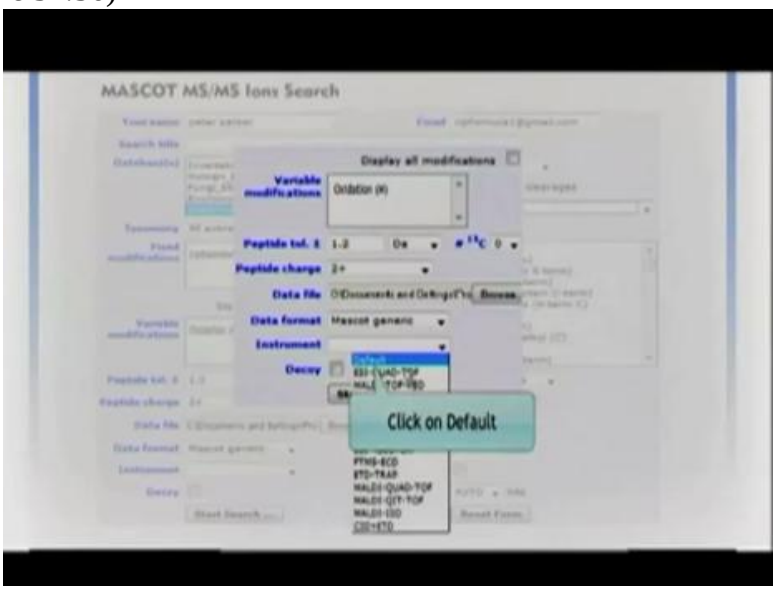
Remaining parameters remain same as PMF. Browse the MS/MS raw data file

(Refer Slide Time 31:48)



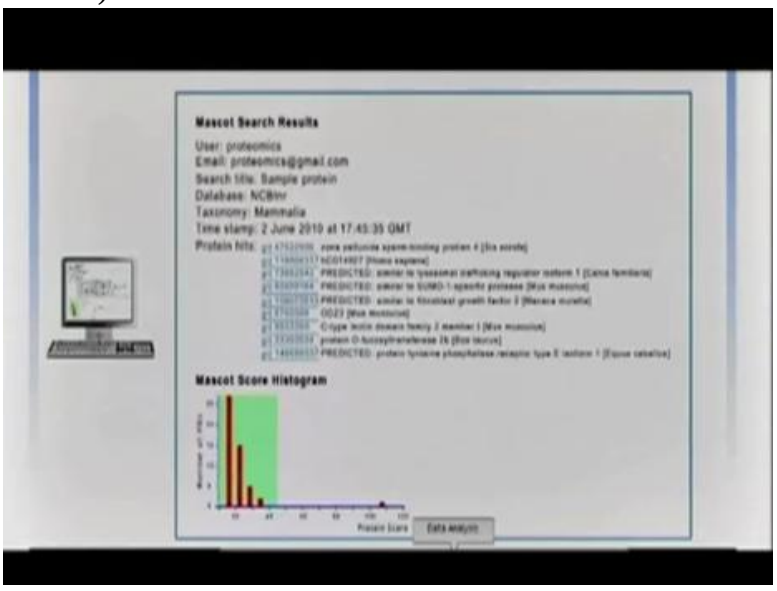
And search the results using Mascot.

(Refer Slide Time 31:56)



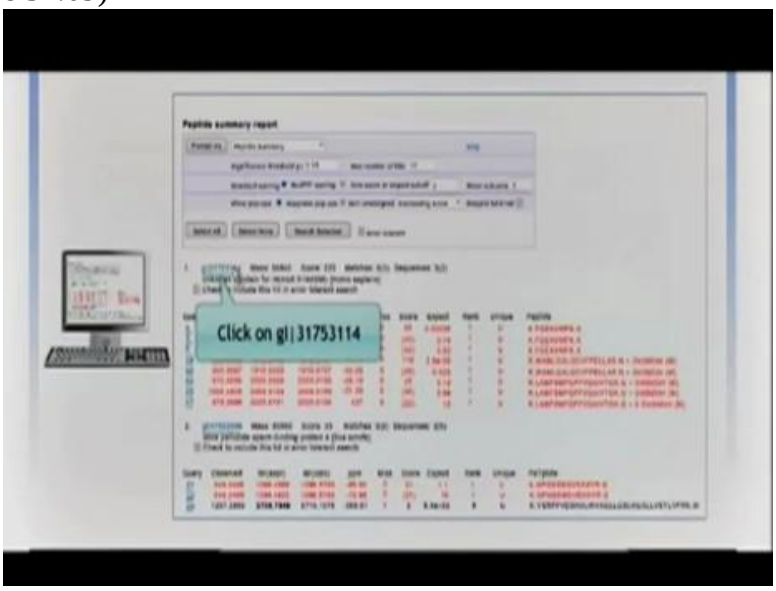
Define the instrument that has been used to generate the raw data. When we don't know the name of the instrument select default

(Refer Slide Time 32:05)



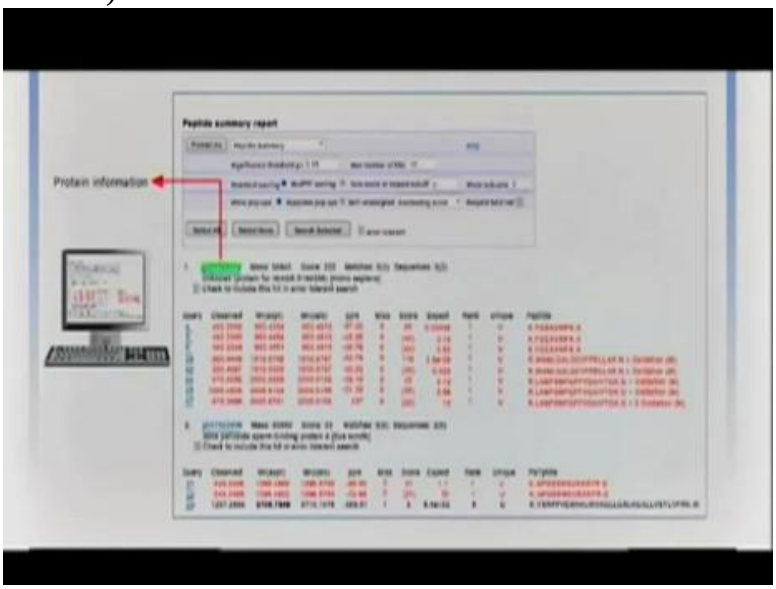
The result output generated is almost similar to PMF.

(Refer Slide Time 32:09)



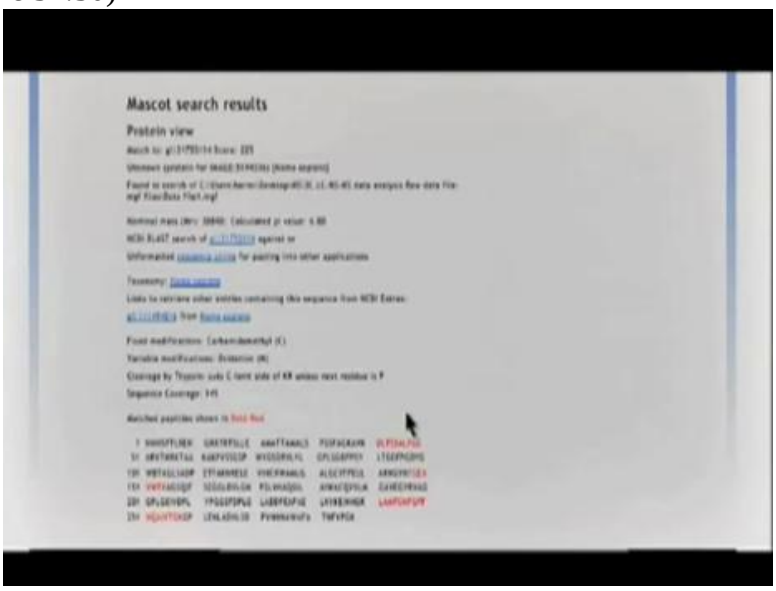
The accession ID indicates the protein information

(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 32:30)



Mascot searches that.

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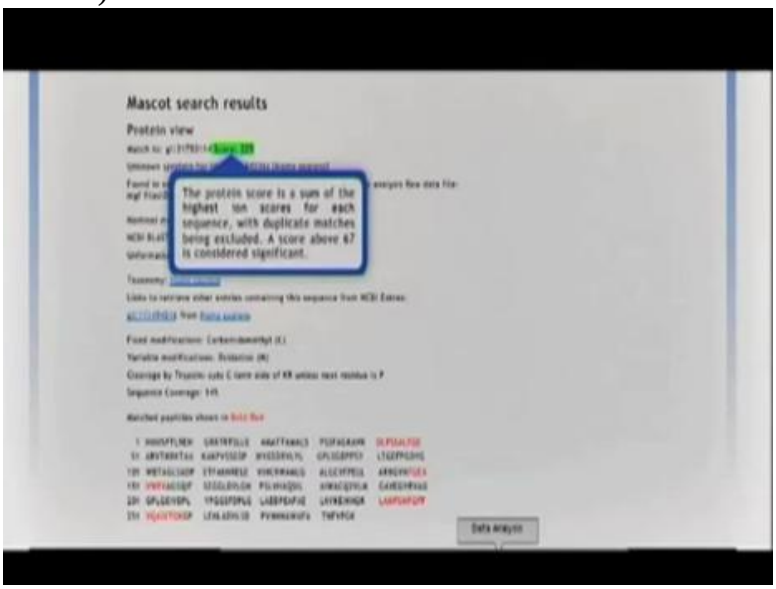
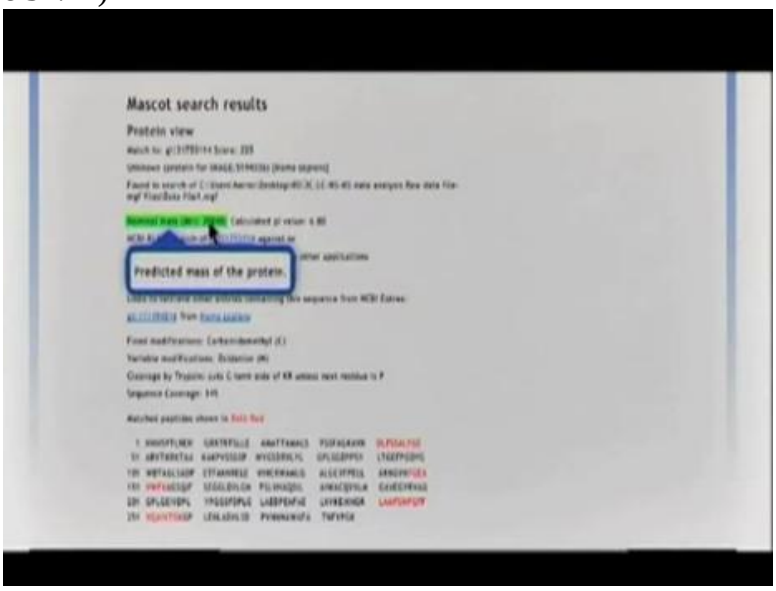


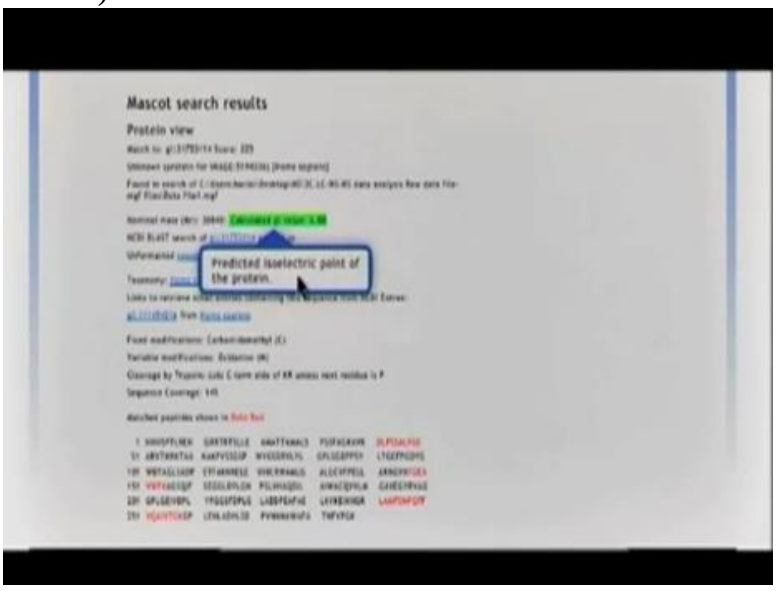
The score, the peptide score is the sum of highest ion scores for each sequence, duplicate matches being excluded

(Refer Slide Time 32:41)



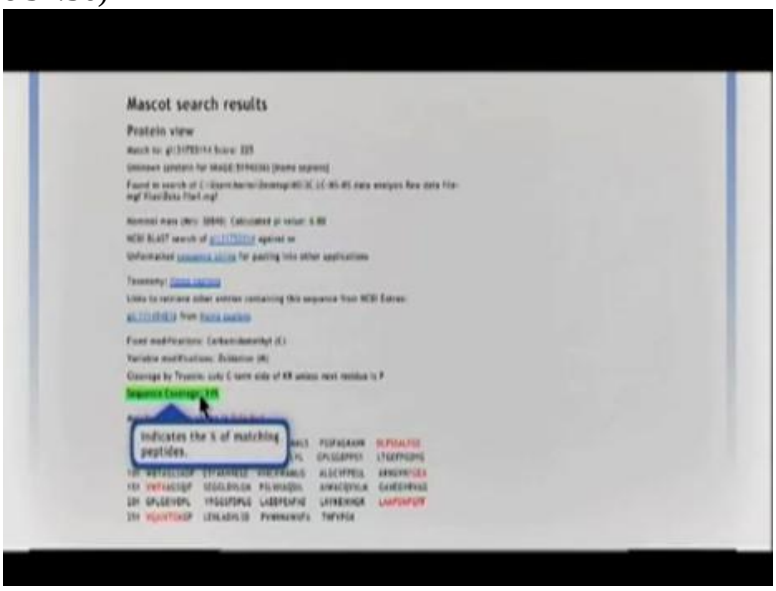
Nominal mass, it is predicted mass of the protein.

(Refer Slide Time 32:48)



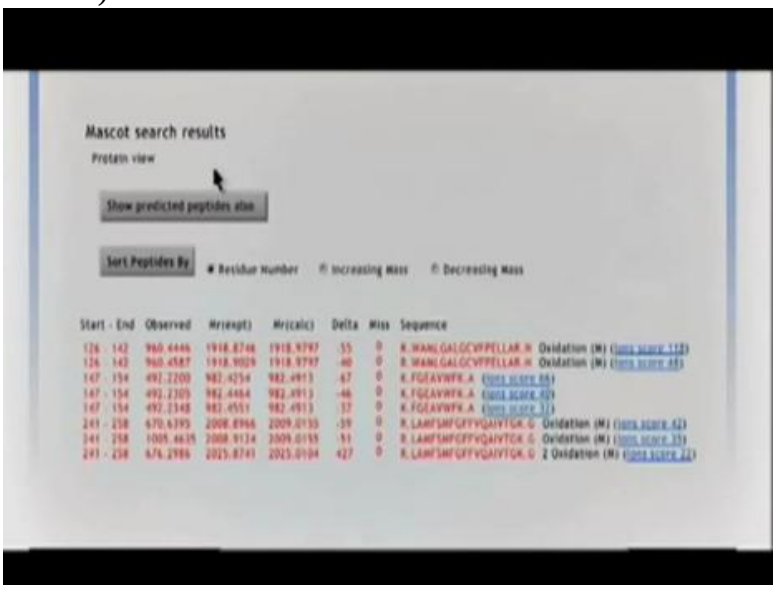
Calculated pI value, isoelectric point of the protein.

(Refer Slide Time 32:56)



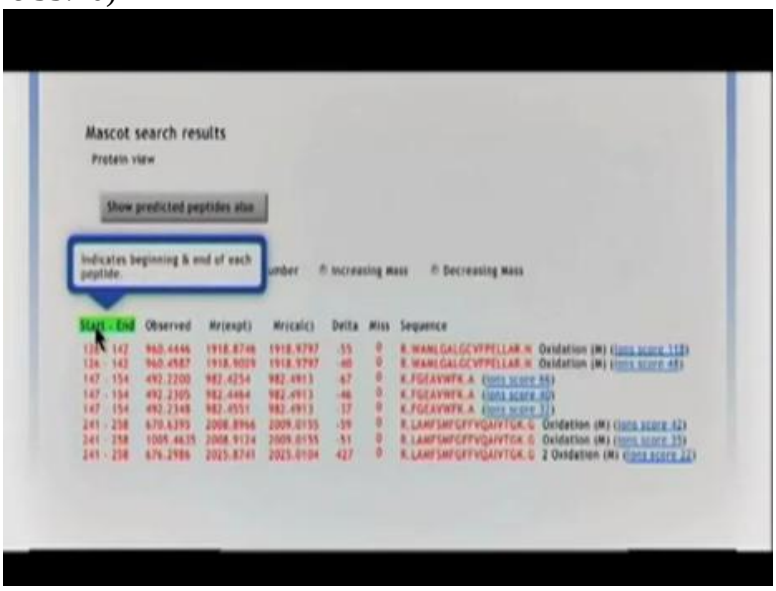
Sequence coverage indicates the percentage of matching peptide. All the peptides are displayed with matching peptides indicated in red.

(Refer Slide Time 33:10)



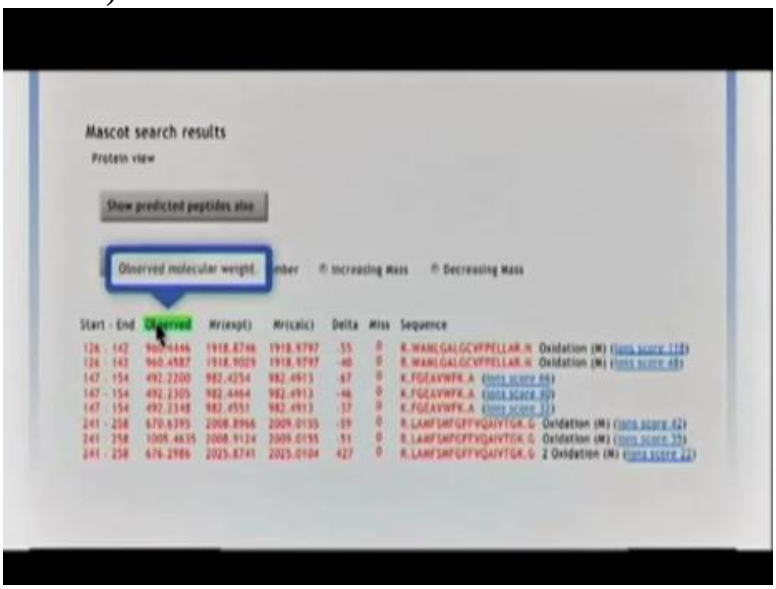
The protein view obtained on

(Refer Slide Time 33:20)



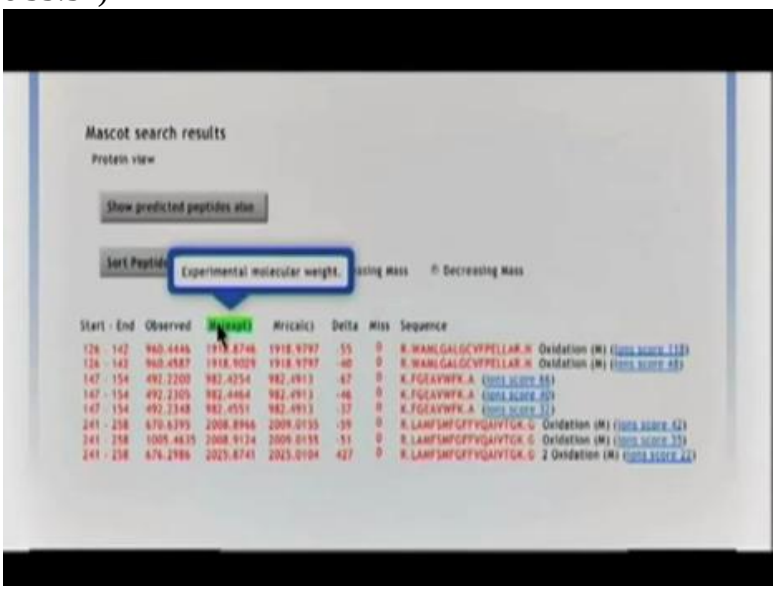
Selecting the particular protein link is very similar to protein view obtained in PMF.

(Refer Slide Time 33:28)



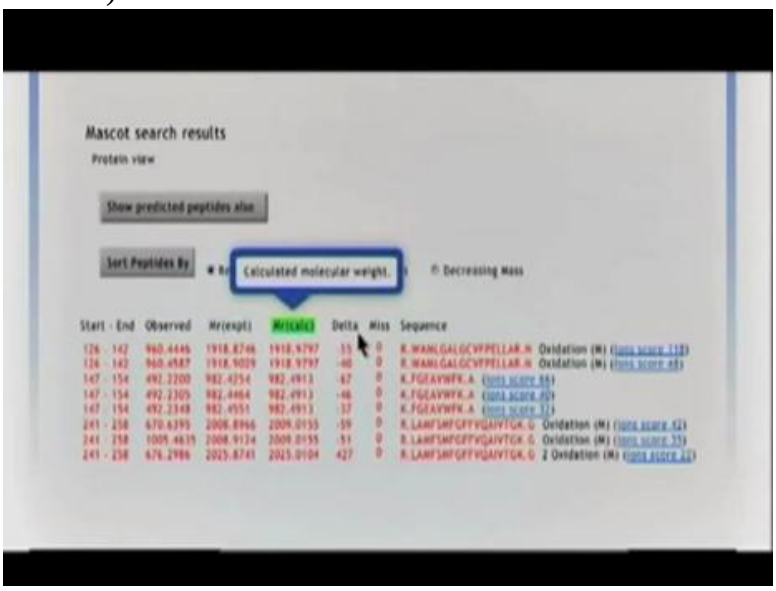
It provides detailed information of each of the matched peptide displayed.

(Refer Slide Time 33:31)



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

(Refer Slide Time 33:33)



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

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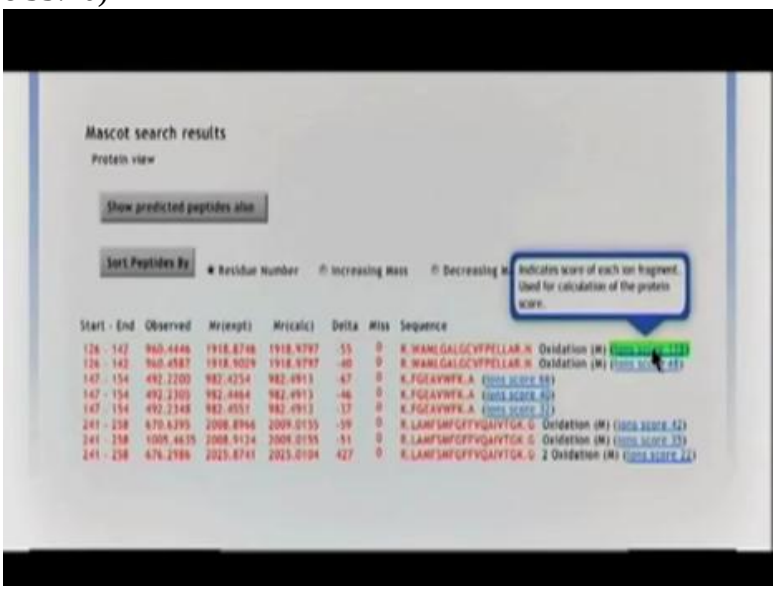
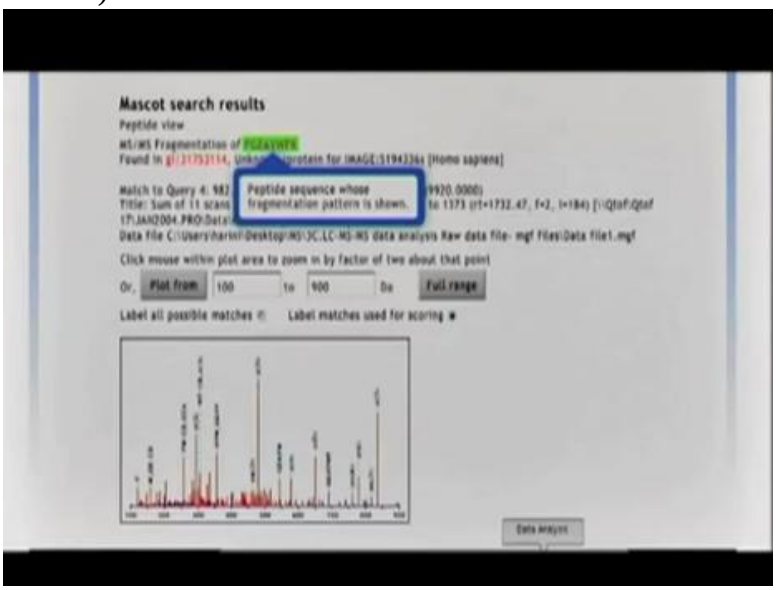


(Refer Slide Time 33:47)



Each peptide in Tandem MS/MS undergoes through second round of fragmentation when it passes through the second mass analyzer before it reaches the detector.

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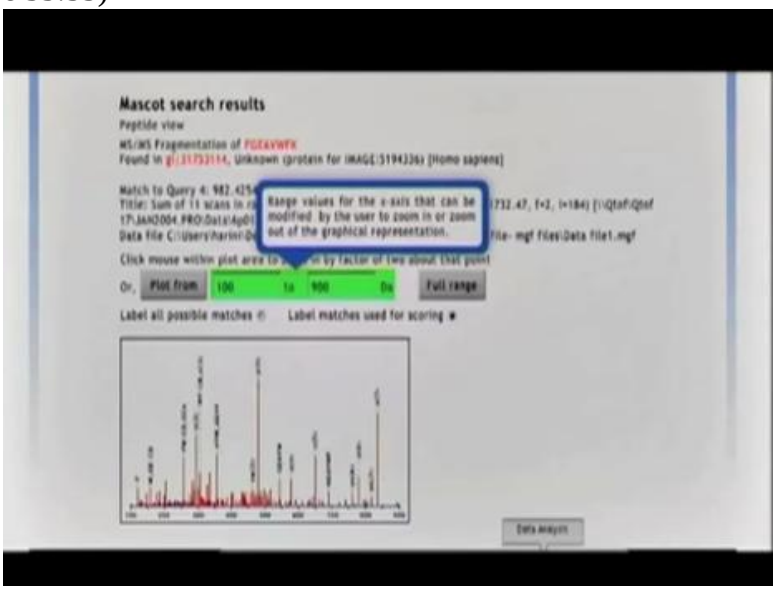
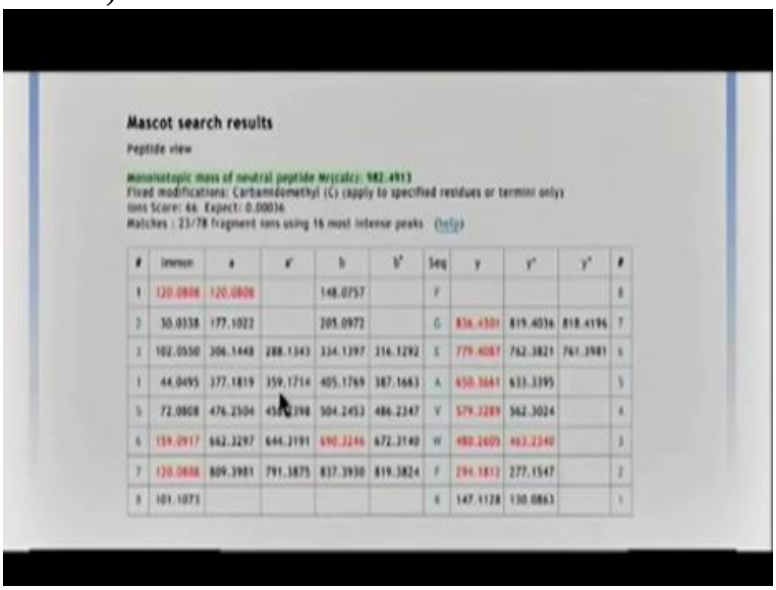


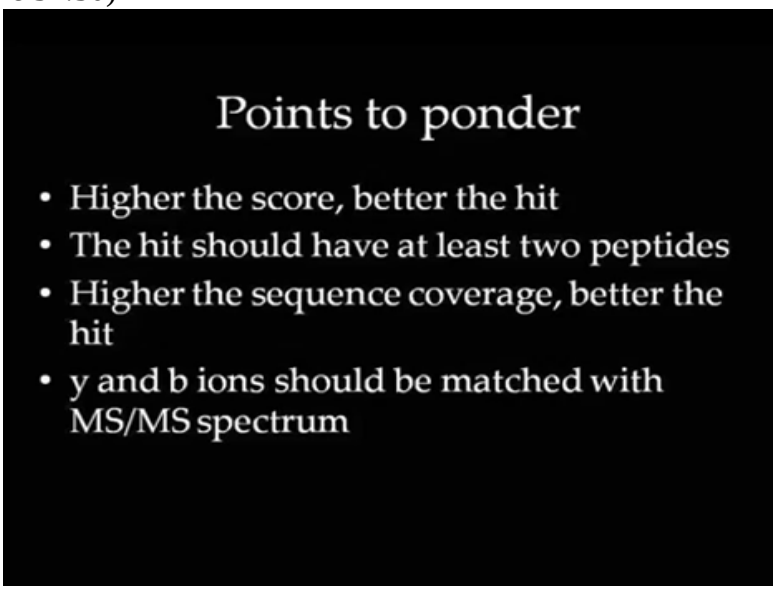
This provides significantly larger amount of information regarding each peptide segment which can be viewed by clicking on the peptide link provided in the summary report. The fragmentation pattern is displayed graphically

(Refer Slide Time 34:13)

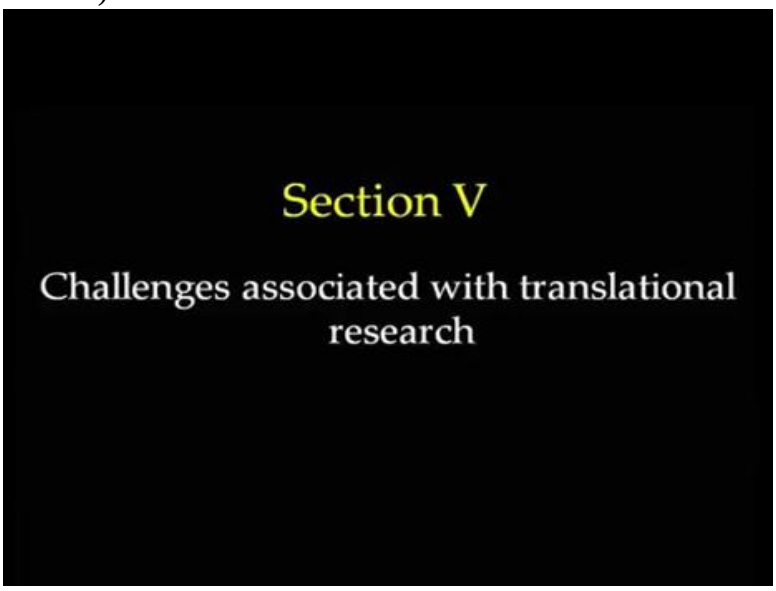


which can be zoomed into as per the requirement

(Refer Slide Time 34:30)



(Refer Slide Time 34:35)



(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

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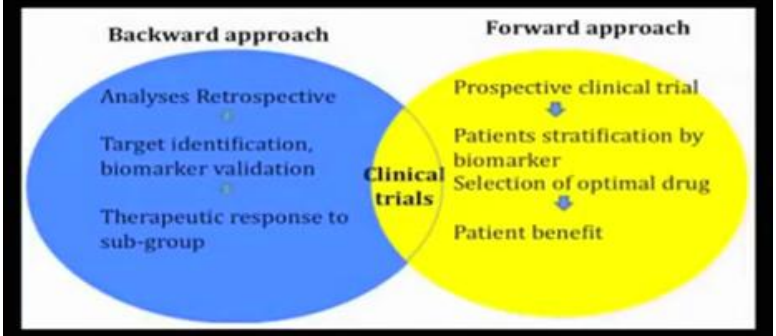
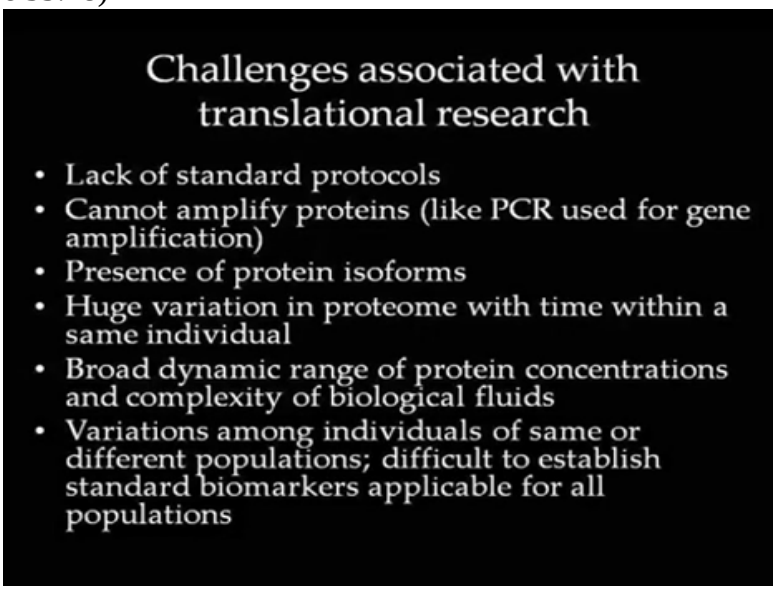
Identification of potential biomarkers by two different approaches- prospective and retrospective



(Refer Slide Time 35:18)



(Refer Slide Time 35:42)