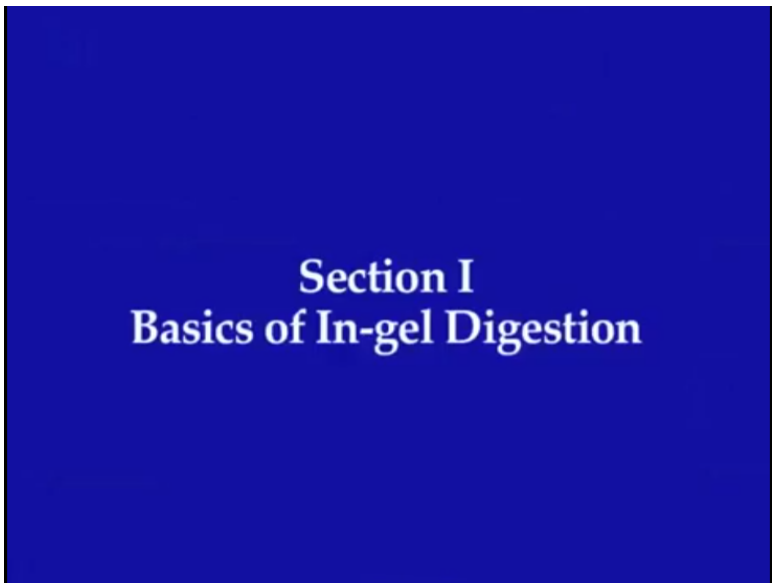
Introduction to Proteomics
Dr. Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology - Bombay

Lecture - 29
In-gel & In-solution Digestion

(Refer Slide Time: 00:18)

(Refer Slide Time: 00:21)

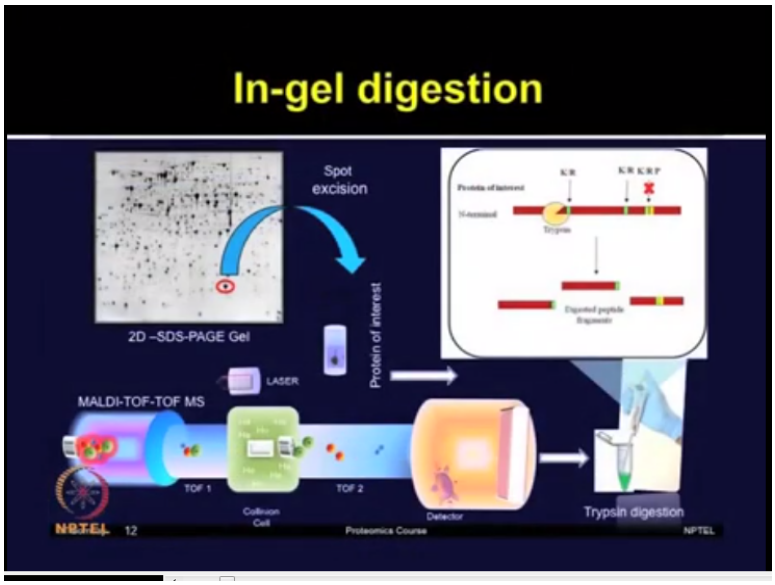


Now let us talk about how to prepare the sample and discuss the steps in more detail.

(Refer Slide Time: 00:30)

The first part will be in-gel digestion of the protein samples. So the mass spectrometric identification of the target protein greatly depends on the efficacy of in-gel digestion process that generates the mixture of peptides from the target protein through proteolytic digestion.

(Refer Slide Time: 00:54)



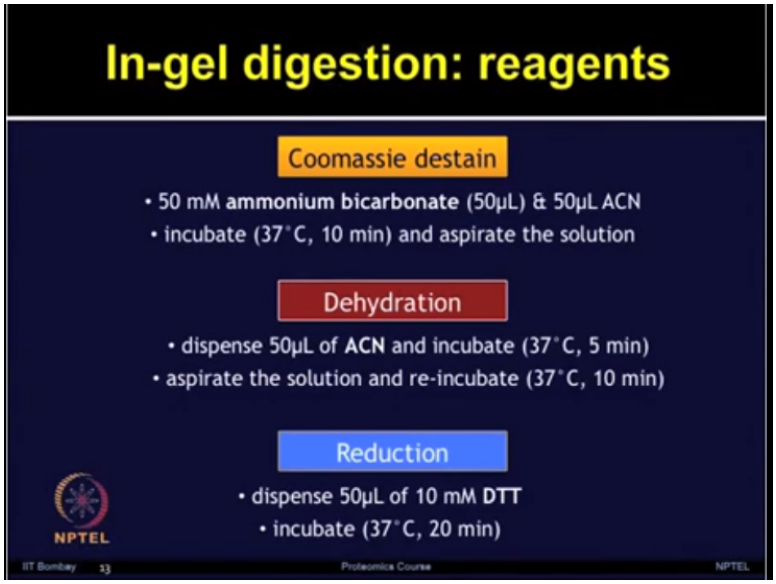
This slide gives you an overview so in the last module we discussed about 2-dimensional electrophoresis. It shows that if you have a spot of interest you can excise that spot from the 2-D gel and then subject that to in-gel digestion followed by the mass spectrometric analysis. In-gel digestion is the multi-step procedure, which includes spot selection, spot excision, removal of the stain, reduction, alkylation, proteolytic cleavage as well as peptide extraction.

So the multiple steps are involved and how good your in-gel digestion is, is going to ensure the success of the spectrum generated from the mass spec. Now although this overview

shows you the process to start with the 2-D gel, but same can be also applied for even the gel free proteomic techniques. If you want to analyze the sample, even from the gel free proteomic based approaches, it is often good idea to separate those protein complex mixture on the SDS-PAGE gel, excise the bands.

And then extract the proteins from that perform the in-gel digestion so that you can simplify the proteome and then you can increase the overall proteome coverage. So similar protocol can be modified and used for various type of applications in the proteomics.

(Refer Slide Time: 02:47)



So this slide gives you various recipe for performing in-gel digestion. The first step is the destaining of the spots or the band because you have stained the gels with the Coomassie brilliant blue or some other stains and first of all, you would like to remove the stains. So stain removal is essential prior to the mass spectrometric analysis.

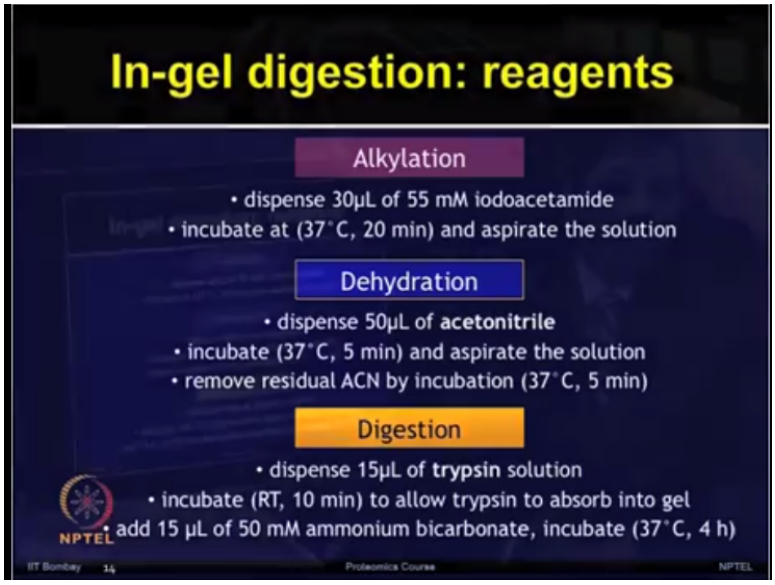
The excised gel pieces should be washed with the bicarbonate buffer and acetonitrile for removal of the staining agent. You can see the recipe in the slide. Will talk more about how to do these experiments when we come to the animation of these steps. Acetonitrile reduces the hydrophobic interaction between protein and the stain while the ionic solution decreases the ionic interaction between negatively charged Coomassie brilliant blue dye and the positively charged protein.

Once the Coomassie dye is staining or the destaining step is performed then we need to dehydrate the gel pieces, which can be done by using addition of acetonitrile. After this

incubation is done then you are ready for performing reduction step. Now why reduction step is required? So after the stain removal, the next steps are including reduction as well as alkylation of protein residues.

So that you can denature the protein into its primary structure. Continuing on to the same theme of in-gel digestion and various steps required to perform such experiment let us now look at the next step, which is alkylation.

(Refer Slide Time: 04:32)



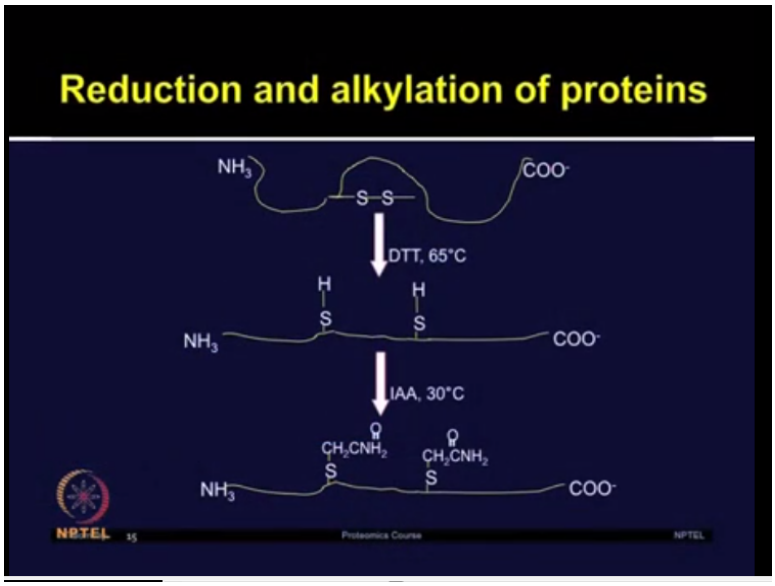
So in the alkylation you need to add the iodoacetamide. The reformation of disulfide bonds may occur so to prevent that iodoacetamide which is an alkylating agent is used here. Again, you need to do the dehydration step as recipes (()) (04:53) in the slide. You can add the acetonitrile and then you are ready to perform the digestion which is usually done by using trypsin. So prior to the MS identification proteins are digested to generate peptides.

There are various enzymes, which one can use for performing this step, but trypsin is most widely used proteolytic enzyme used for the protein digestion. It breaks the peptide bonds at the carboxy terminus at basic amino acids such as arginine and lysine. Once digestion is done which is usually the overnight step then one need to do the peptides extraction of the digested proteins.

So peptides generated through the proteolytic cleavage can be extracted by using recipe including formic acid or trifluoroacetic acid TFA in the 50% acetonitrile solution. Now

coming back to the importance of reduction and alkylation of the proteins. We mentioned that we need to add DTT and IAA in various steps during the in-gel digestion process.

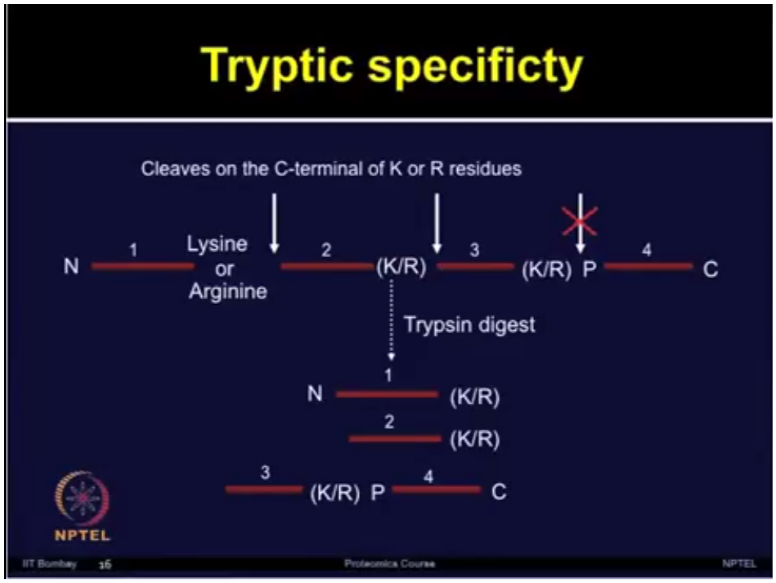
(Refer Slide Time: 06:20)



So DTT is important for treatment of proteins to break the disulfide bonds, which we have also talked in the sample preparation when we discussed earlier. Now iodoacetamide it adds the iodoacetamide group to the sulfhydryl group and prevents dye sulfide bond formation. So these steps are quite important in in-gel digestion process. So coming to the specificity of trypsin.

First of all, I discussed why they need to do the proteolytic digestion. So you want to generate the peptides with the molecular weight within the math range of mass spectrometer. So you always want to simplify the process for even very superior analytical instruments so that you can increase the efficiency of the process.

(Refer Slide Time: 07:13)



The enzymatic digestion are performed with various enzymes, but typically with the trypsin, which cleaves at the C-terminal of lysine and arginine residues, but exceptions can occur with the proline. If proline is present, then that breakage will not happen. So one can use the modified trypsin, which is a serine endopeptidase; however, it cleaves at the proline lysine and proline arginine bonds at the much lower rate.

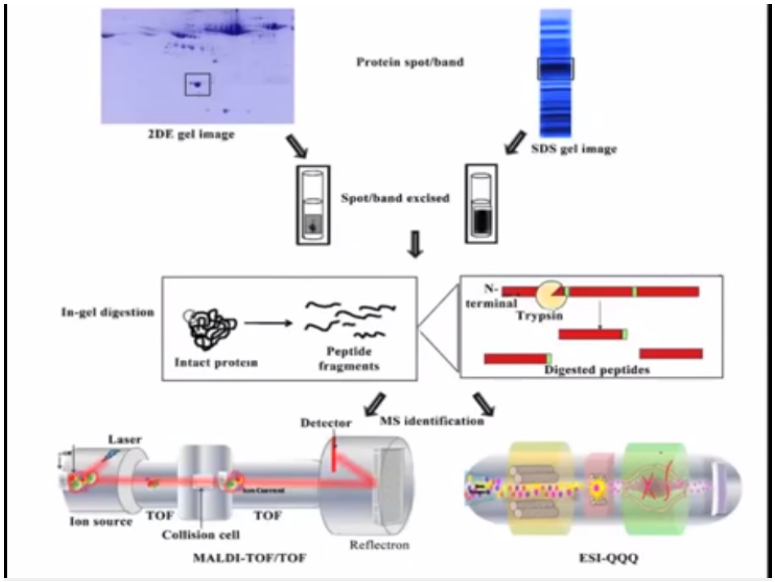
You can see the cleavage process and the specificity in the slide where it shows if you have the lysine or arginine residue, it can break the bonds, but when there is a proline residue present there then it cannot cleave. So the in-gel digestion of proteins isolated by the gel electrophoresis remains a core area in the mass spectrometry or in any of the proteomics applications.

(Refer Slide Time: 08:20)

So 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 make the specific requirements of particular proteomic experiments. The in-gel digestion procedure is compatible with the downstream mass spectrometry analysis whether you want to continue with the MALDI-TOF or you want to do the LC-MS based mass spectrometry analysis.

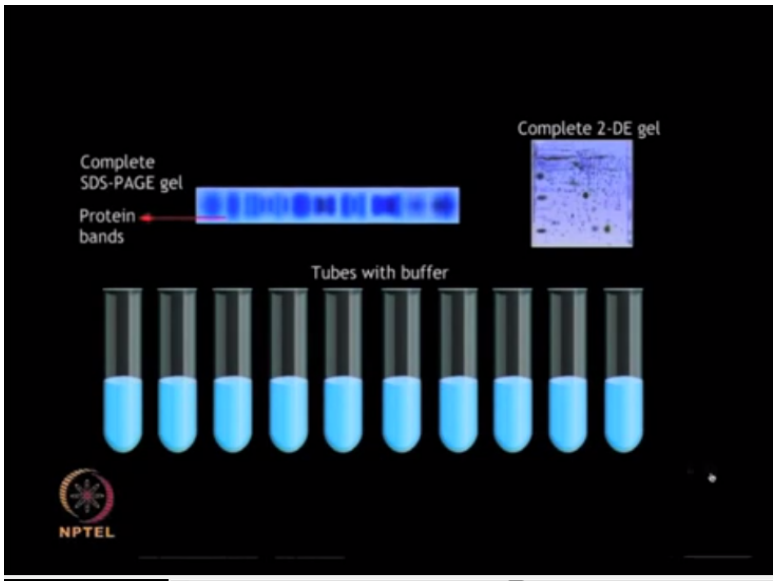
(Refer Slide Time: 08:54)

(Refer Slide Time: 08:57)



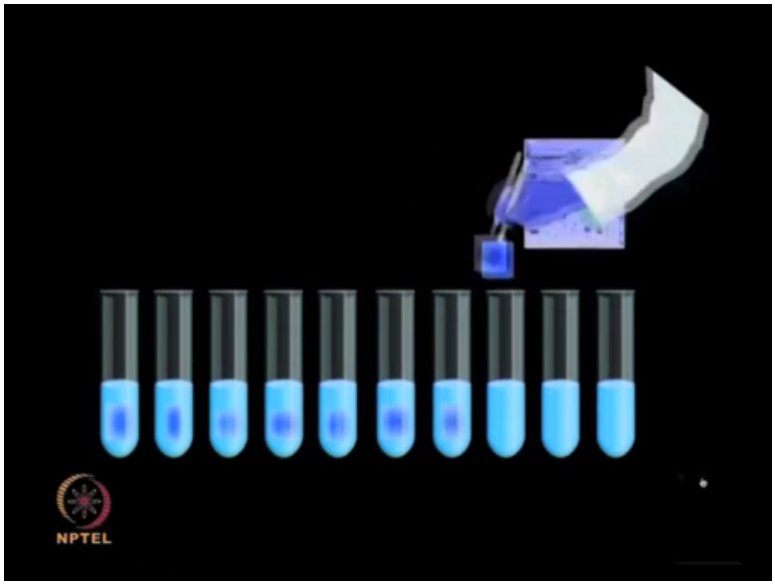
In-gel digestion, the protein separated by 2-D are analyzed and significant spots are excised, processed and taken for mass spectrometric analysis. Prior to the mass spectrometric analysis, it is important to cleave the protein in the gel by trypsin to make them smaller peptides so that it can come out of the gel and is easy to be analyzed using mass spectrometry.

(Refer Slide Time: 09:23)



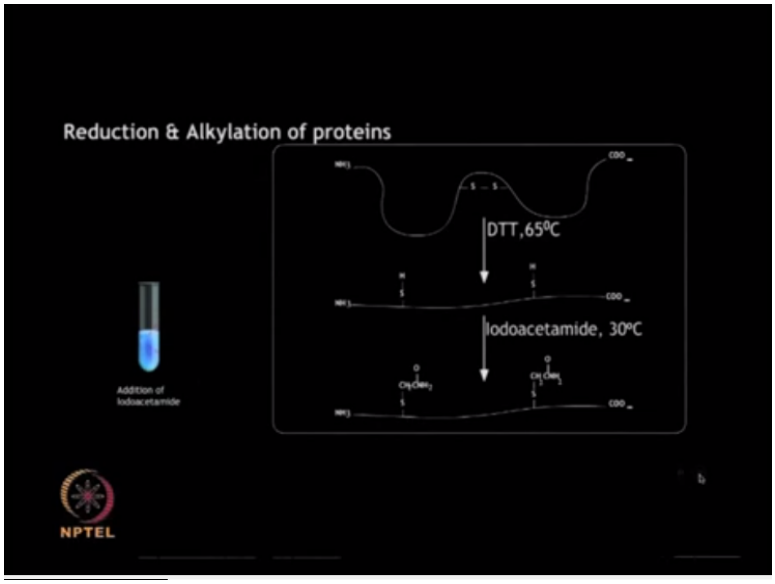
Electrophoretic separation of a protein mixture results in distinct protein bands. These proteins can be used for analytical purposes by carrying out in-gel digestion.

(Refer Slide Time: 09:45)



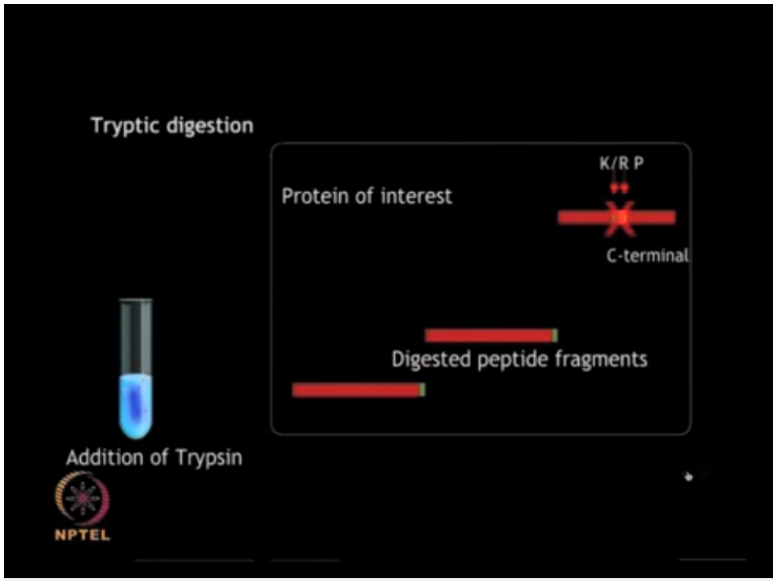
Entire gel is fragmented into small pieces and each piece is dissolved in a suitable buffer. To simplify the complex proteome, it is good idea to chop down the overall separated proteins into the smaller pieces.

(Refer Slide Time: 10:02)



The protein solution is treated with a reducing agent like dithiothreitol DTT, which cleaves the disulfide bond in the protein. This is followed by treatment with iodoacetamide or IAA, which alkylates the sulfhydryl group and thereby prevents the reformation of disulfide bonds.

(Refer Slide Time: 10:34)



After cleavage of the disulfide bonds, the protein is treated with a proteolytic enzyme. The most commonly used enzymes include trypsin. Trypsin cleaves the protein at specific residues arginine and lysine and generates smaller peptide fragments. This tryptic digest is used for further purification and analysis.

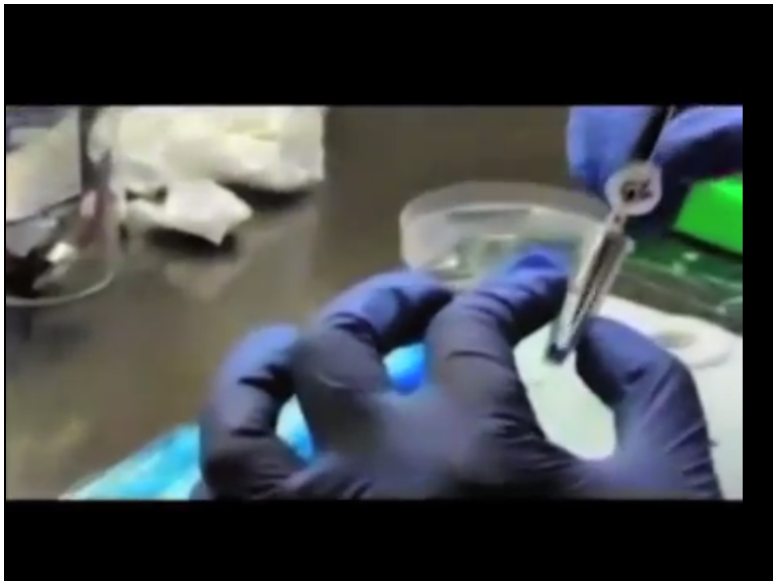
(Refer Slide Time: 11:11)

Prior to the in-gel digestion, the 2-D gels were washed with the distilled water for 4 hours to remove the SDS from the gel.

(Refer Slide Time: 11:44)

Clean the glass plate and place the gel on the plate. Excise the selected spot using sterile scalpel.

(Refer Slide Time: 11:56)



Place the excised spot into fresh Eppendorf tube. Clean the glass plate again. Place the excised spot on the clean glass plate.

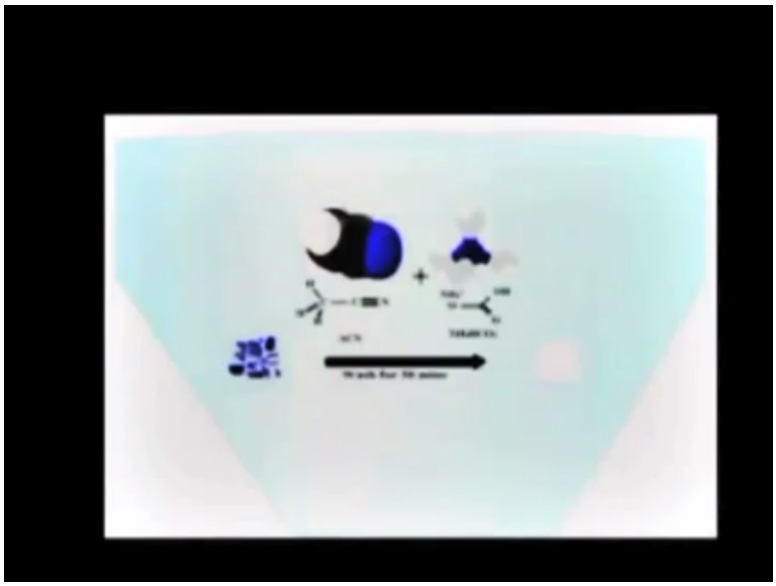
(Refer Slide Time: 12:23)

Slice the big gel pieces into small pieces and use them for in-gel digestion. Now, place the excised gel in the clean sterile Eppendorf tube.

(Refer Slide Time: 12:53)

Add the stain removal solution to the spot. Keep it on the shaker for 30 minutes at room temperature. After 30 minutes, discard the solution.

(Refer Slide Time: 13:52)

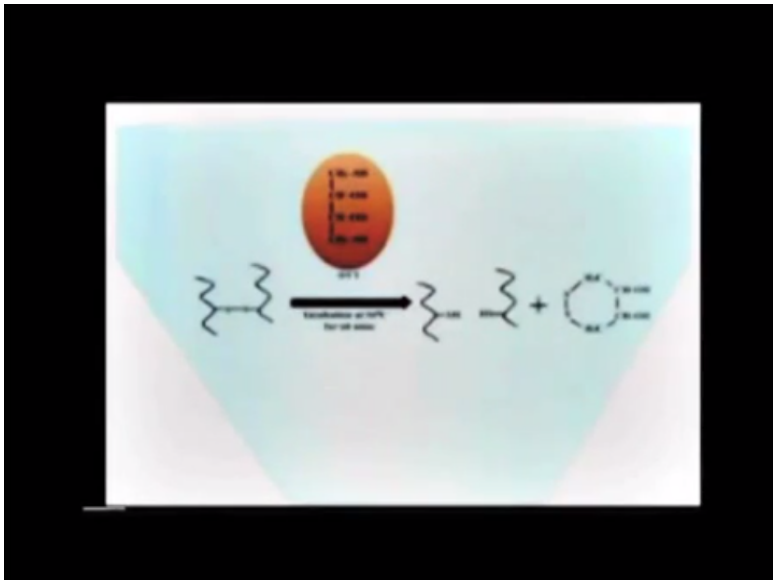


During the treatment, these staining solution acts on the gel pieces causing it to swell and be stained by reducing the interaction between protein and dye. Add dehydration solution to the gel pieces. Rotate for 30 minutes at room temperature. After 30 minutes, discard the solution. After 30 minutes, remove the solution. Repeat the step twice to ensure complete removal of stain from the gel.

(Refer Slide Time: 15:35)

Add reduction solution to the gel pieces and place it at 56 degree centigrade for 60 minutes.

(Refer Slide Time: 16:01)



Here we will see how reduction solution with DTT breaks the disulfide bonds. Discard the solution. Wash the gel pieces with rehydration solution and after that add dehydration solution and discard the solution after vortexing.

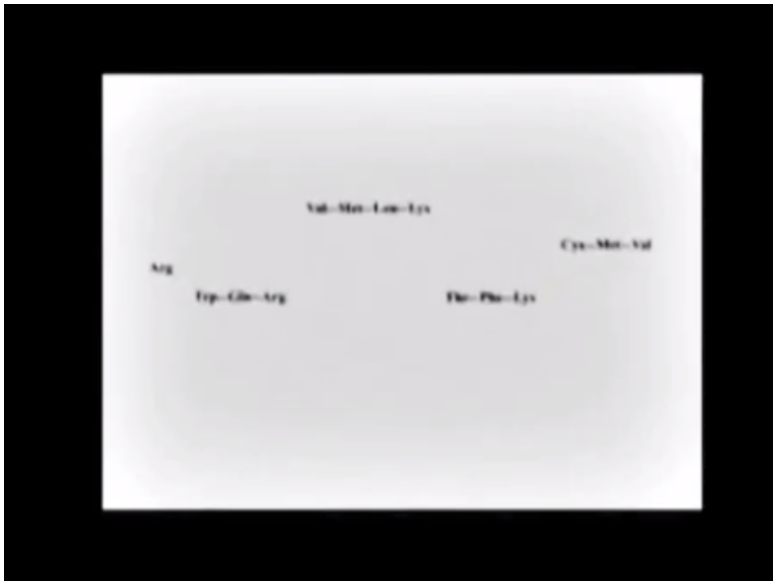
(Refer Slide Time: 17:27)

To the same gel pieces add alkylation solution and keep it in dark for 20 minutes at room temperature.

(Refer Slide Time: 17:48)

Alkylation solution having IAA react with free sulfhydryl groups and prevents the reformation of disulfide bonds. Remove the alkylation solution. Add dehydration solution, vortex it for 10 minutes to wash the gel pieces. Discard the solution and dry the gel pieces completely. Trypsin solution was prepared and added to the dry gel pieces. Keep it overnight at 37 degree centigrade in dry bath.

(Refer Slide Time: 19:19)

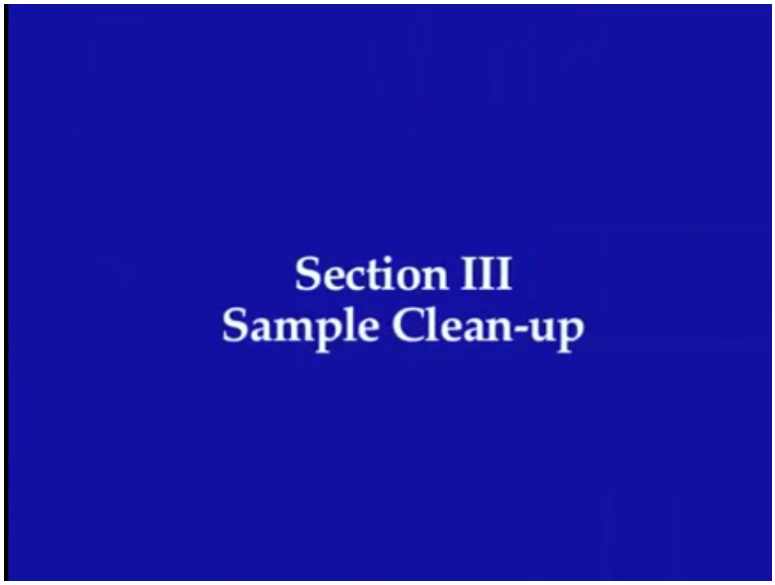


Trypsin is a proteolytic enzyme which cleaves the protein or the carboxyl terminal of lysine or arginine and leads to the formation of small peptides of 8 to 35 Daltons amino acid length. Trypsin activity was stopped by keeping the reaction mixture in ice. Extraction solution having acetonitrile in 0.1% trifluoroacetic acid was added to the gel pieces. Vortex vigorously for 10 minutes.

(Refer Slide Time: 20:12)

The supernatant was collected and extraction process was repeated twice. Preserve the samples in -20 degree centigrade till further use.

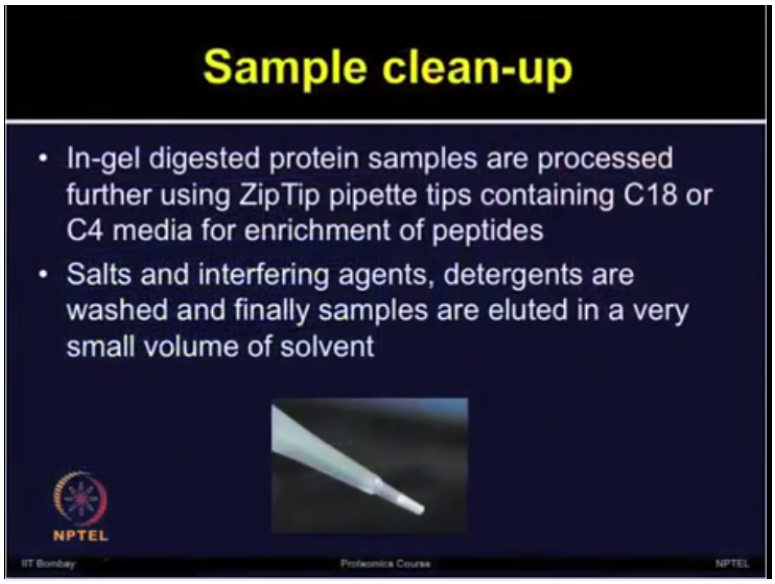
(Refer Slide Time: 21:10)



So 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 we have done the in-gel digestion, you can directly use these tryptic digest for further mass spectrometry analysis, but it is often recommended that in between you add one more step, which is sample clean-up.

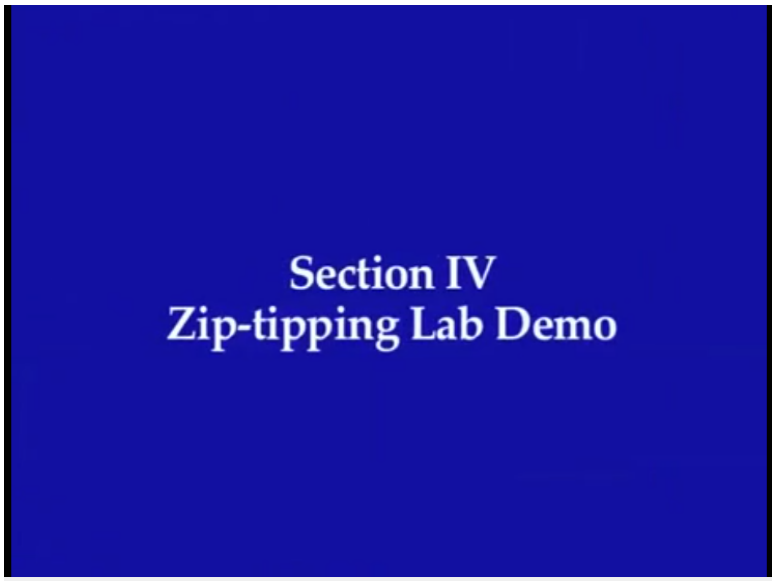
You do not want your columns or your MALDI instrument to get clogged due to the salt or some other interfering residues present in the mixture.

(Refer Slide Time: 21:52)



So it is recommended that one should use a clean-up step in between. So the in-gel digested protein they can be cleaned up by processing further using 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.

(Refer Slide Time: 22:24)

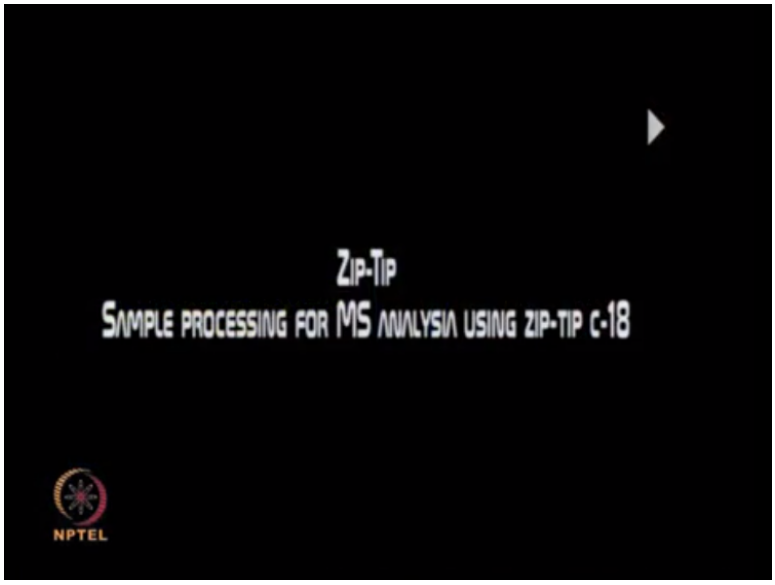


So the ZipTip is very small tip like device for removal of salts as well as other interfering components from the protein sample and is performed before injecting the sample for the mass spectrometry analysis.

(Refer Slide Time: 22:44)

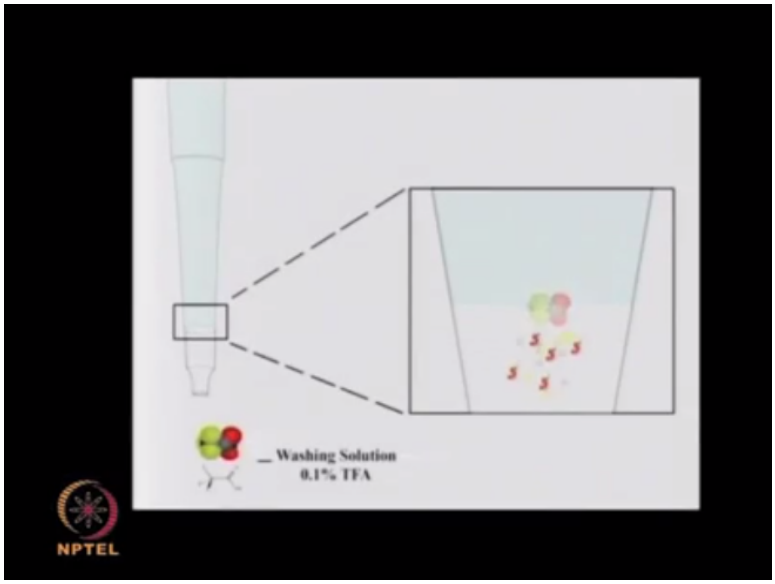
The ZipTip can be incorporated into high-throughput robotic devices or multi-channel pipettes for the high-throughput applications. Let me show you this video for the sample cleanup by using ZipTips.

(Refer Slide Time: 23:00)



In-gel digested protein samples can be further processed by using ZipTip.

(Refer Slide Time: 23:10)

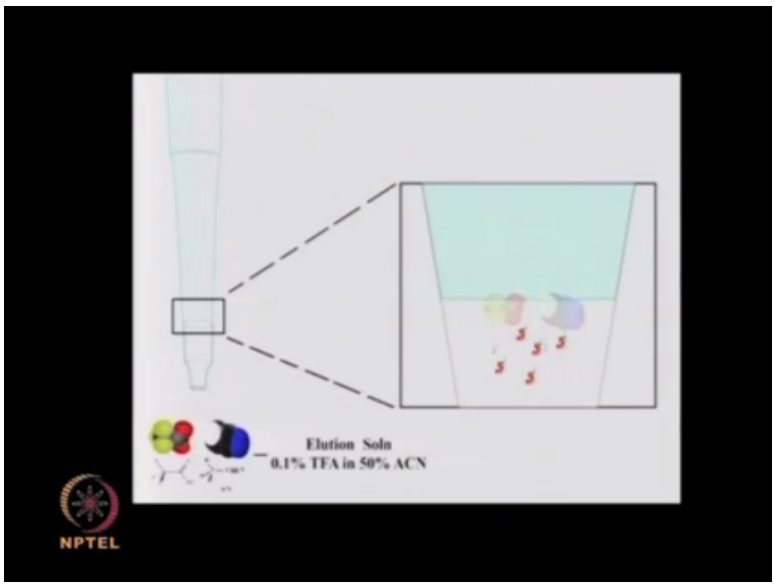


This animation shows the washing solution of 0.1% TFA passing through the ZipTip column. Now load the sample of your interest onto the ZipTip by pipetting 5 to 10 microliter of samples and this step has to be repeated 10 to 15 times. You can do the binding of peptides to this activated ZipTip by aspirating and dispensing 10 cycles.

(Refer Slide Time: 24:00)

So samples are passed through the activated ZipTip where they are captured in particular bed of chromatography media. Now wash this C18 tip thrice with 10 microliter of 0.1% TFA to remove the salt and other interfering components. So these steps ensure the salts and detergents are washed and finally samples can be eluted in a very small volume of solvent.

(Refer Slide Time: 24:44)

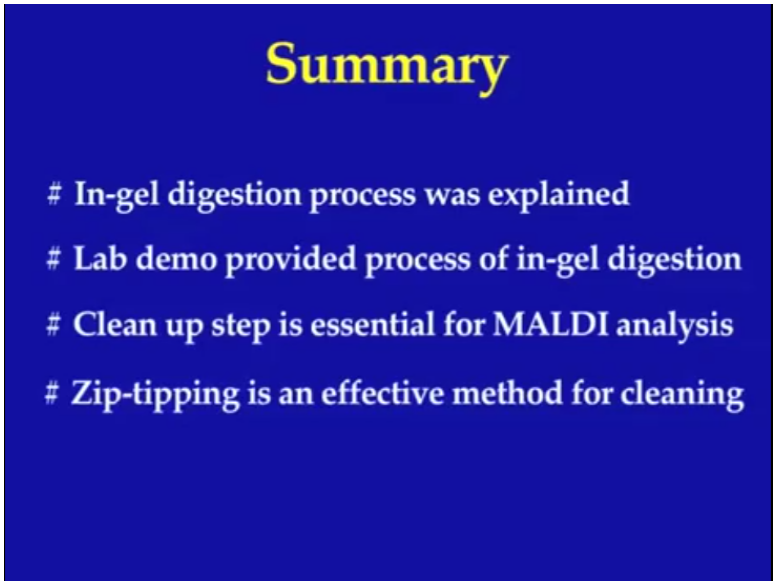


Now elute the sample from the ZipTip with 10 microliter of 50 to 70% acetonitrile in 0.1% of trifluoroacetic acid.

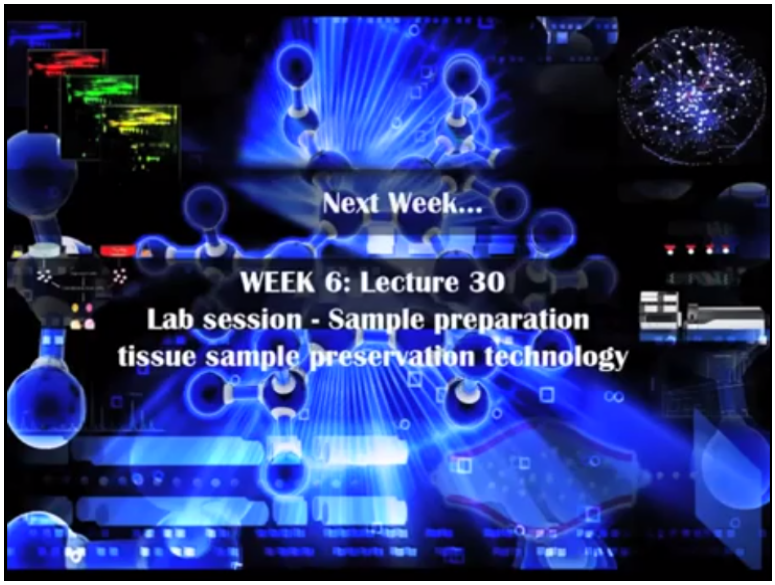
(Refer Slide Time: 25:05)

After the elution is done, then you can keep these process samples in cryo-boxes and is stored in -20 degree centigrade in freezers. The in-gel digested samples, which have been processed by using ZipTip can be further analyzed by using mass spectrometry.

(Refer Slide Time: 25:34)



(Refer Slide Time: 25:41)



(Refer Slide Time: 25:45)