Introduction to Proteomics
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Lecture – 26
MALDI sample preparation and analysis

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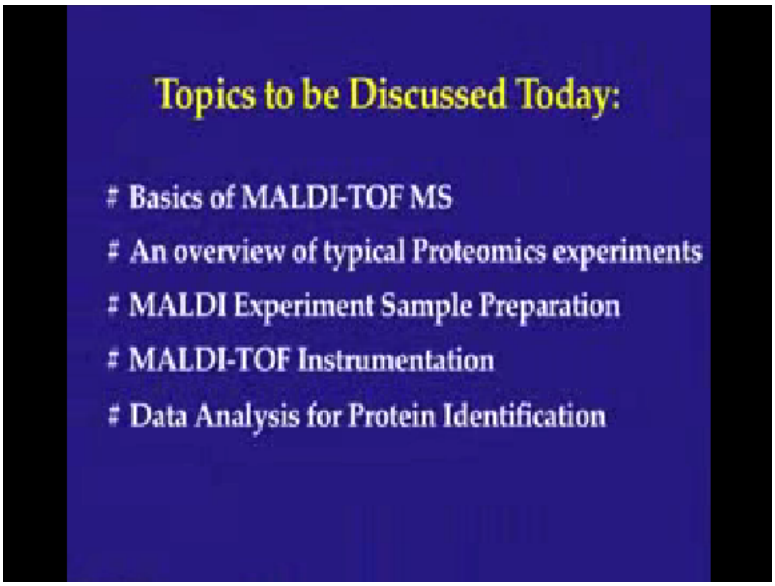

Welcome to the proteomics course. Today we will talk about matrix-assisted laser desorption ionisation, time of flight, MALDI-TOF. In previous lecture, we talked about basics of mass spectrometry, the various combinations of mass analyzers and ionization sources. Now it is time for us to combine those and start discussing these in more detail. So let us focus on the MALDI-TOF which is one of the very widely used technique in proteomics.

It provides a high support platform for similar applications including molecular weight determination, protein identification as well as post-translational modification studies.

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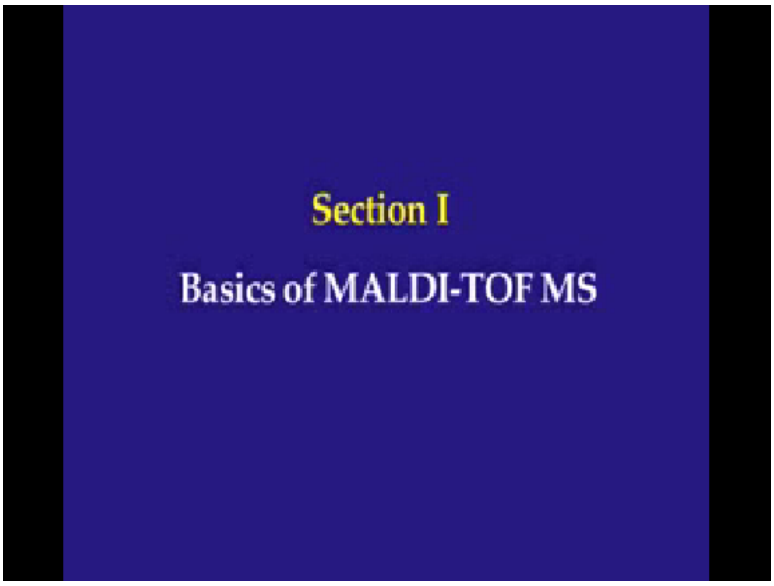

Let us first start about basics effects of MALDI-TOF. So MALDI is an efficient process for generating gas phase ions of peptides and proteins for mass spectrometric detection. MALDI is one of the most widely used ionization technique currently applicable in the proteomics area. The ionization method was independently developed by 2 scientists, Koichi Tanaka and Hillenkamp.

Tanaka also received the Nobel Prize for his noble contribution into soft ionization technique such as MALDI. So let us go through the some of the basic concepts involved in the MALDI-TOF. We can split that in 2 parts, one is MALDI which is ionization source and other is TOF which is a mass analyzer.

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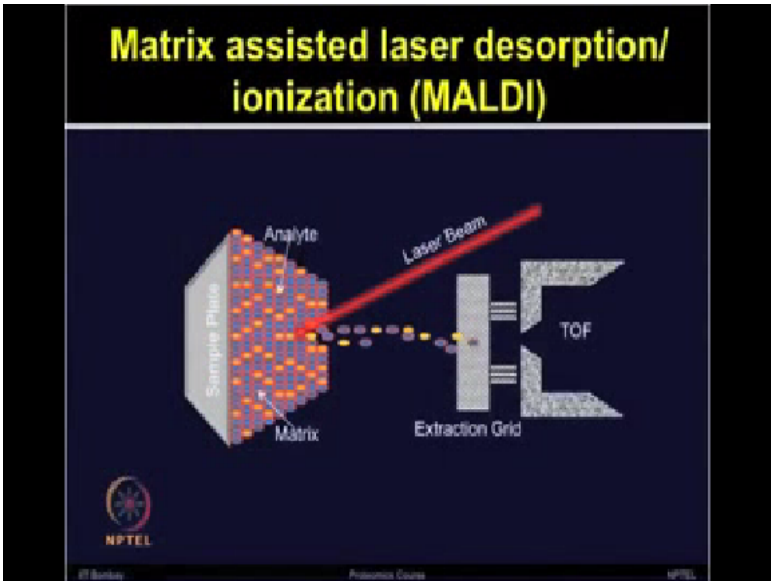

Let us first talk about matrix-assisted laser desorption/ionization or MALDI. So analyte or the proteins of interest are mixed with the matrix which is usually an aromatic compound. The various type of matrices available which we will talk in more detail when we come to the sample preparation and matrix selection but just for your reference, we can use 2,5-Dihydroxybenzoic acid.

We can use sinapinic acid and there are several other choices. Once you have selected a matrix for the experiment, then analyte and matrix can be dissolved in an organic solvent after which then it can be placed on the metallic target. As you can see in the slide, the first left section shows you how to place the analyte and matrix together on the sample plate. Now once you have placed the matrix and the analyte on the target plate, you can put that in the vacuum chamber and apply high-voltage.

Now these crystals are targeted with the short laser beams as you can see in the slide. Then rapid sublimation can convert analyte into the gas phase ions. Now these ions once generated, they can accelerate away from the target plate through the mass analyzer which is time of flight, TOF tube and we can reach towards the detector.

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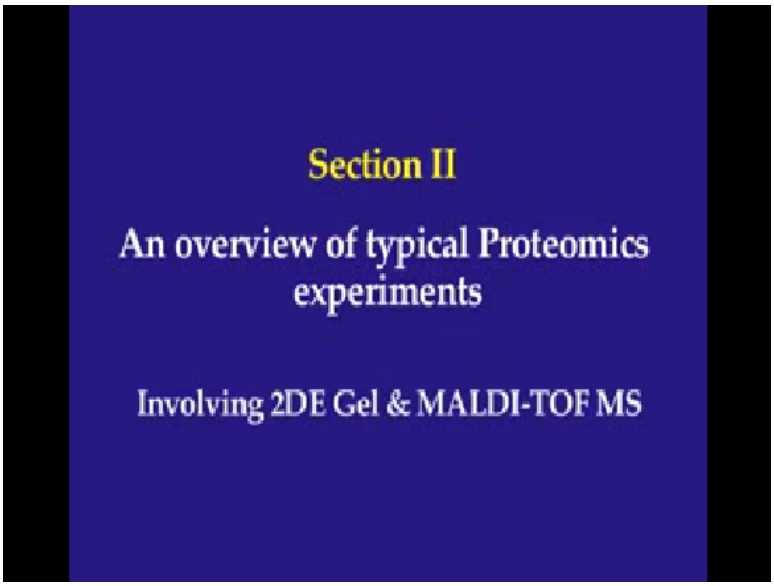

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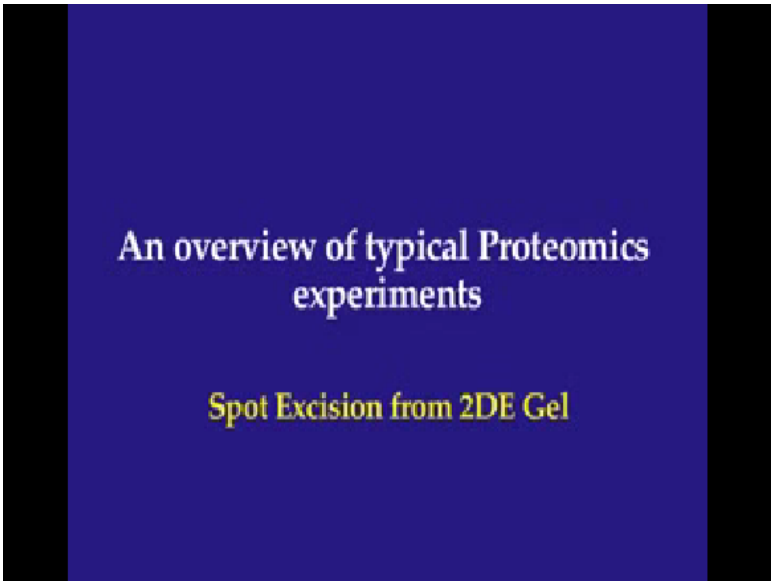

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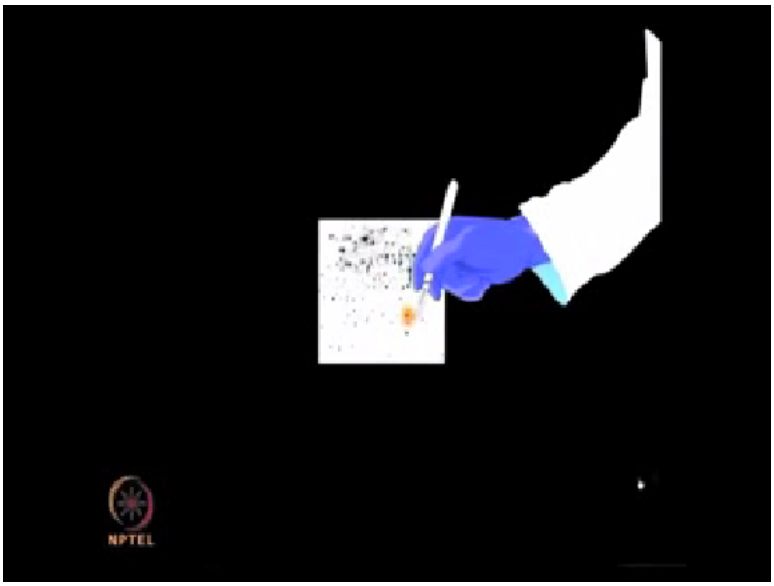

Sample preparation and spotting. The protein sample must be prepared suitably before it can be analyzed by the mass spectrometer. If you run a 2-D gel, so first of all the purified protein of interest need to be excised from the gel on which it has been electrophoresed and dissolved in the suitable buffer. So depending upon the application, if you have purified a protein, you can separate that on the gel and then cut their band or if you have a mixture of the proteins in 2-D gel, you can just excise that particular spot.

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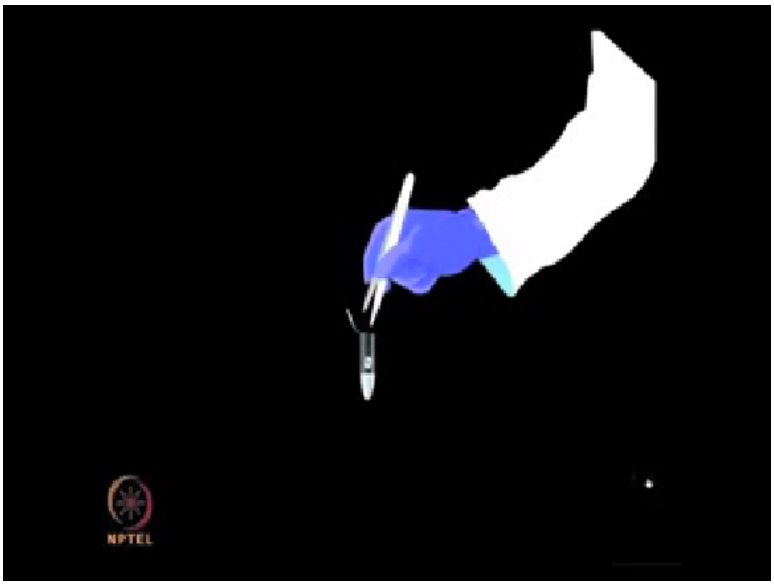

Spot can be dissolved in the suitable buffer.

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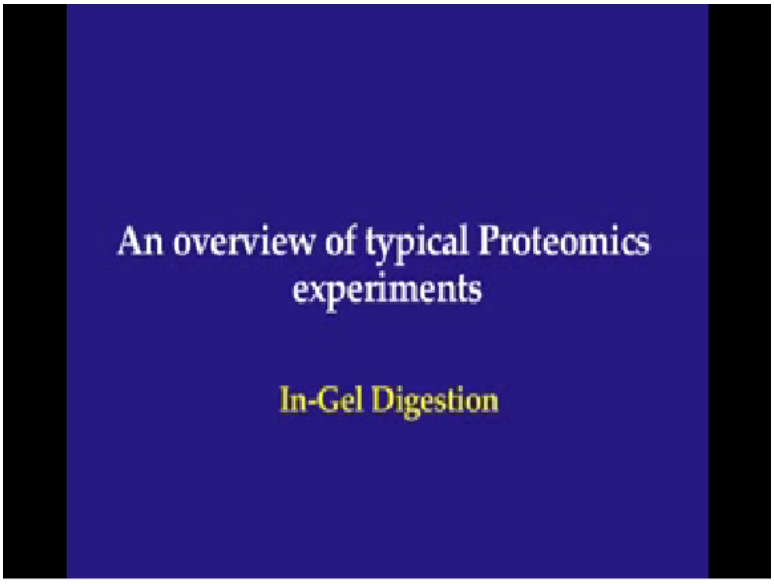

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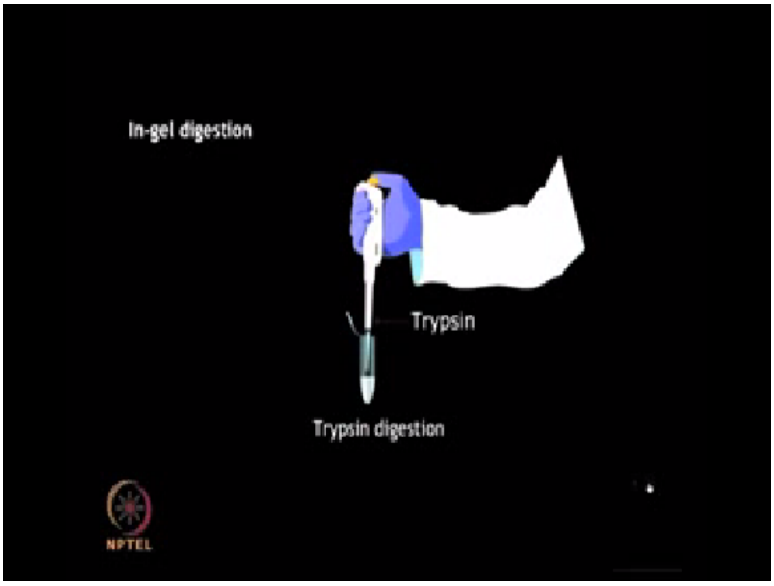

Trypsin is then added to this mixture in order to carry out digestion of the protein. Trypsin cleaves the protein at the C terminal of the arginine and lysine residues but that is not always universal.

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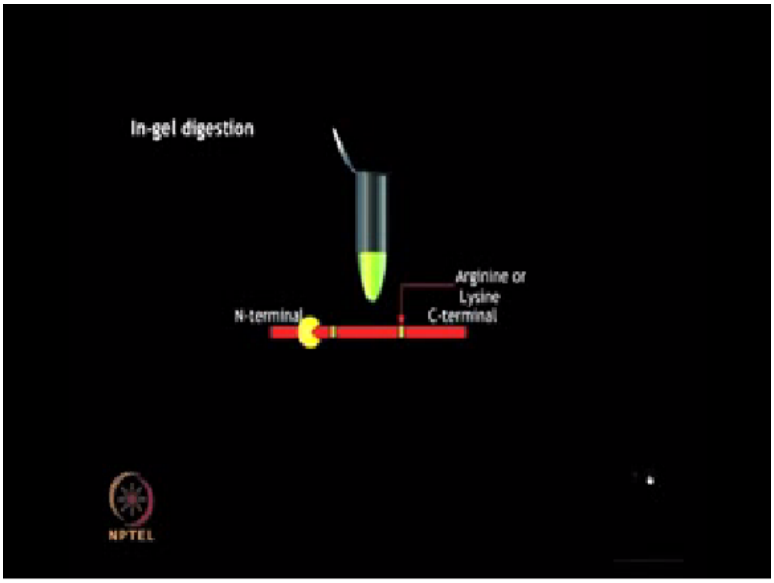

If you have a proline present immediately after, then it will hinder that.

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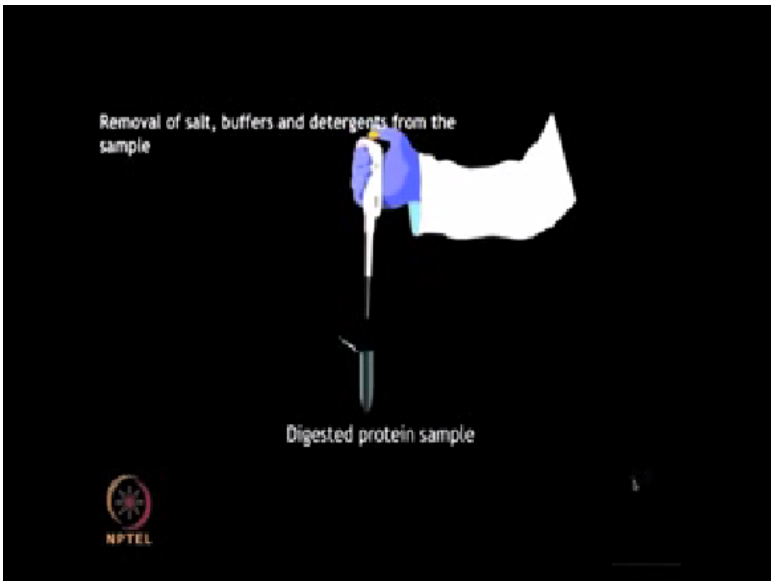

But overall the protein is digested into smaller fragments of manageable size.

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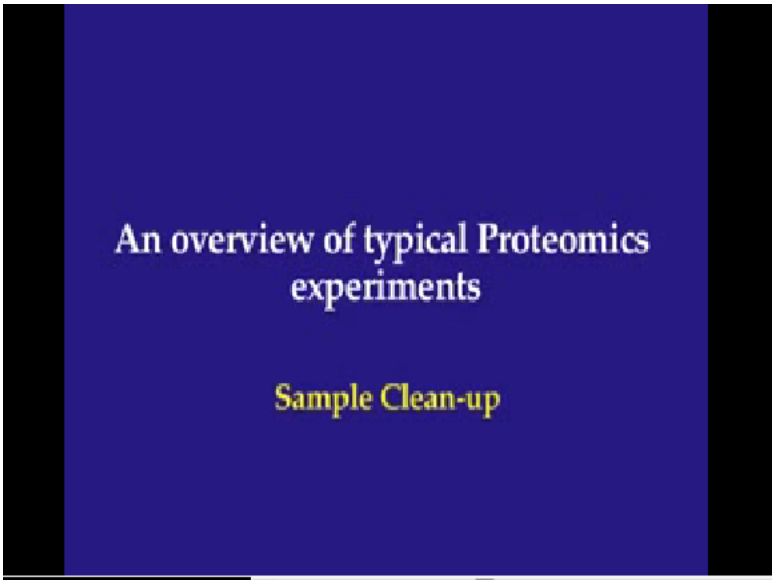

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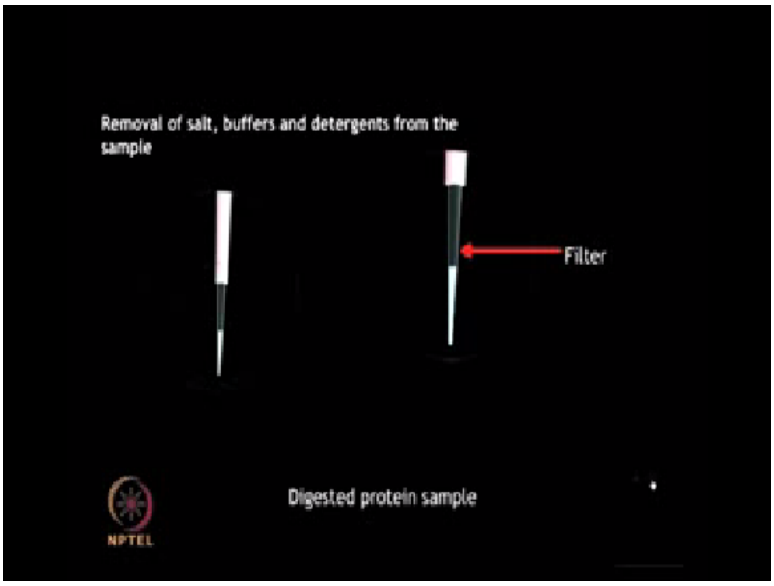

Once the protein sample has been digested, all the salt, buffer and any detergent must be removed from the sample. So after doing (()) (06:29) and before proceeding further mass spectrometry analysis, in between an efficient step is to use some filters or ZipTip which can eliminate some of these contaminants and salts. It offers several advantages such as quick purification, sample enrichment and ensures that there is no contamination.

So there are multiple advantages of using ZipTips; however, it can purify only limited volume of the sample and also it adsorbs some amount of the protein sample thereby leading to losses.

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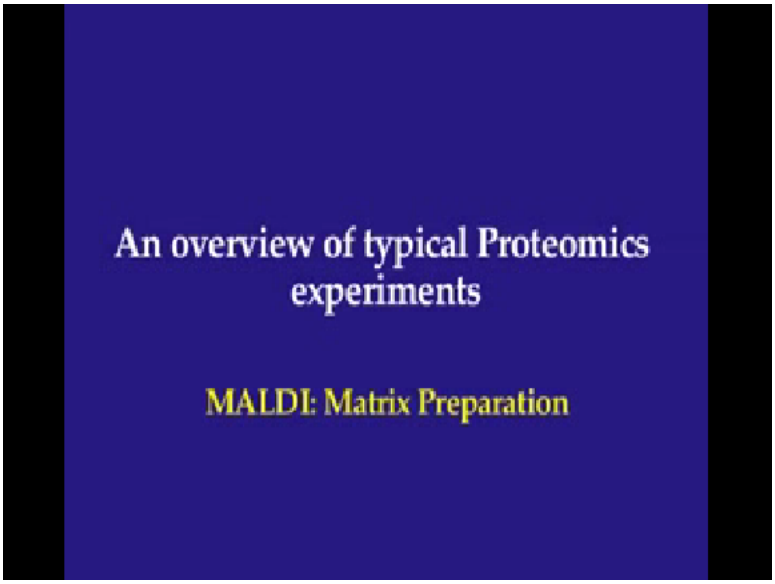

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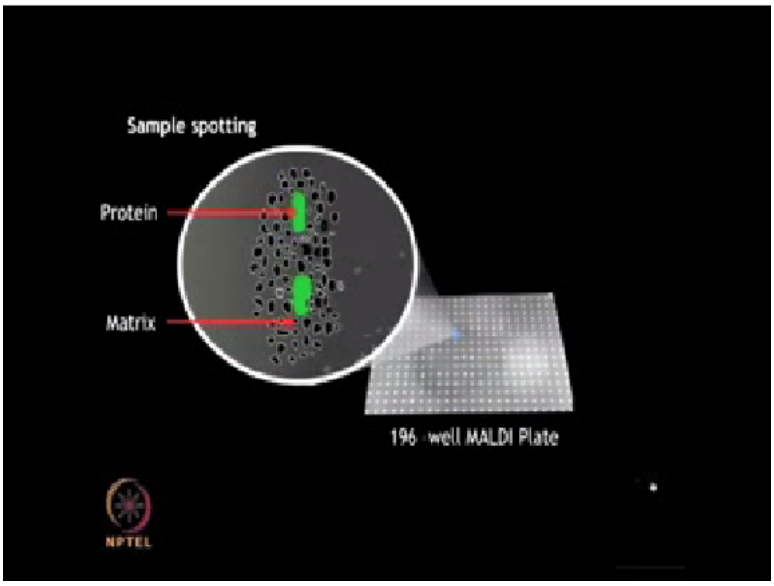

The purified protein sample can be mixed with an aromatic matrix compound such as alpha-cyano-4-hydroxycinnamic acid or sinapinic acid in the presence of an organic solvent. The components are mixed thoroughly and then the solution containing the organic matrix with embedded analyte of interest can be spotted onto a metallic MALDI sample plate. MALDI gets here opportunity to analyse large number of samples in high throughput fashion.

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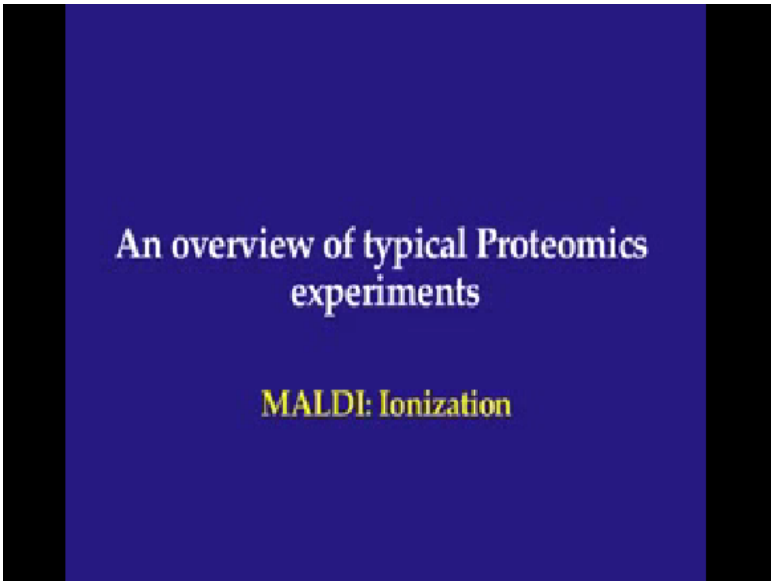

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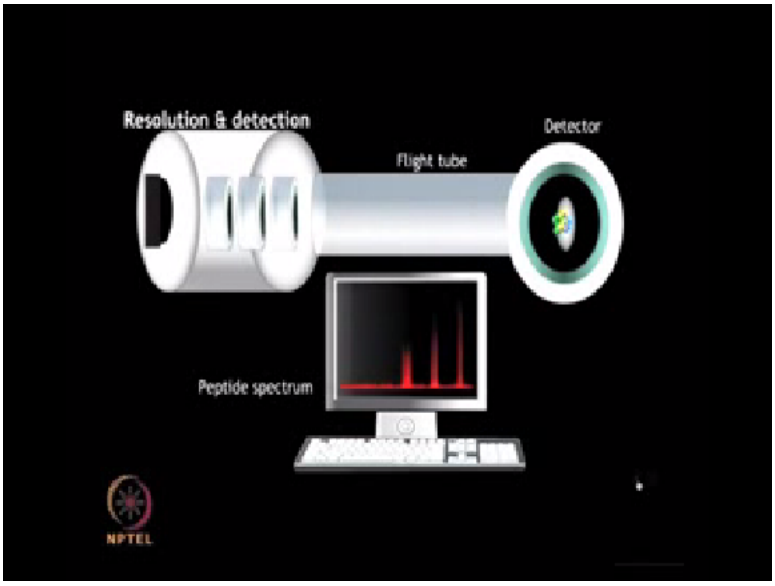

The target plate containing the spotted matrix and analyte can be further placed in a vacuum chamber with high-voltage and short laser pulse is applied. The laser energy gets absorbed by the matrix and is transferred to the analyte molecules which undergo rapid sublimation resulting in gas phase ions. The gas phase ions generated are accelerated and travel through the flight tube at different rates.

The lighter ions move rapidly and reaches the detector first while the heavier ions migrate slowly. These ions are resolved and detected on the basis of their mass-to-charge ratio and mass spectrum is generated. Parameters such as geometric design, power supply quality, calibration method, sample morphology, ion B-velocity, etc. all of these factors affect the accuracy of mass detection.

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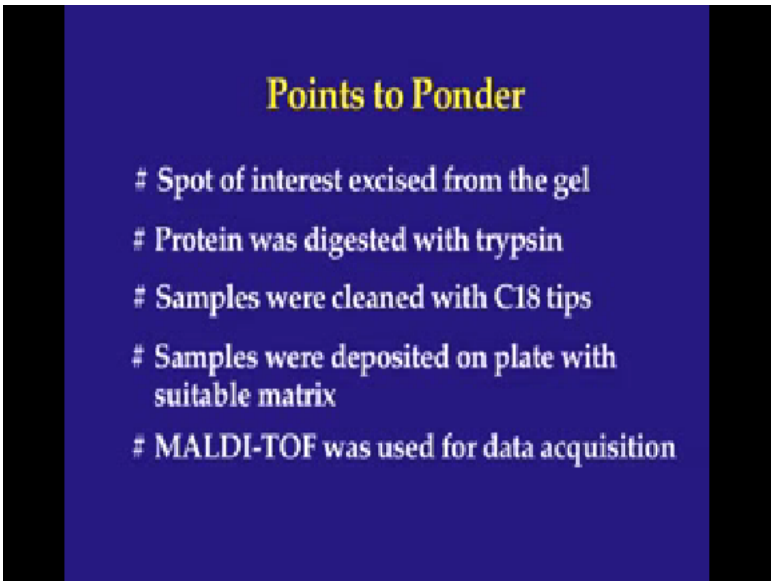

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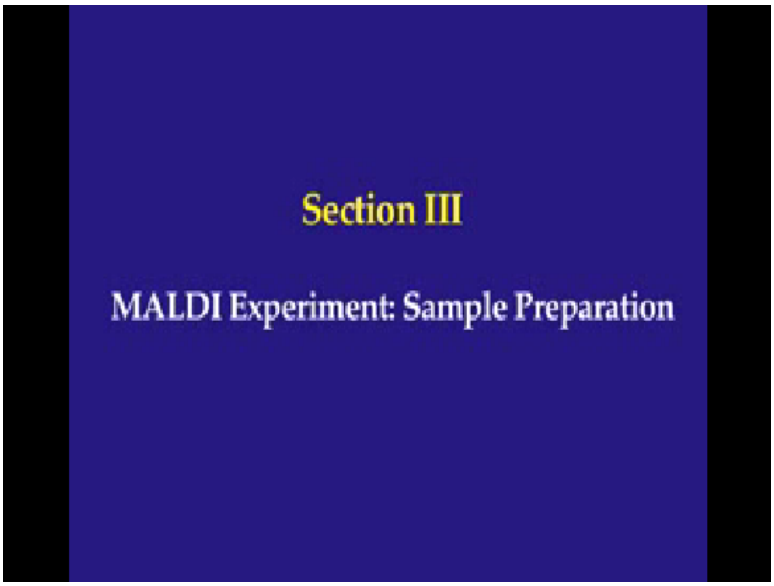

So now you know how to perform the cleaning step by using ZipTips. Now you have the sample ready and you have selected the matrix. So now let me show you this various steps involved before you can actually start the MALDI experiment.

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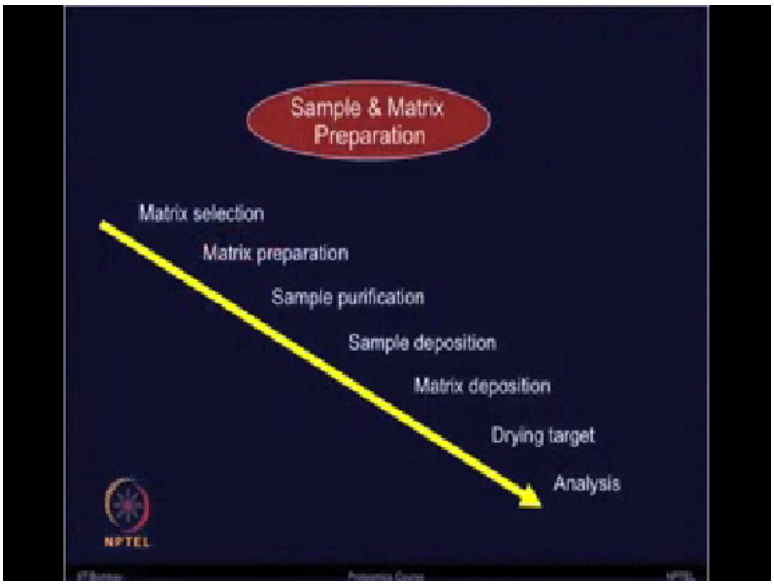

So you need to select the matrix. You need to prepare matrix. You have already done the sample purification. Now sample need to be deposited on the MALDI plate. Either you can mix with the matrix or you can do these 2 separately. There are various combinations one can try and then once both sample and matrix is deposited on the MALDI target plate, then you are ready to do the drying and then plate can be used for MALDI-TOF instrument for further analysis.

Let us first talk about matrix selection. So the important step in MALDI-TOF analysis is 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. So these matrices are low molecular weight organic compound with low vapour pressure and volatile nature.

Most of the matrices are acidic in nature, so it can easily excite the photon and ionise 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 a certain major features of these matrices commonly used for the various applications.

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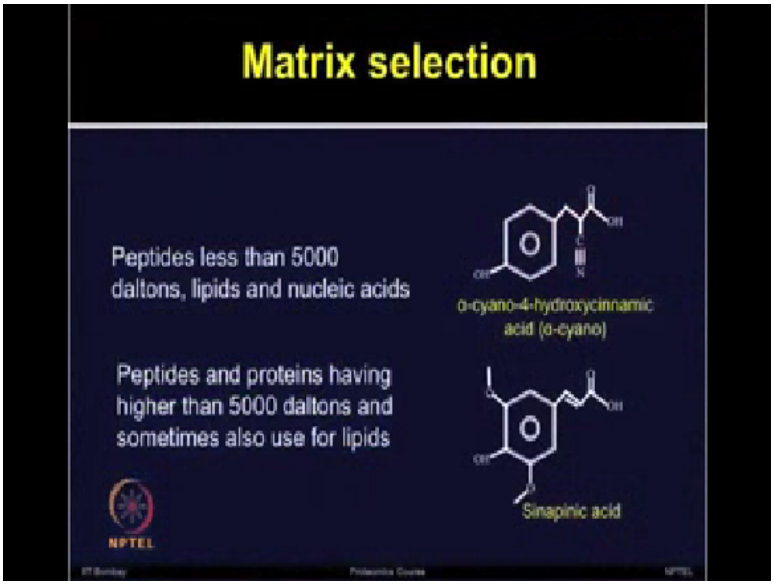

So one is the alpha-cyano-4-hydroxycinnamic acid. Then you have peptides less than 5000 daltons or lipids and nucleic acid, one can use this matrix. One can also use sinapinic acid if peptides and proteins are having more than 5000 daltons and it can also be sometime used for the lipids. Then you have options such as 2,5-Dihydroxybenzoic acid also known as DHB.

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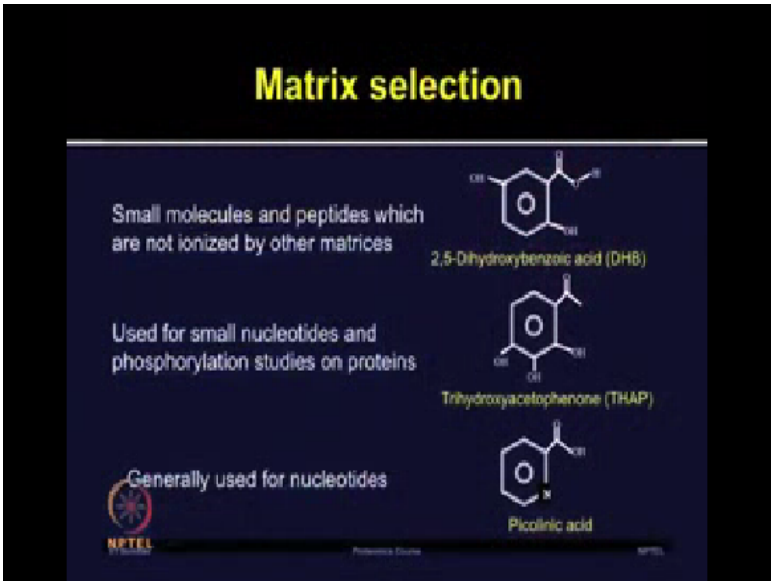

Small molecules and peptides which are not ionised by the other molecules can be analysed by using this matrix. Trihydroxyacetophenone, THAP, this is used for small nucleotides and also used for phosphorylation and specialised applications. Then we have picolinic acid which is generally used for the nucleotides. So these are only few representative matrices.

As you can see, there are many options available for selecting the matrix depending on the molecular weight and the type of applications. But regardless of these, these matrices absorb energy from the laser source and converts both matrix and analyte into the gaseous phase. Matrix can also analyze analyte molecule by providing energy which comes from the laser bombardment.

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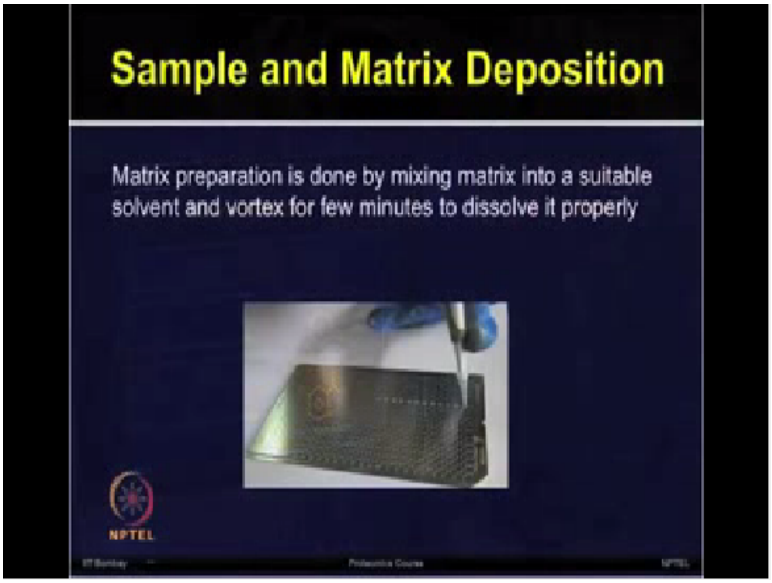

Now once you have selected a matrix, matrix can be prepared by mixing it into a suitable solvent and vortex it for a few minutes so that it can dissolve properly. Now you are ready with both your analyte, the protein which you want to analyse as well as the matrix which you have selected for your application. Now one needs to think how to deposit that 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 a fund of tube and then the mixture is directly deposited by using micropipette onto the MALDI plate but one can also try various combinations. Even one approach, the sample is first deposited 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 is to apply that with the sandwich base method in which first small amount of Matrix is deposited on the plate, then you add the protein sample and again the matrix is

spotted on top of it so that you have enough matrix below and the above of the analyte. So one can try different combination of placing the matrix and the analyte and then once you have placed all of this sample of interest on the MALDI plate, then you are ready to dry the target plate.

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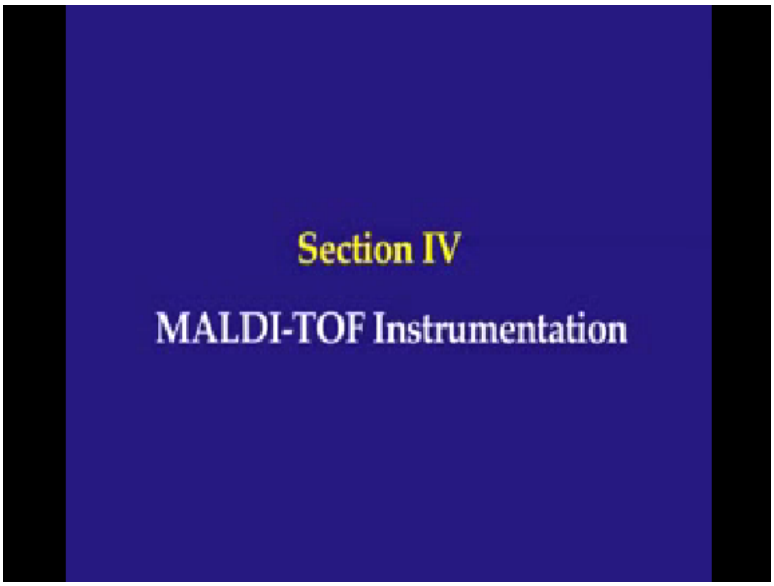

So after spotting is done and MALDI plate is dried almost 30 minutes, then the instrument can be turned on and MS analysis can be performed. Now there are various type of configuration of these instruments available as well as there are various type of commercial software which help to operate the hardware.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

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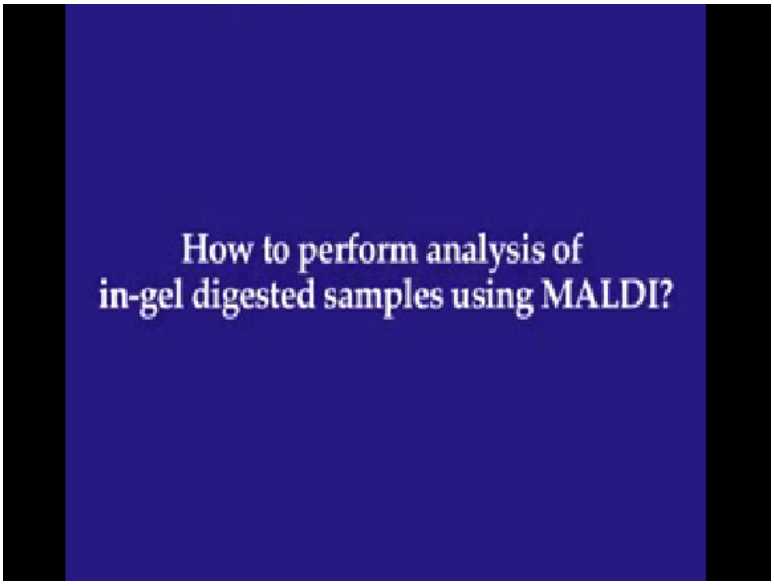

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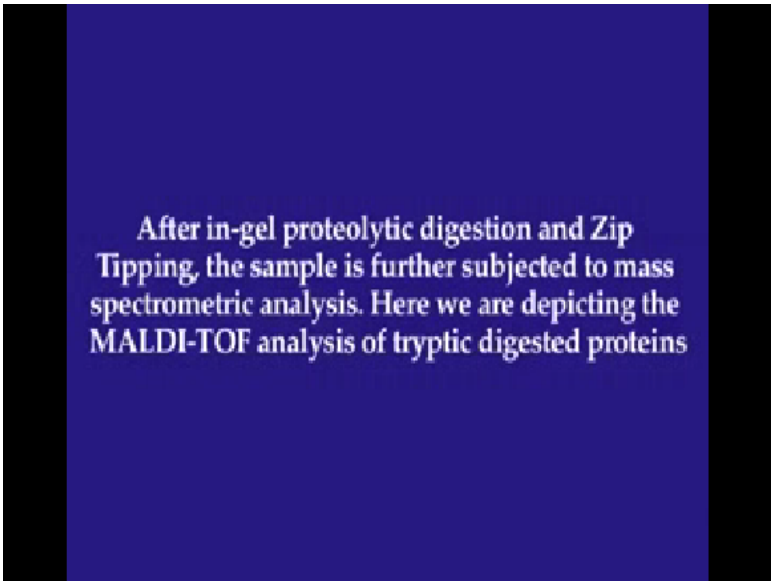

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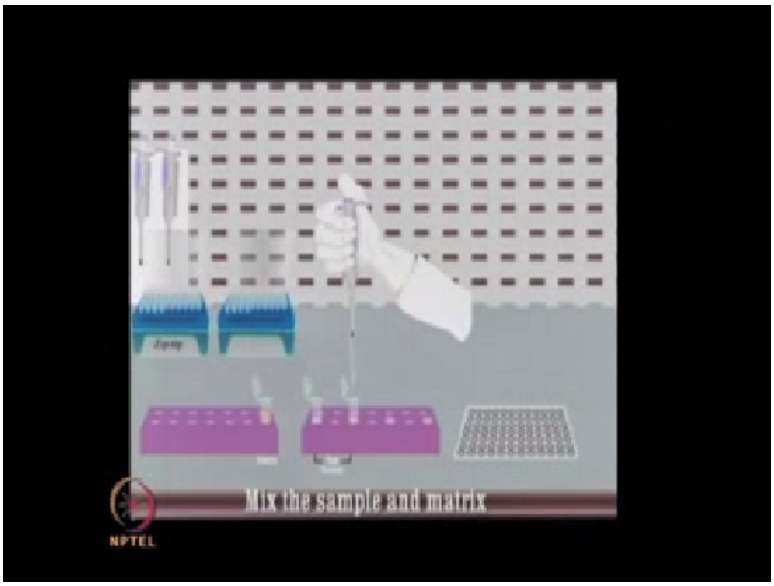

MALDI is performed in 2 steps. In first step, the compound for the analysis should be dissolved in a solvent containing small organic molecules known as matrix.

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This mixture is dried before analysis and liquid solvent used in the preparation of solution is removed (()) (17:13) by depicting the matrix preparation as well as instrumentation, we will try to give you overview of the MALDI-TOF instrumentation.

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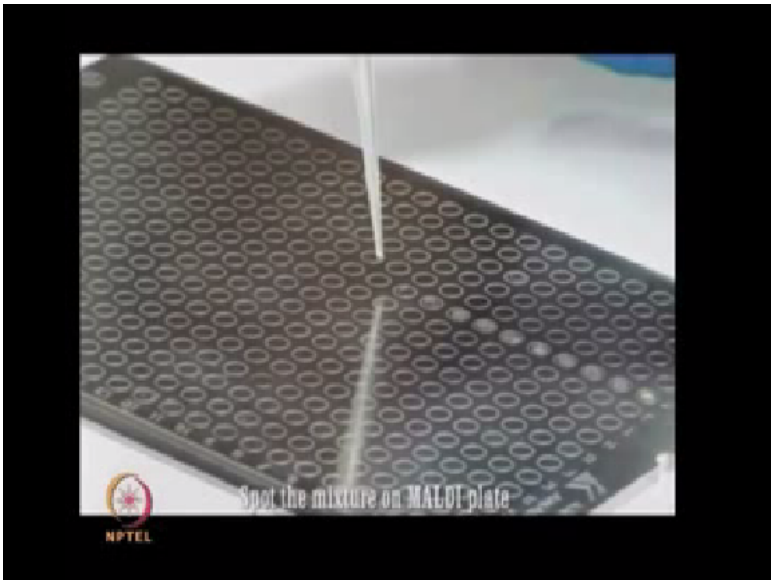

To spot the mixture on the MALDI plate, how uniformly you can plate these mixtures on the MALDI plate insures your good spectra and data quality later on.

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Completed on the MALDI plates, the samples are allowed to dry for 30 minutes after which the instrumentation is switched on and MS analysis can be performed. While these steps are happening, you need to ensure that instrument is on.

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So click on the software and open the acquisition window and then click on the open door. Insert MALDI target plate, face up with the cut-off corner to the front and now by using software, close the door. The door of insertion chamber is now closed.

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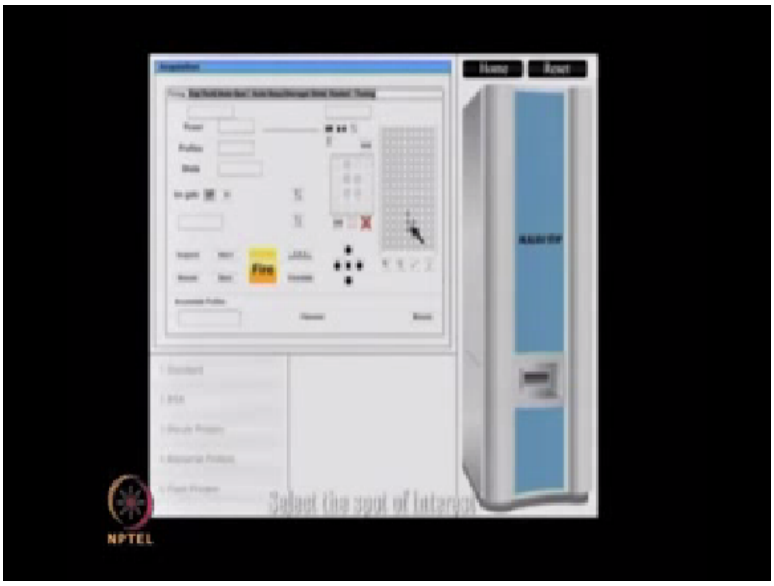

You can select the plate, you can view the overall plate on the screen and then select the spot which you want to analyse. So click on the yellow target in the acquisition window and select "go to the location". You can now do the laser bombarding and peptide spectrum is generated.

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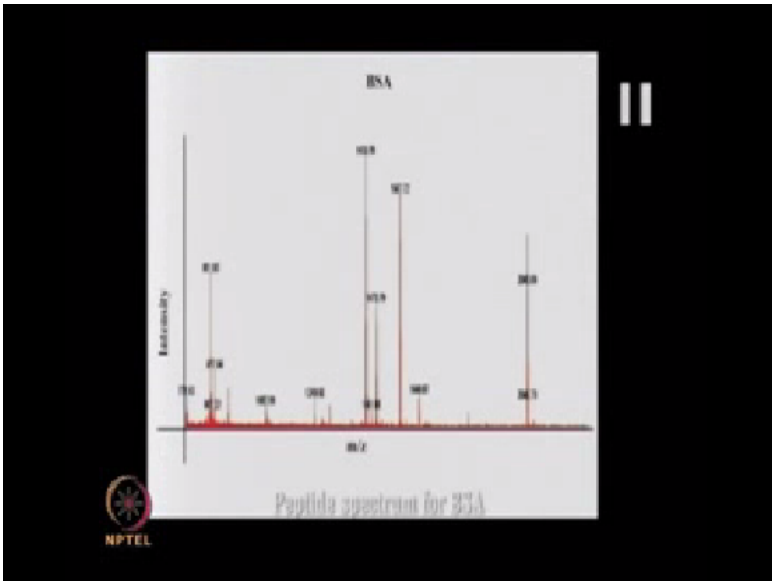

We have shown here one standard protein, bovine serum albumin. So you have to look at various locations where you can get best spectra from that spot and then you can freeze it. Same process can be performed for different spots and different regions.

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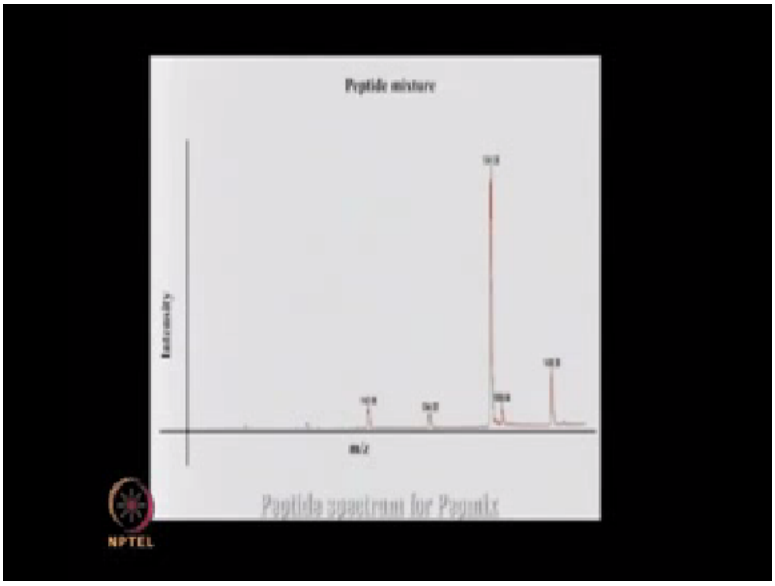

Now we have shown here are spectra for the Pep mix. So now you are clear with how to perform the MALDI-TOF experiment. Now let us add one more mass analyser, so now we have a configuration of MALDI-TOF-TOF.

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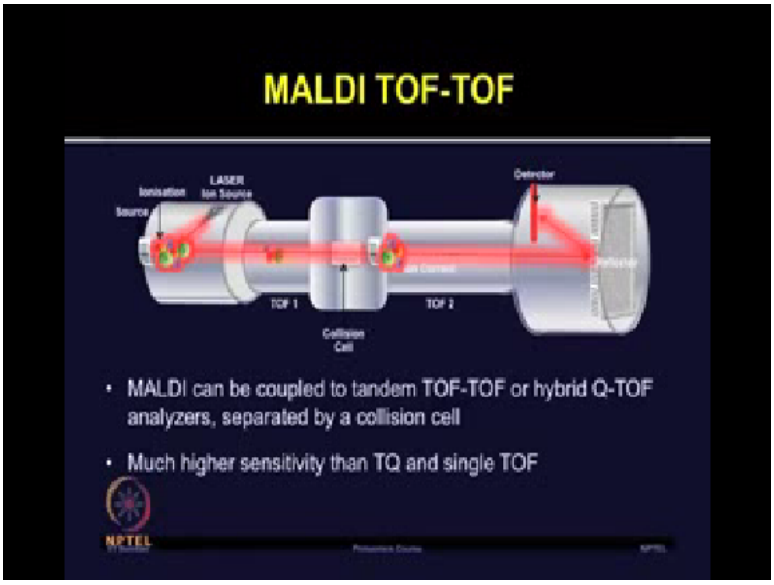

So MALDI can be coupled to the tandem time of flight in combination with another time of flight, so TOF-TOF or with hybrid quadruple time of flight analyzers which are separated by the collisions cells. Now for proteomic application, it is recommended to use the TOF-TOF or Q-TOF. The peptide ions are accelerated through the first time of flight tube as you can see in the slide and then they are dissociated by introducing an inert gas into the collision cell.

This process allows the collision-induced dissociation spectra from the MALDI produced from the precursor ions. Now these hybrid configurations are more sensitive than the triple quad and the single time of flight. So the combination of TOF-TOF allows the protein identification through the peptide mass fingerprinting and high throughput analysis of the protein or proteome is possible with the hybrid TOF analyzers.

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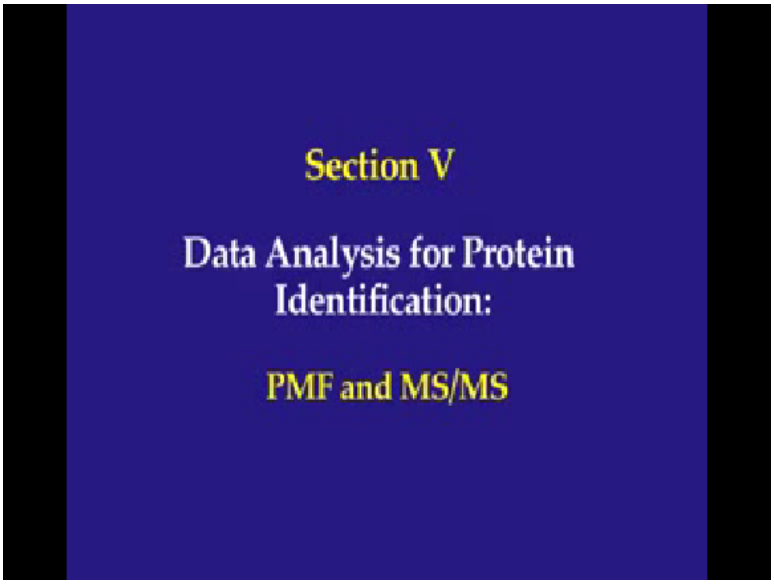

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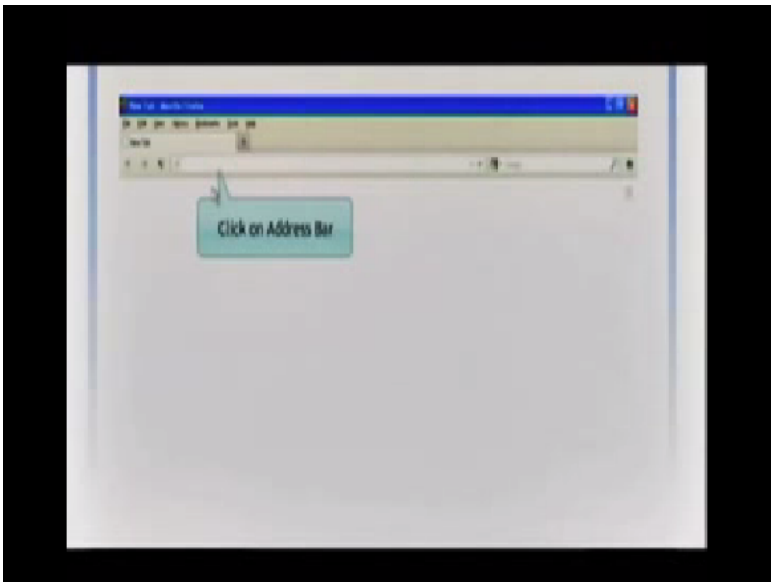

Open the matrix signs browser window to carry out online data analysis.

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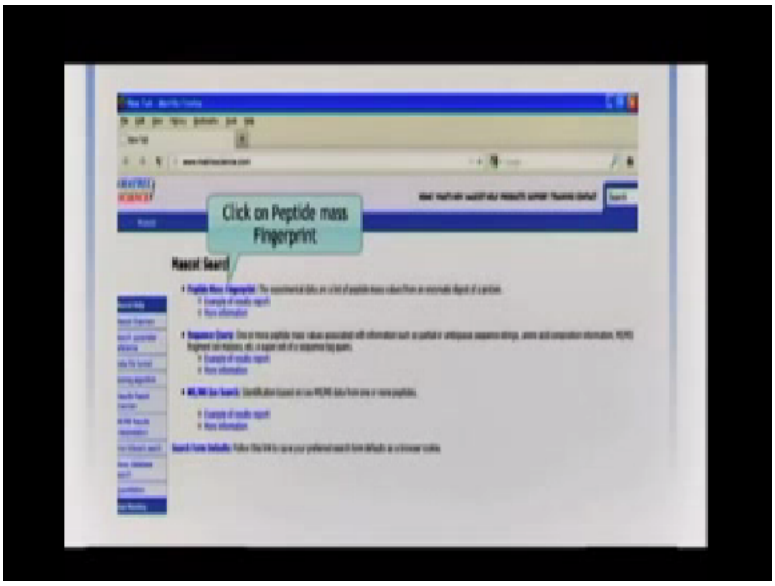

For peptide analysis, click on peptide mass fingerprinting.

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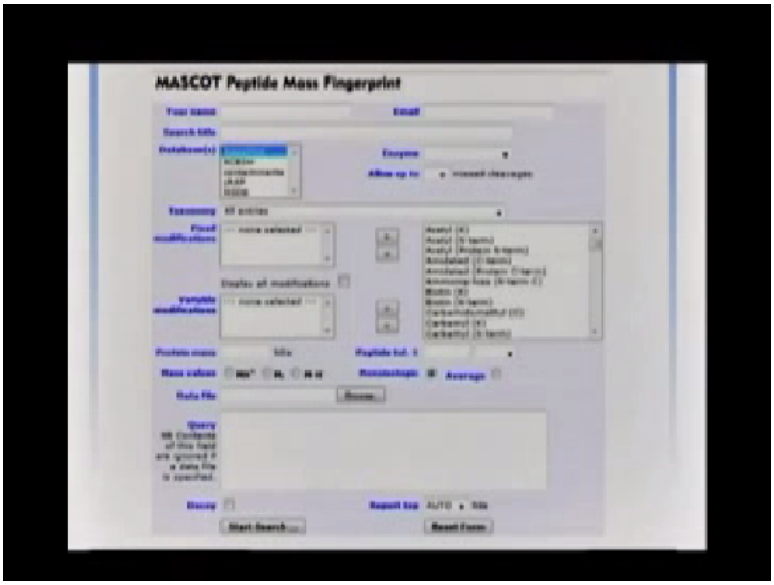
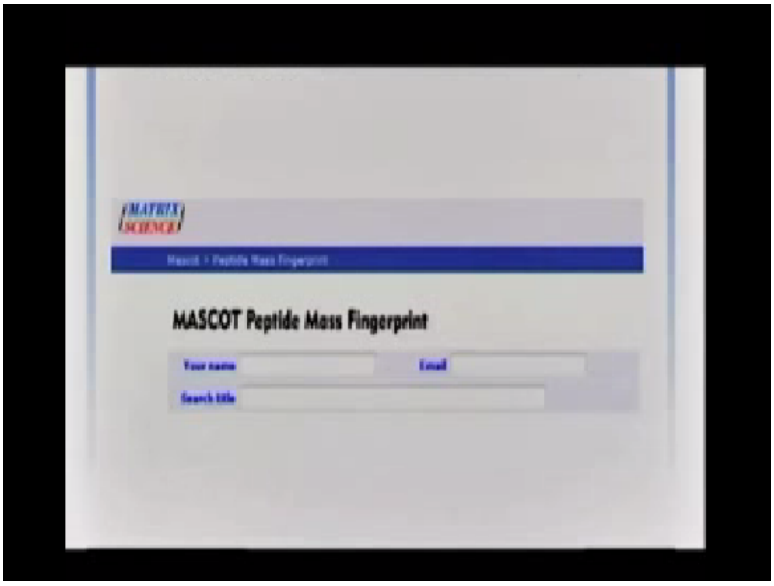


MASCOT peptide mass fingerprint.

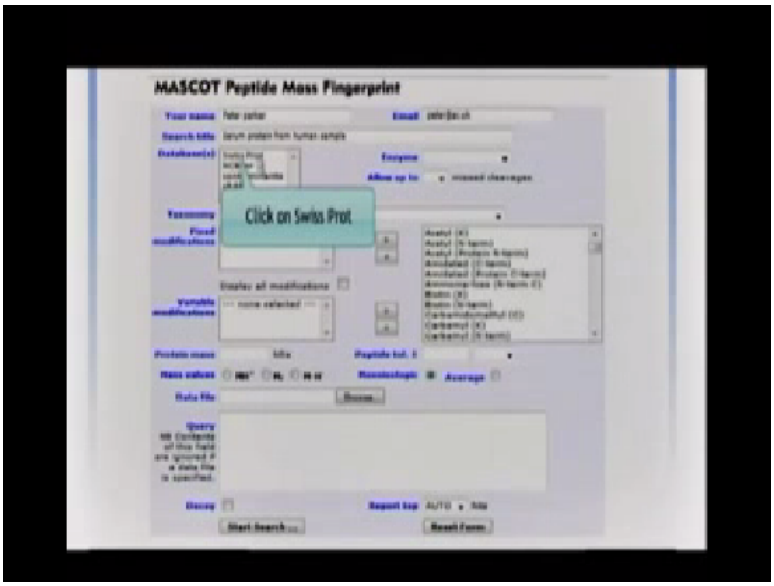
(Refer Slide Time: 21:47)



Please enter the user ID and details to acquire information in case of any network loss.

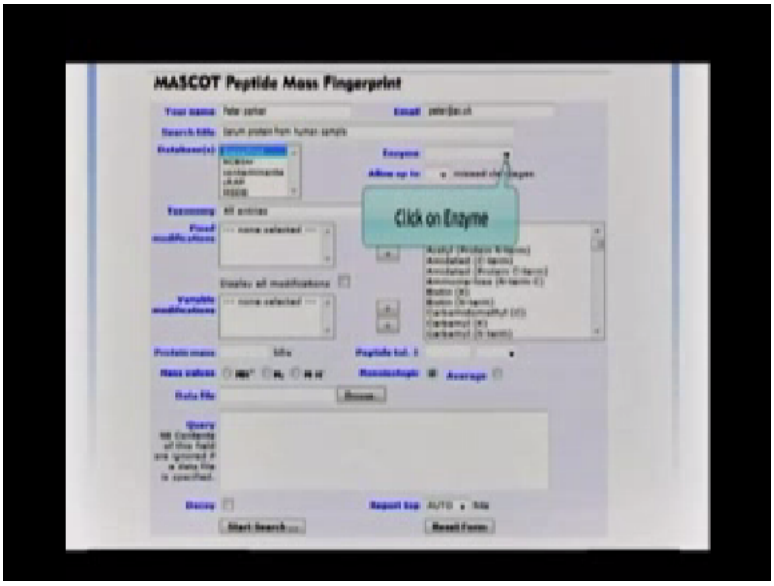
The following parameters should be selected.

(Refer Slide Time: 21:56)



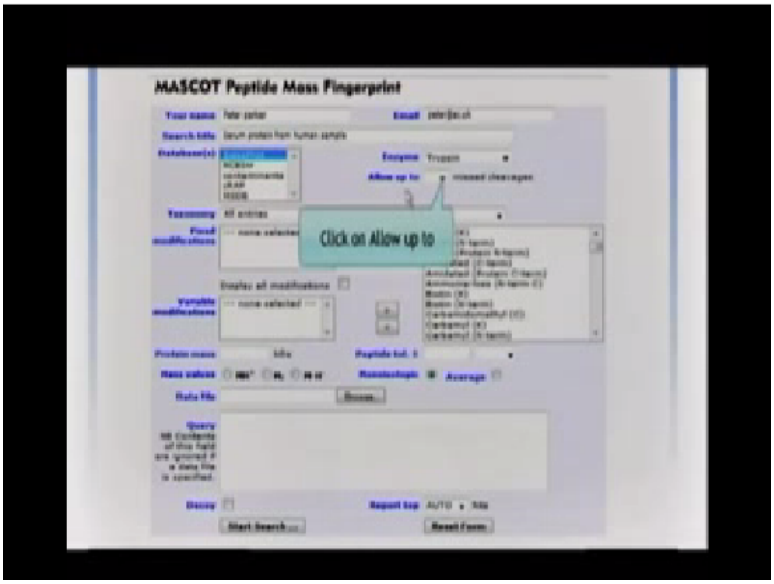
Database, primary databases include were SPROT and NCBI, Select Swiss Prot database for data analysis.

(Refer Slide Time: 22:12)



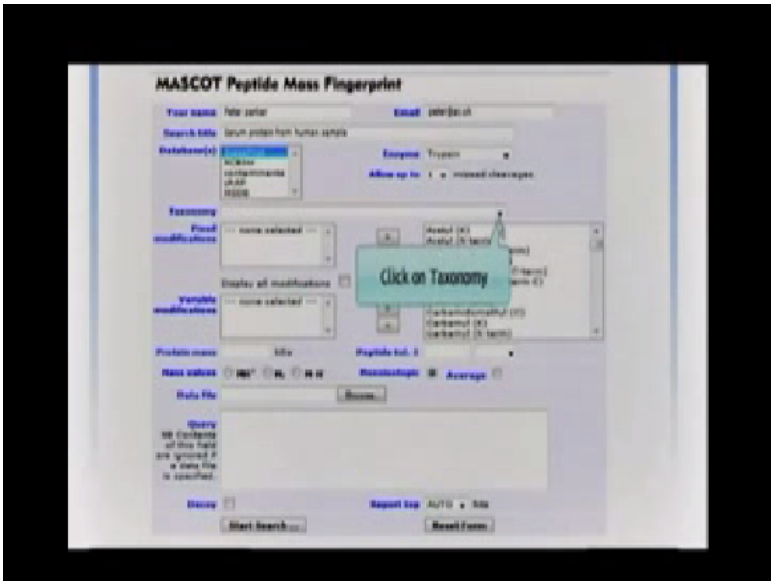
Enzyme, click Enzyme as Trypsin.

(Refer Slide Time: 22:18)



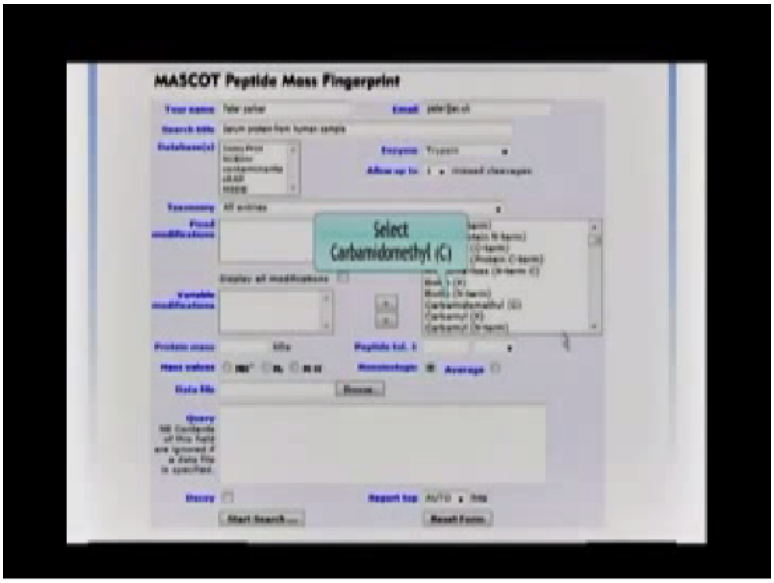
Missed cleavages. Missed cleavages are allowed up to 1.

(Refer Slide Time: 22:25)



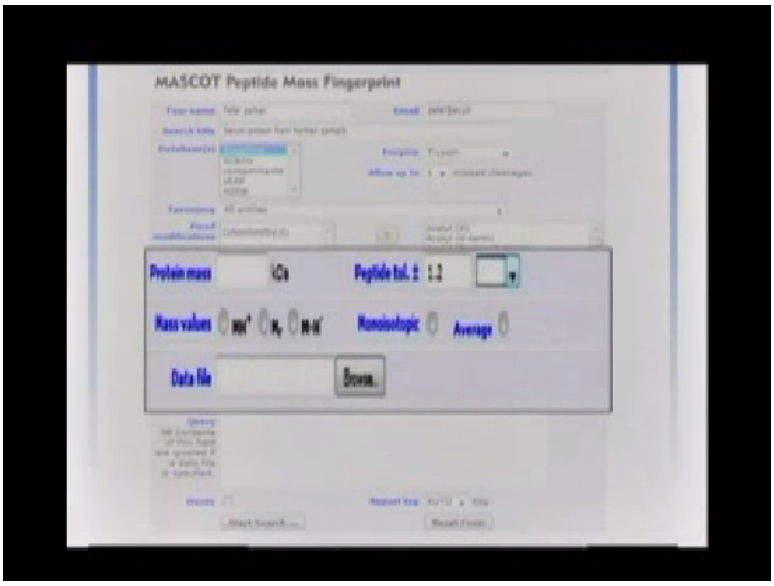
Next is taxonomy. The protein extracted from the biological specimen has to be assigned to a particular species or a group of species to which the sample belongs. When you are not sure of organism, select all entries.

(Refer Slide Time: 22:41)



Switch modification, select carbamidomethyl, which modifications are applied collectively across the database to account for change in mass of specific residue. Now scroll down and select oxidation. These are mass changes suspected to occur during sample handling and accounted for by increasing the number of primary sequences compared against experimental masses, include it as variable modification.

(Refer Slide Time: 23:17)

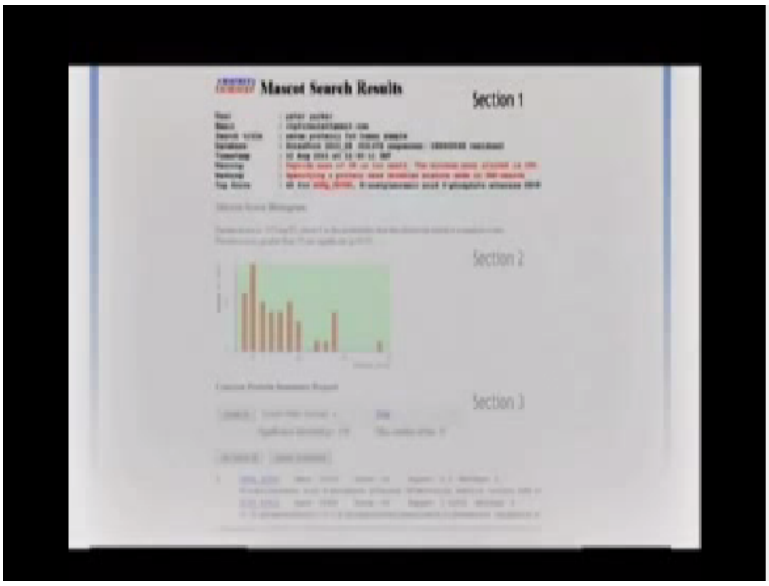


Depending upon user needs, the parameters can be changed. Protein mass is the mass of intact protein and is optional. Set peptide tolerance as +-1.2 daltons. Mass values, it specifies the type of charge to be examined. Select MH+. Select monoisotopic. Report, top 10 hits. Select decoy for statistical analysis. Copy the m/z value and paste in the selected box for MASCOT search. Click on search.

(Refer Slide Time: 24:01)

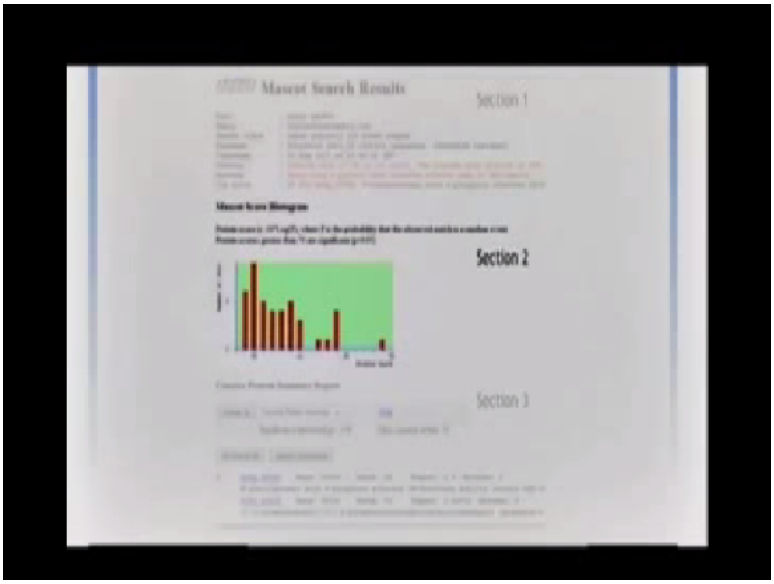
The output can be seen in 3 sections.

(Refer Slide Time: 24:03)



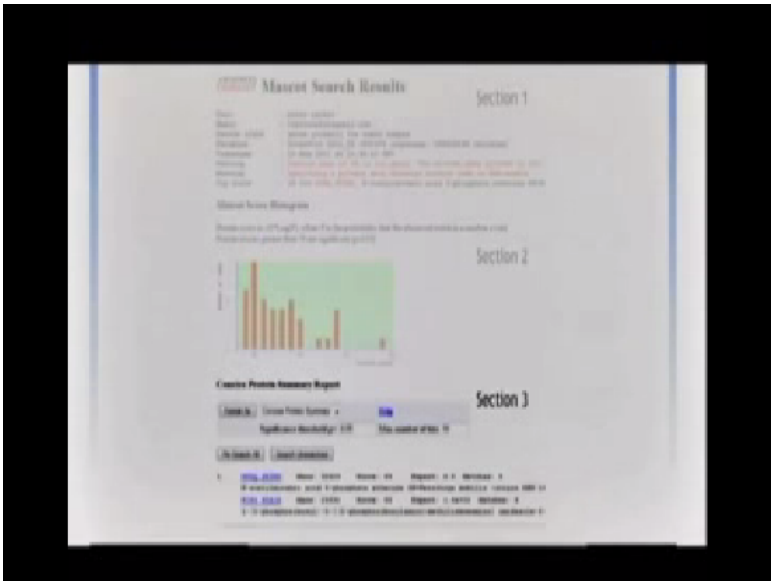
In section 1, the summary of set parameters defined by user.

(Refer Slide Time: 24:14)



Section 2, MASCOT score histogram. The number of protein heads with score is plotted along the graph.

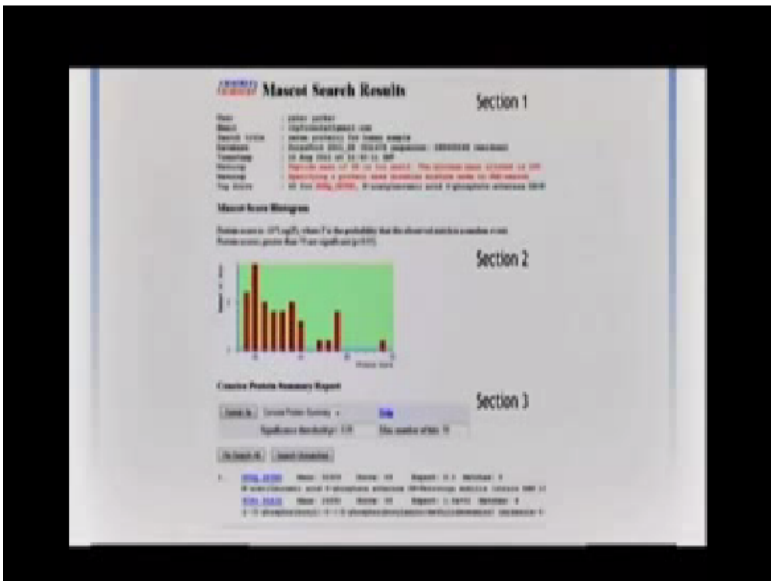
(Refer Slide Time: 24:23)



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

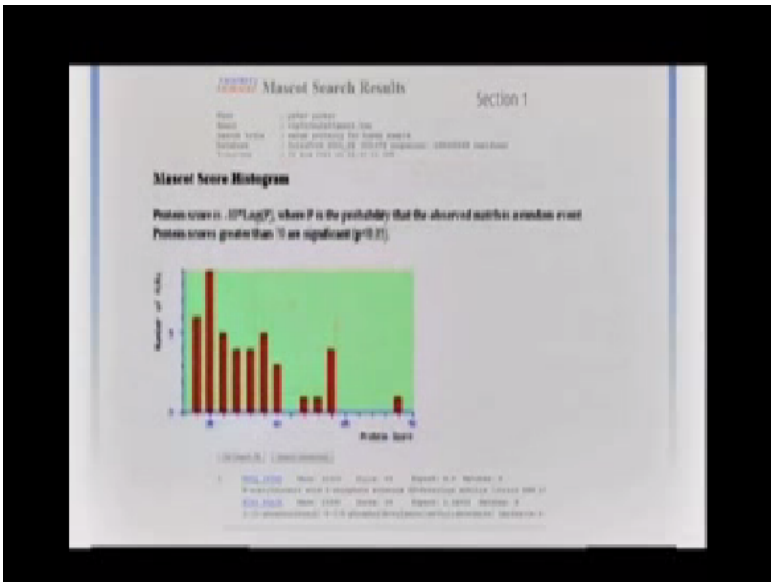
(Refer Slide Time: 24:38)

(Refer Slide Time: 24:39)



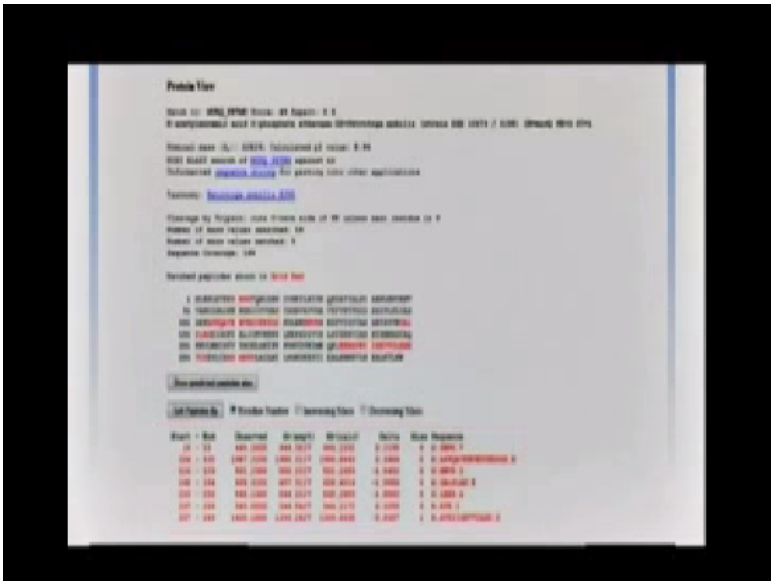
Data analysis. If the search parameters are now the best fit, the software generates an error message. Depending on the error message, user needs to change the parameter settings and do the search again.

(Refer Slide Time: 24:52)



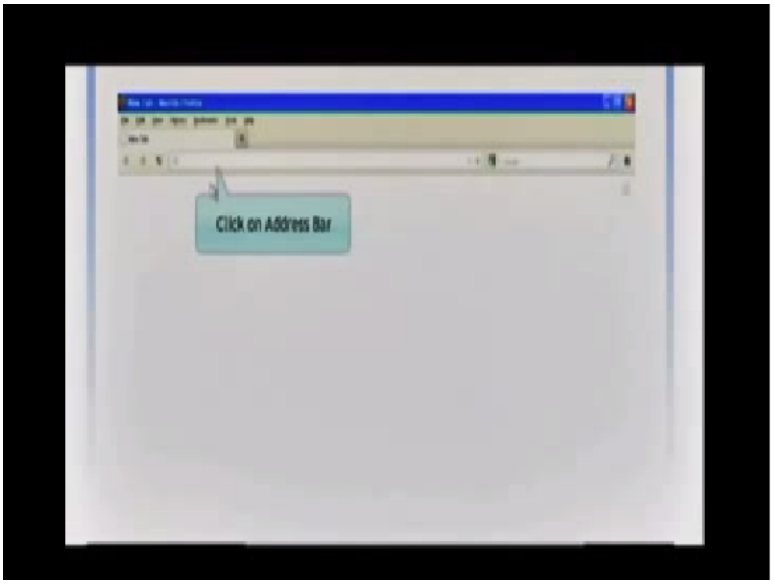
In section 2, MASCOT score histogram, the number of protein heads and their score is displayed along the graph.

(Refer Slide Time: 25:01)

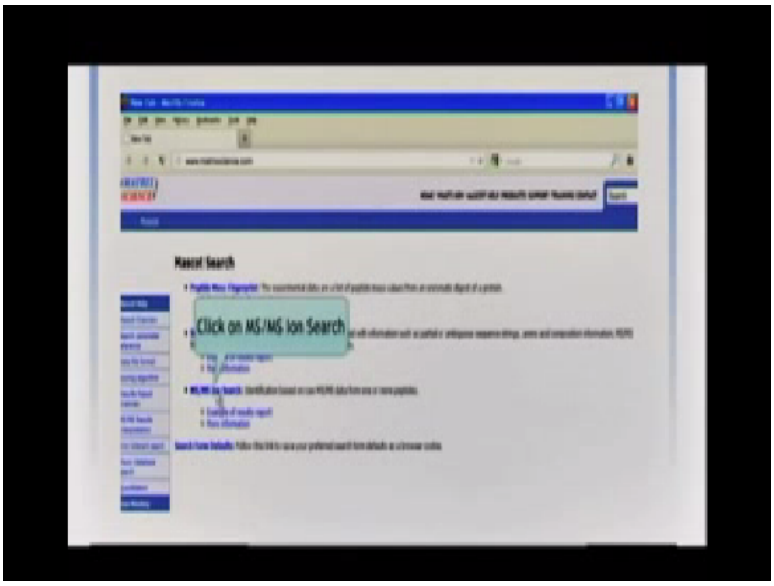


Routine view section displays matching of the query peptide to the protein sequence in the database. The sequence types the matched region, what is the expected and calculated value of the query peptide and sequence details.

(Refer Slide Time: 25:17)

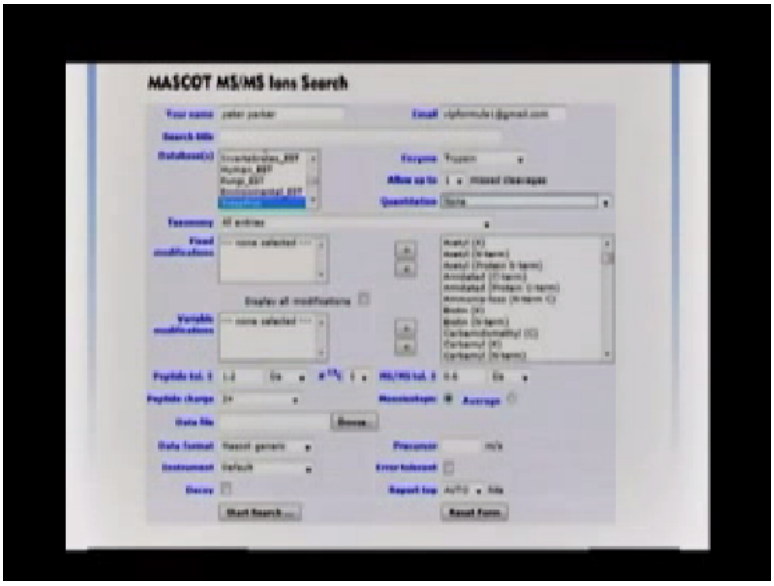


(Refer Slide Time: 25:24)



For better protein identification and to increase protein score, the idea of each peak generated is carried out to generate MS-MS data. For such data analysis, MS ion search option is selected from the matrix signs browser window.

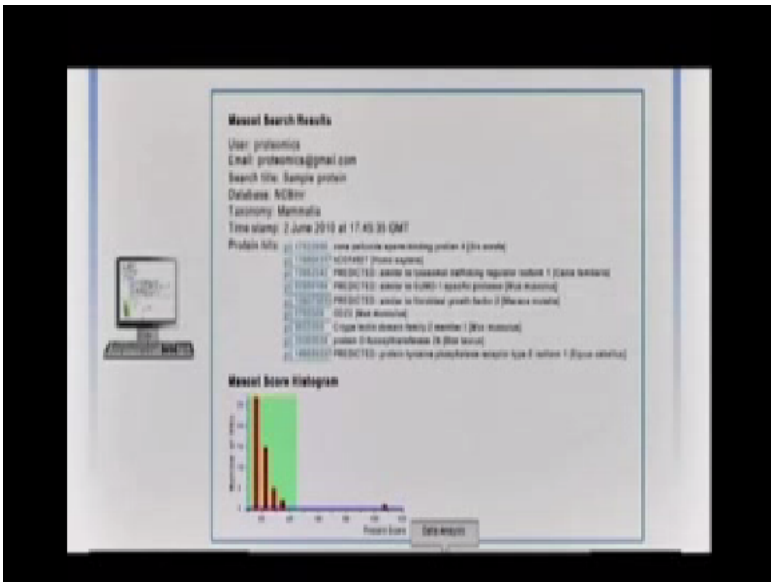
(Refer Slide Time: 25:30)



In MS/MS search tool, more input parameters like quantitation, MS/MS tolerance, peptide charge, instrument, etc. in addition to fields for PMF and best other parameters are similar to that of the peptide mass fingerprint. Depending upon the process carried out for data generation, a selection in the quantitation must be made. In case of label field quantitation, select none in quantitation tab. Remaining parameters remain same as PMF.

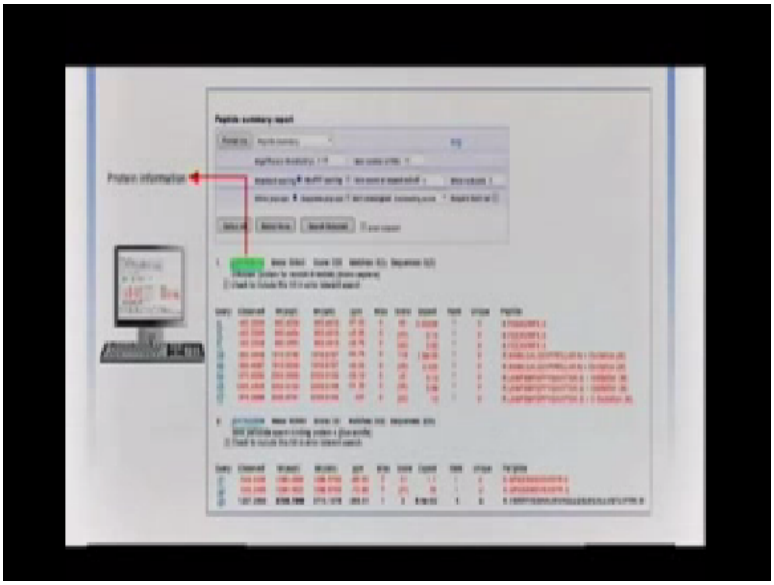
Browse the MS/MS raw data file and search the result using MASCOT. Define the instrument that has been used to generate the raw data. When we do not know the name of the instrument, select default.

(Refer Slide Time: 26:23)



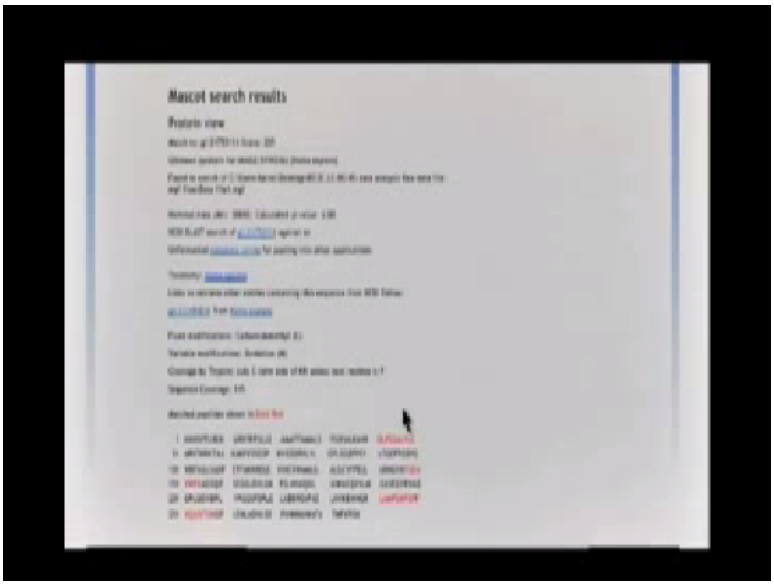
The result output generated is almost similar to PMF output.

(Refer Slide Time: 26:29)



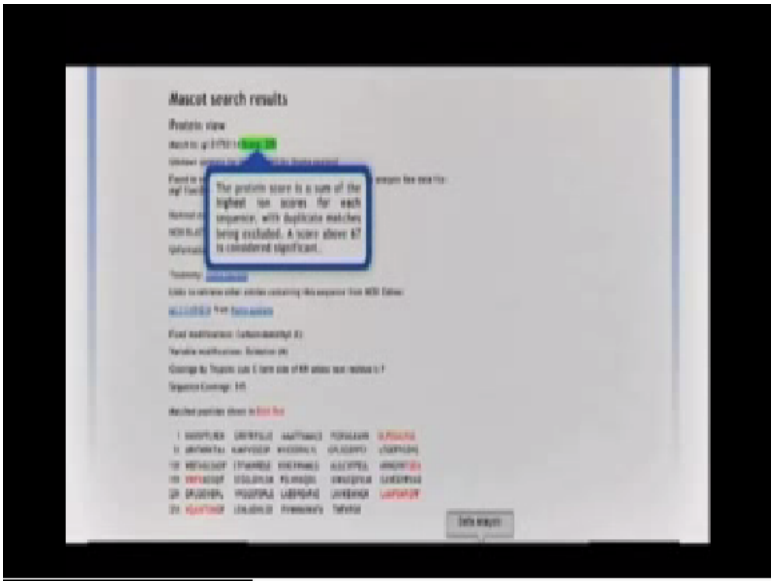
The accession ID indicates the protein information obtained from the database marked as green. The protein head outside the green box indicates P value less than 0.05 which is statistically significant, whereas hits inside the green box indicates random matches.

(Refer Slide Time: 26:49)



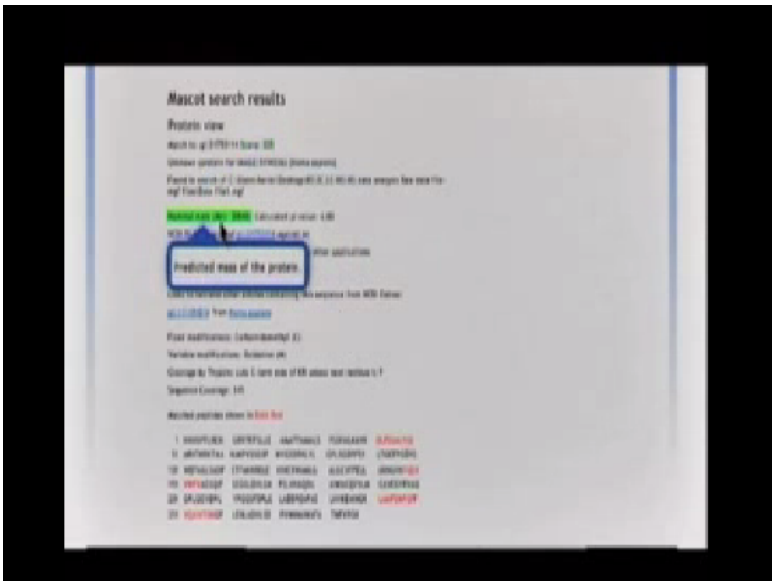
MASCOT search results.

(Refer Slide Time: 26:54)



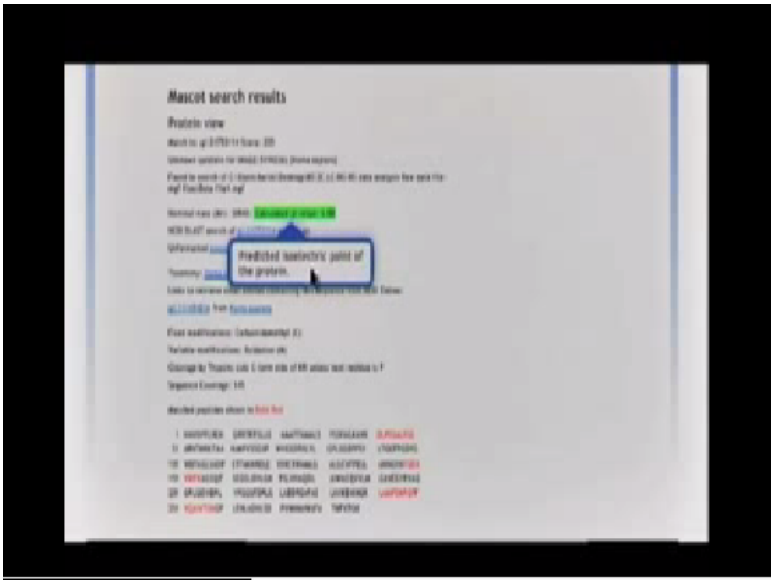
Score, the peptide score is a sum of highest ions score for each sequence with duplicate matches being excluded.

(Refer Slide Time: 27:00)



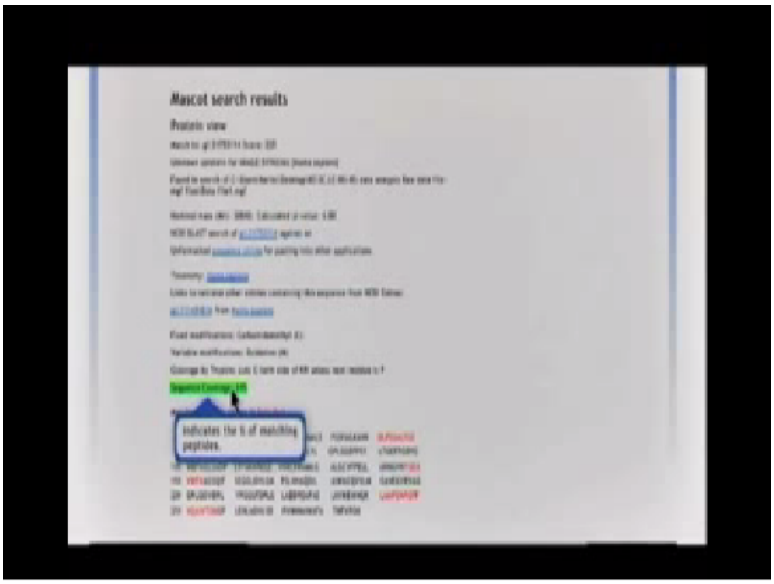
Nominal mass, a predicted mass of the protein.

(Refer Slide Time: 27:08)



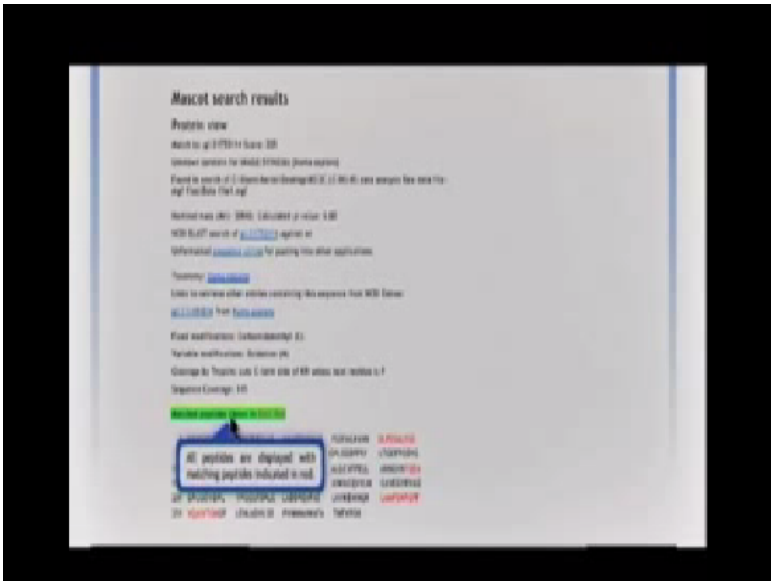
Calculated TI value predicted as to electric point of the protein.

(Refer Slide Time: 27:16)



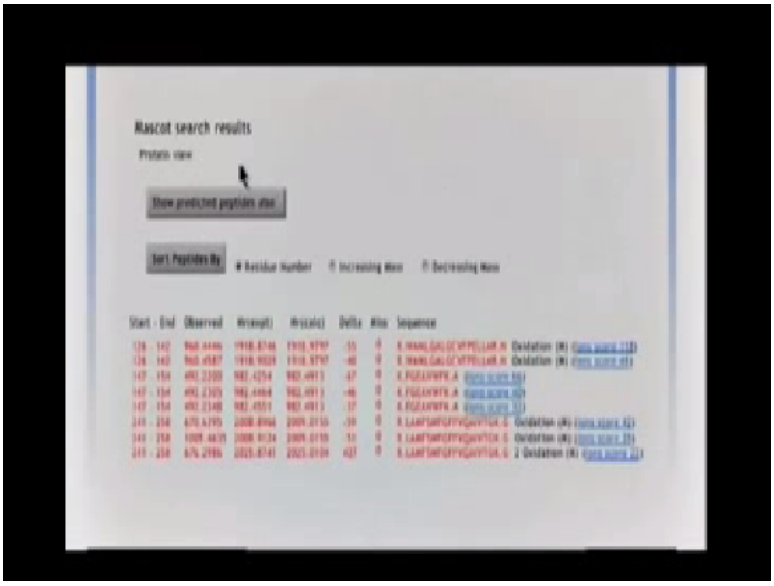
Sequence coverage indicates the percent of matching peptide.

(Refer Slide Time: 27:23)



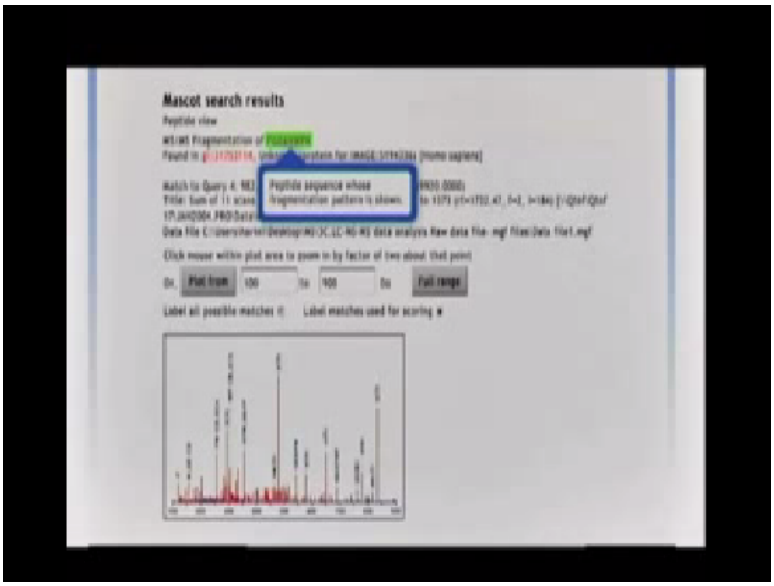
All the peptides are displayed with matching peptide indicated in red.

(Refer Slide Time: 27:30)



The protein view obtained on selecting the particular protein length is very similar to protein view observed in PMF. It provides detailed information about each of the matched peptide displayed. The start and end position of amino acid calculated an experimental molecular weight, number of missed (()) (27:52) cleavages, sequence of each peptide segment and their corresponding ion scores are shown. The highest ion scores are used for computing the final protein score.

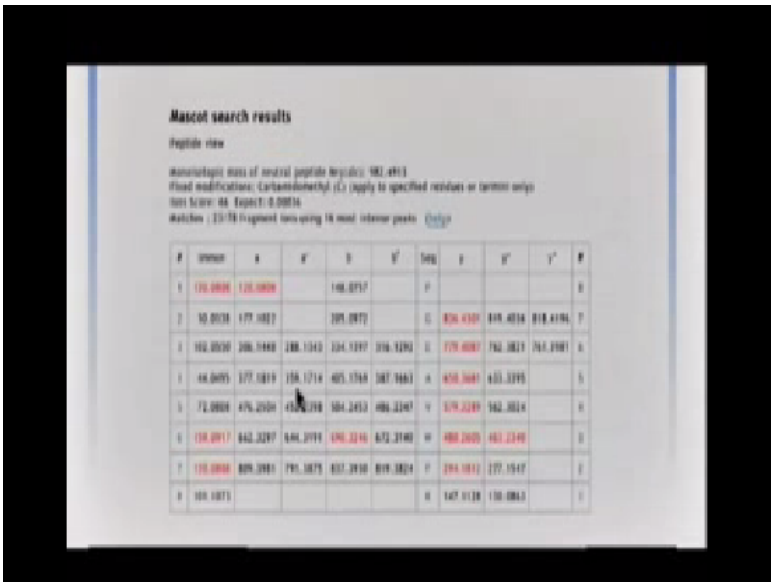
(Refer Slide Time: 28:06)



Each peptide (()) (28:06) MS/MS undergoes a second round of fragmentation when it passes through the second mass analyzer before it reaches the detector. This provides significantly larger amount of information regarding each peptide fragment which can be viewed by clicking

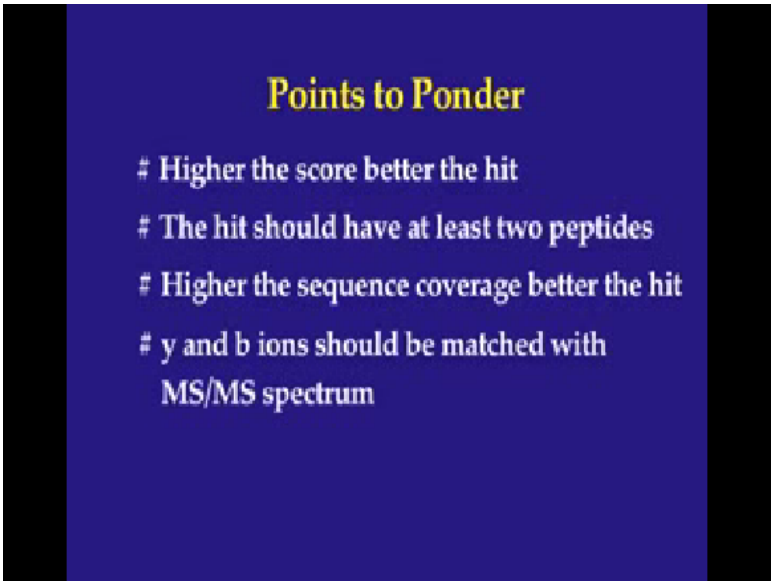
on the peptide length provided in the summary report.

(Refer Slide Time: 28:34)

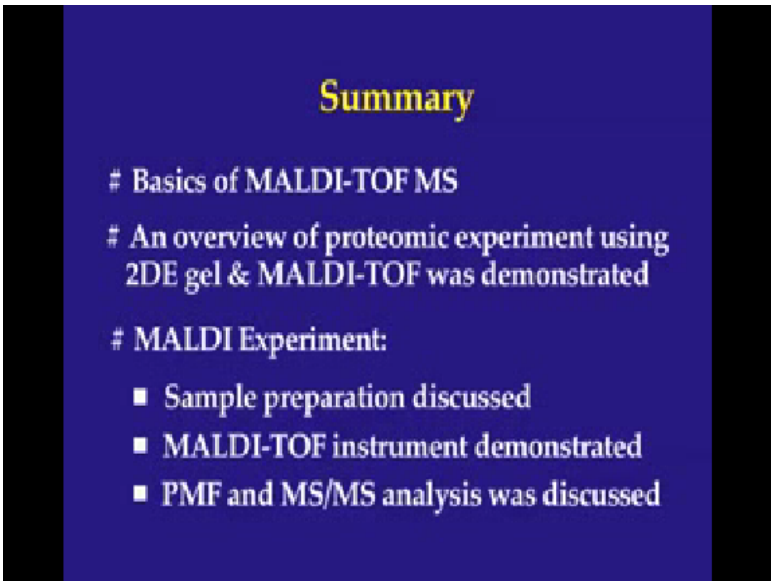


The fragmentation pattern is displayed graphically which can be zoomed into as per the requirement.

(Refer Slide Time: 28:36)



(Refer Slide Time: 28:50)



(Refer Slide Time: 29:05)

(Refer Slide Time: 29:10)

References

1. Garaguso I, Borlak J. Matrix layer sample preparation: an improved MALDI-MS peptide analysis method for proteomic studies. Proteomics. 2008;8(13):2583-95.

2. Schuiberger AM, Moss JA. Optimized sample preparation for MALDI mass spectrometry analysis of protected synthetic peptides. J Am Soc Mass Spectrom. 2008;19(4):614-9.

3. Dave KA, Headlam MJ, Wallis TP, Gorman JJ. Preparation and analysis of proteins and peptides using MALDI TOF/TOF mass spectrometry. Curr Protoc Protein Sci. 2011;Chapter 16:Unit 16.13.

4. Drevinek M, Dresler J, Klimentova J et al. Evaluation of sample preparation methods for MALDI-TOF MS identification of highly dangerous bacteria. Lett Appl Microbiol. 2012;55(1):40-6.

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6. Biswas SI, Rolain JM. Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. J Microbiol Methods. 2013;92(1):14-24.