Mass Spectrometry Based Proteomics
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Mod 01 Lecture Number 04

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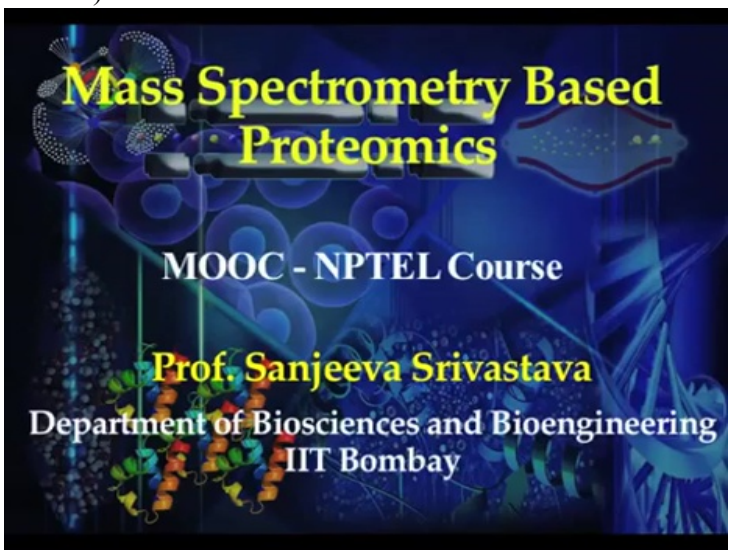

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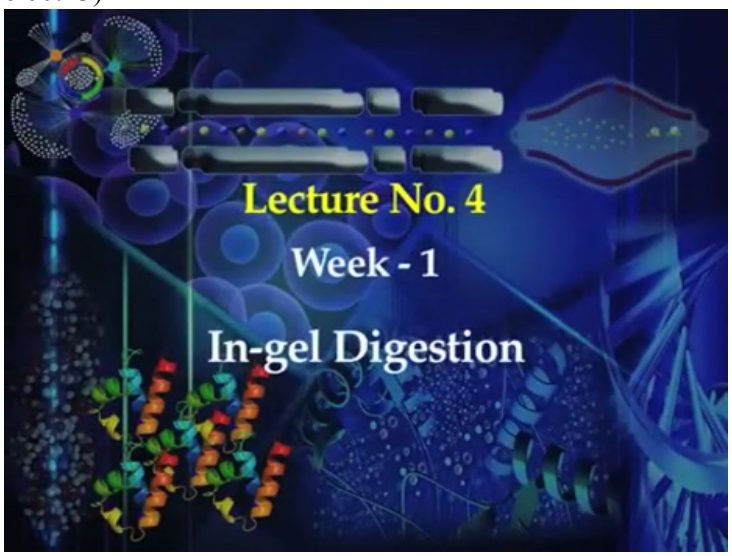

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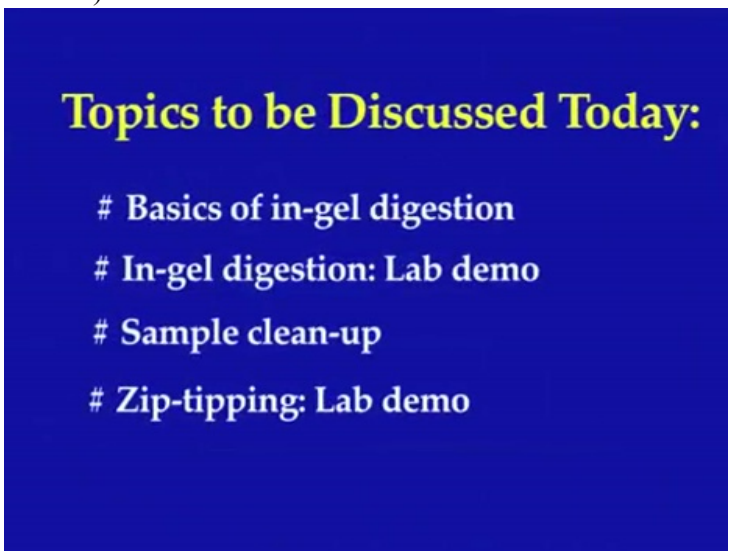

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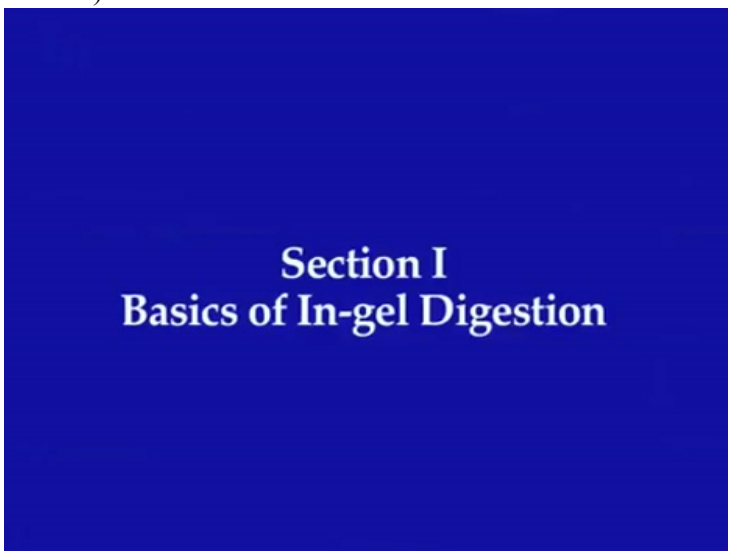

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Now let's talk about how to prepare the sample. We discuss these steps in more detail.

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

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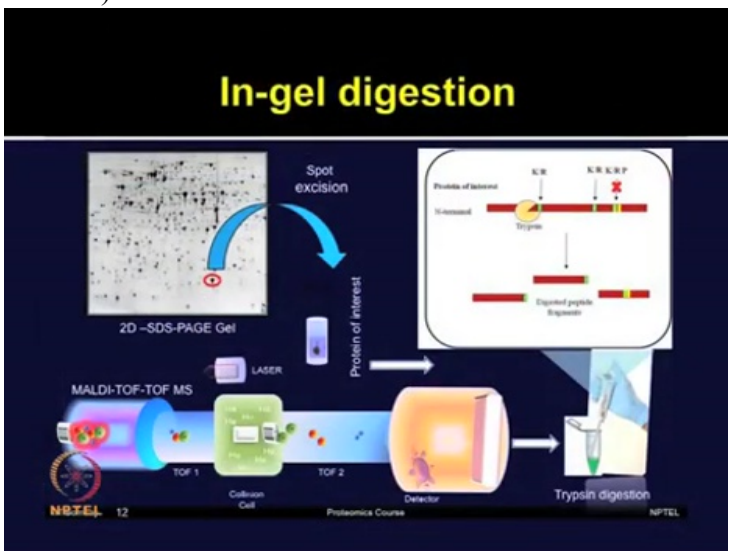

This slide gives you an overview. Since in the last module, we discussed about two-dimensional electrophoresis. It shows that if you have a spot of interest you can excise that spot from the 2-DE gel and subject that to in-gel digestion followed by mass spectrometric analysis.

In-gel digestion is a multi-step procedure, which includes spot selection, spot excision, removal of the stain, reduction, alkylation, proteolytic cleavage as well as peptides extraction.

There are multiple steps involved and bow good in-gel digestion is going to ensure the success of the spectrum generated from the mass spec. Now although this overview shows the process to start with the 2-DE gel but the same can be also applied for even the gel-free proteomic techniques.

If you want to analyze a sample even from the gel-free proteomics based approaches it is often a good idea to separate those complex protein 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 types of applications in the proteomics.

This slide gives you various recipes for performing in-gel digestion. The first step is de-staining of the spots or the band …

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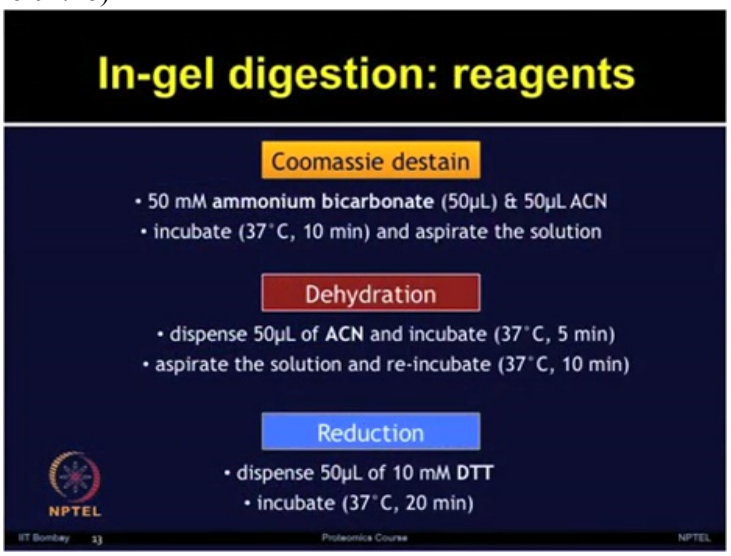

.. . because you have stained the gels with Coomassie brilliant blue or some other stains First of all you would like to remove these stains So the stain removal is essential prior to the mass spectrometric analysis.

The excised gel pieces should be washed with bicarbonate buffer and acetonitrile for removal of the staining agent. You can see the recipe in the slide. We will talk more about how to do these experiments when we come to the animation of these steps.

Acetonitrile reduces the hydrophobic interaction between the protein and the stain while the ionic solution decreases the ionic interactions between negatively charged Coomassie brilliant blue dye and the positively charged protein.

Once the Coomassie de-staining or the de-staining step is performed then we need to dehydrate the gel pieces which can be done by using the addition of acetonitrile.

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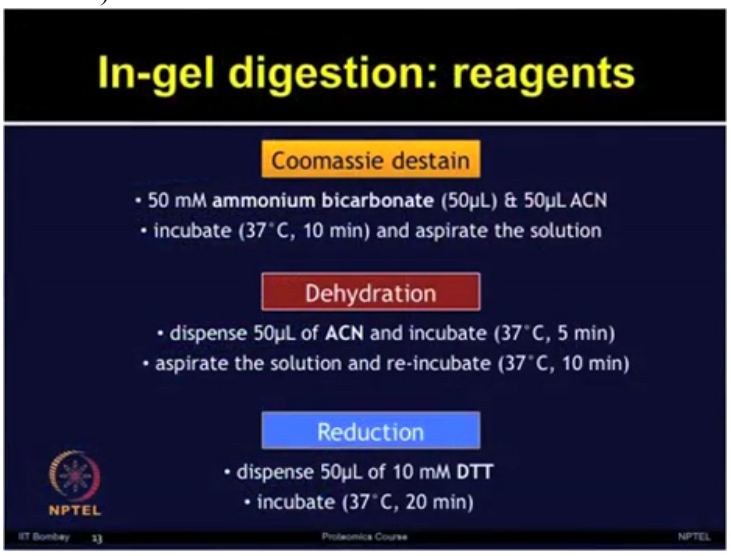

After this incubation is done then you are ready for performing reduction step.

Now why reduction step is required? After the stain removal the next steps are including the reduction as well as alkylation of protein residues so that we 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's now look at the next step which is alkylation.

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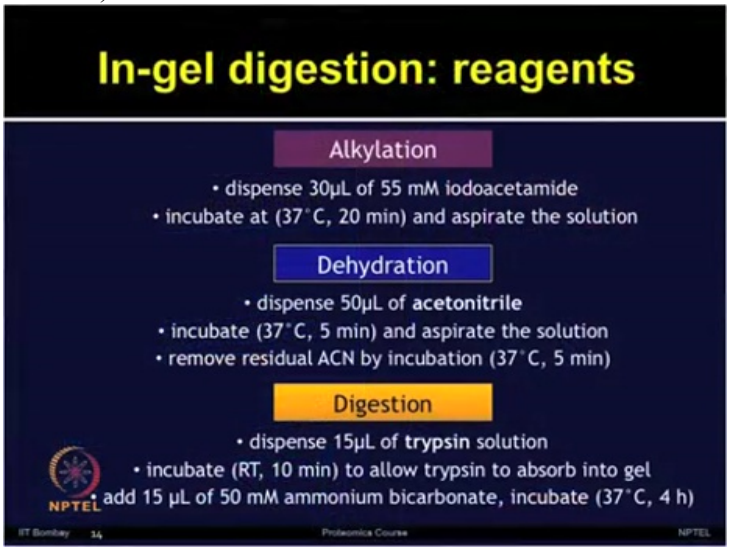

So in the alkylation you need to add the iodoacetamide. The reformation of disulphide bonds may occur. So, to prevent that, iodoacetamide which is an alkylating agent is used here. Again you need to do dehydration step as recipe is shown in the slide.

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 carboxyl terminals of basic amino acids such as Arginine and Lysine.

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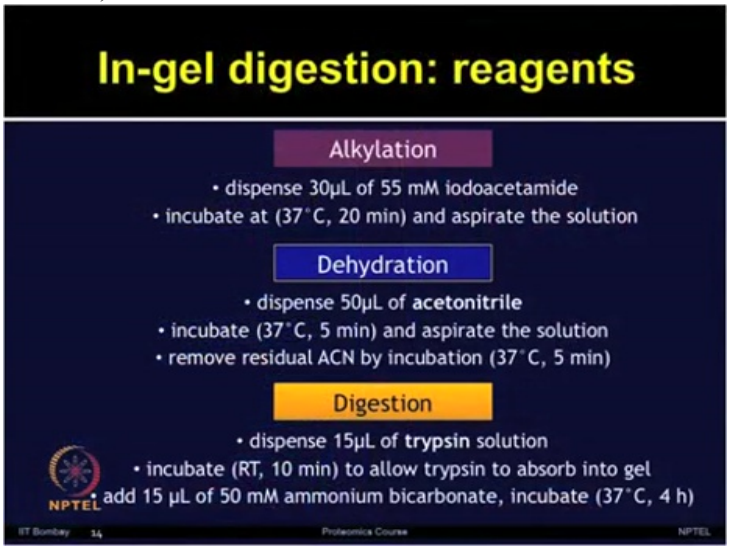

Once digestion is done which is usually the over-night step then one needs to do the peptides extraction of the digested proteins. So the peptides generated through the proteolytic cleavage can be extracted by using recipe including formic acid or TriFlouroAcetic acid, TFA in the 50% acetonitrile solution.

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.

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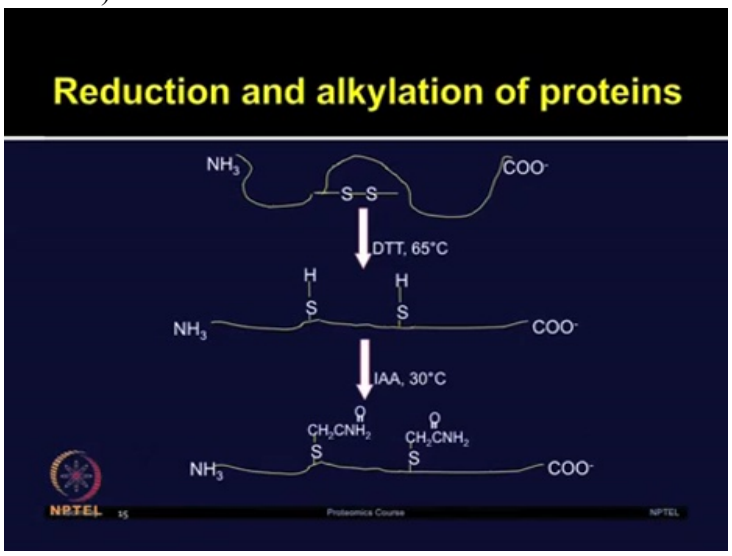

So, DTT is important for the treatment of proteins to break the disulphide bonds which we have also talked in the sample preparation when we discussed earlier.

Now iodoacetamide, it adds the iodoacetamide group to sulphydryl group and prevents disulphide bond formation. So these steps are quite important in in-gel digestion process.

So coming to the specificity of trypsin, first of all I will discuss why we need to do the proteolytic digestion. See, you want to generate the peptides with the molecular weight within the mass range of mass spectrometer.

So you always want to simplify the process for even very superior analytical instrument so that you can increase the efficiency of the process.

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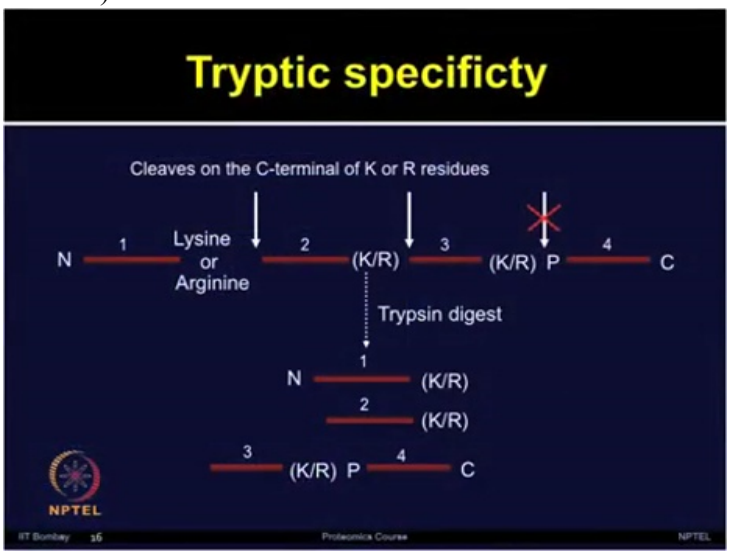

The enzymatic digestion are performed with various enzymes but typically with the trypsin which cleaves at the C-terminal of lysine and arginine residues but exceptions can occur with the proline.

If proline is in then that breakage will not happen So, one can use a modified trypsin which is serine indopeptidase. However it cleaves at proline-lysine and proline-arginine bonds at a much slower rate.

You can see the cleavage process and the specificity in the slide where it shows if you have lysine or arginine residues it can break the bonds. But when there is a proline residue present there then it cannot cleave.

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

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So the following video which we are going to watch, it 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 experiment.

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

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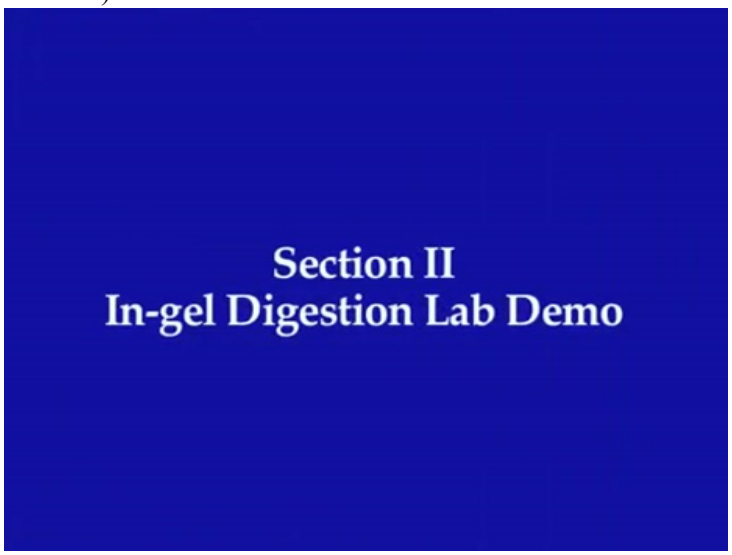

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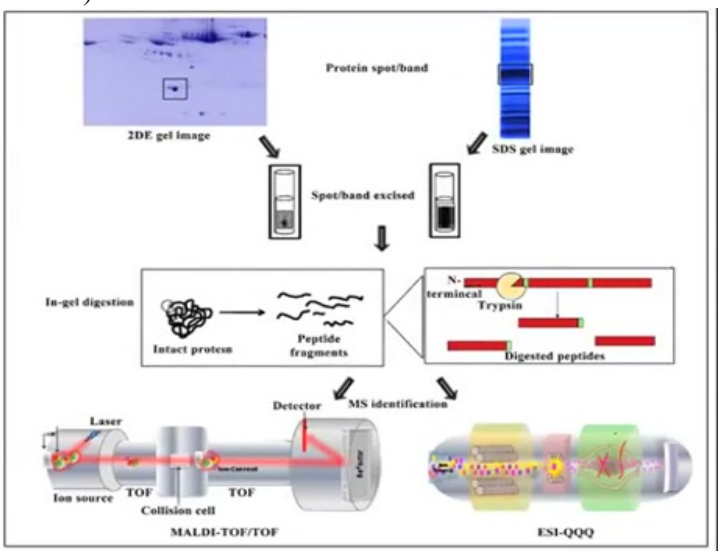

In-gel digested proteins separated by 2D are analyzed and significant spots are excised, processed and taken for the mass spectrometric analysis.

Prior to the mass spectrometric analysis, it is important to clean 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 the mass spectrometry.

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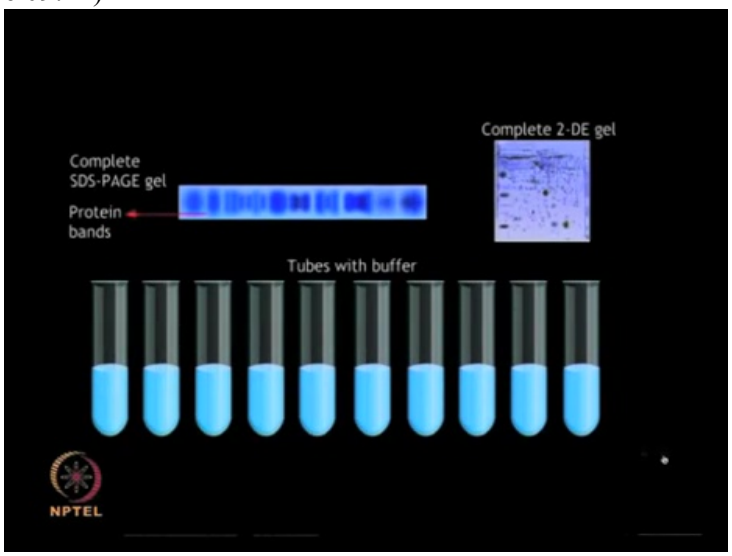

Electrophoretic separation of the protein mixture results in distinct protein bands.

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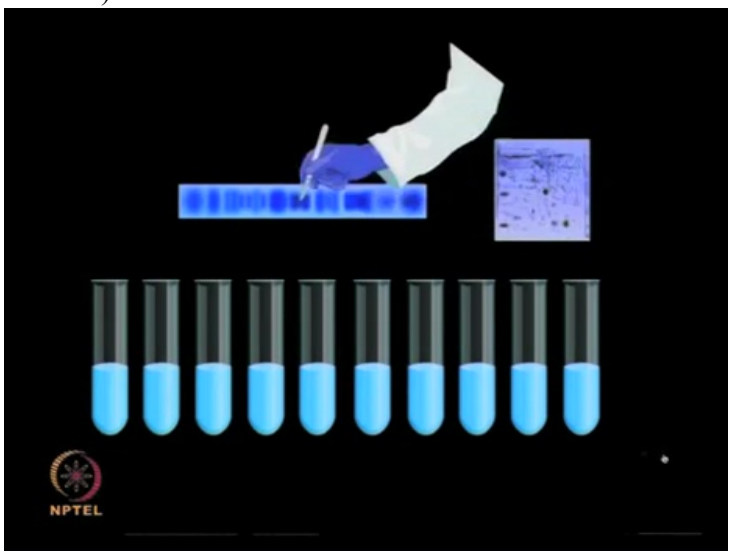

These proteins can be used for analytic purposes by carrying out in-gel digestion.

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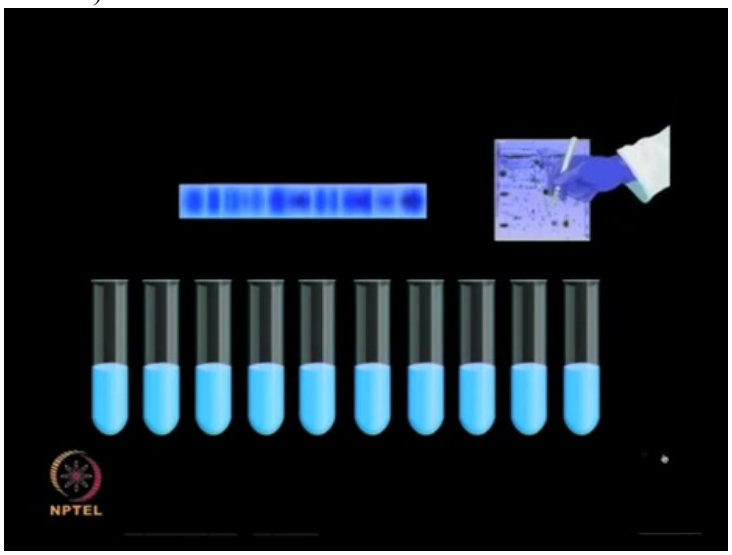

Entire gel is fragmented into small pieces and each piece is.

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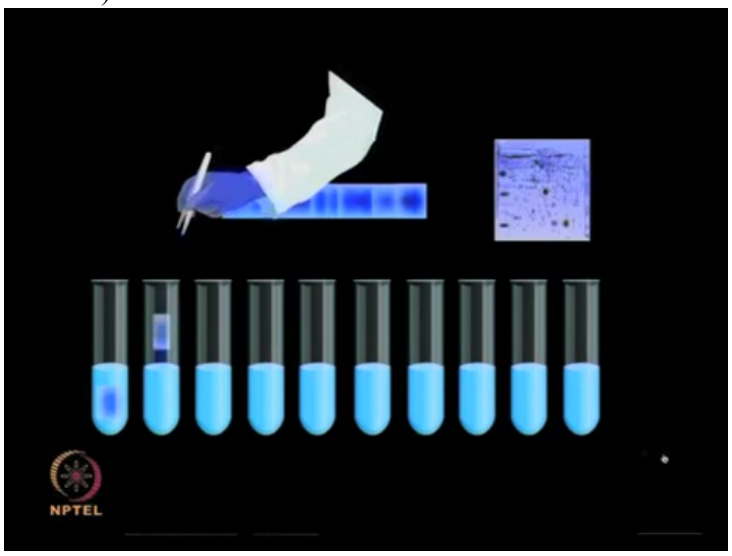

…dissolved in a suitable buffer

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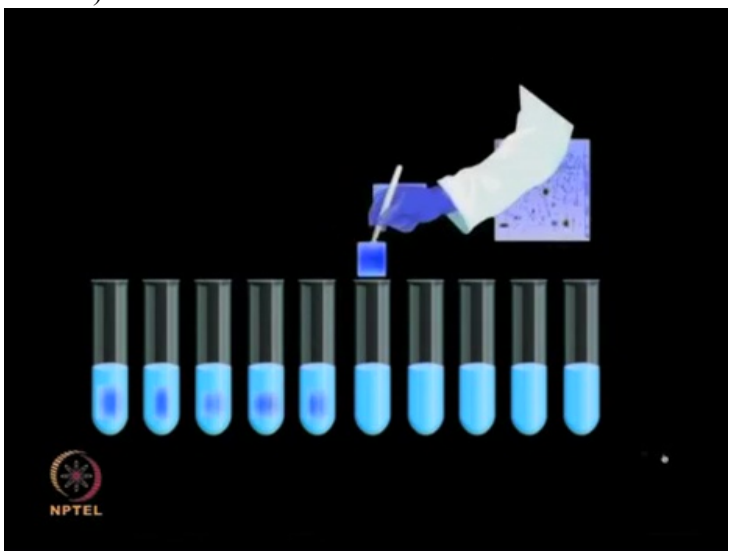

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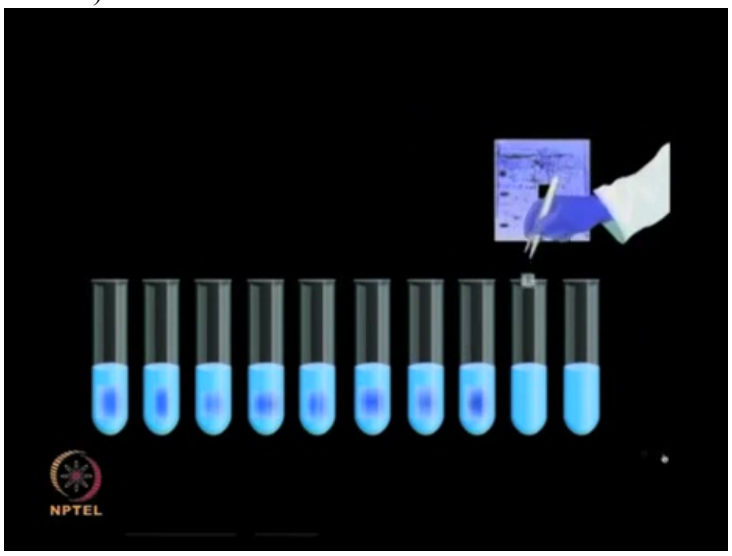

To simplify the complex proteome, it is good idea to chop down…

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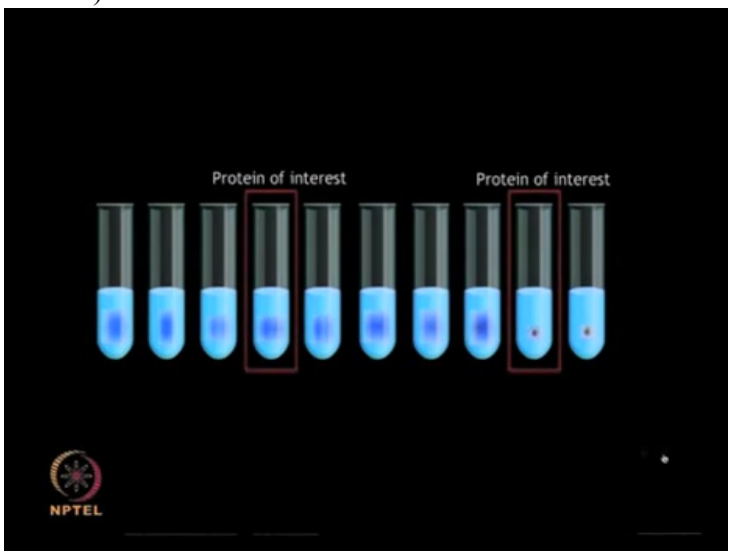

… the overall proteins into the smaller pieces The protein solution is treated with …

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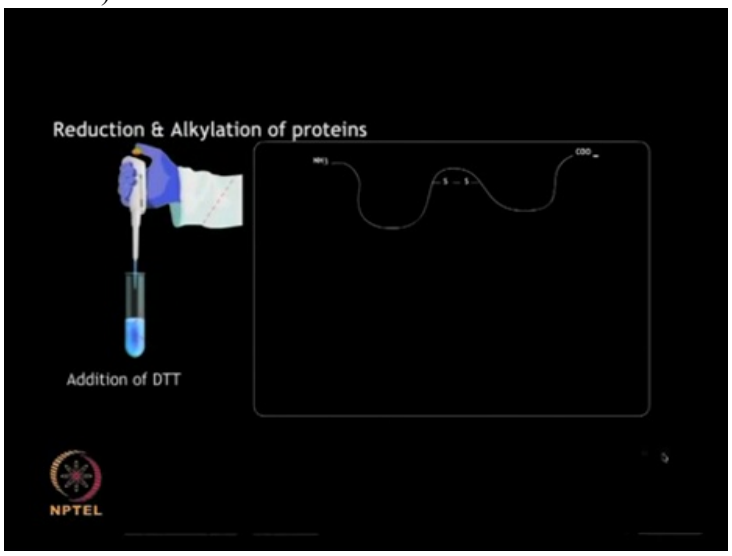

…a reducing agent like Dithiothreitol DTT which cleaves the disulfide bond in the protein

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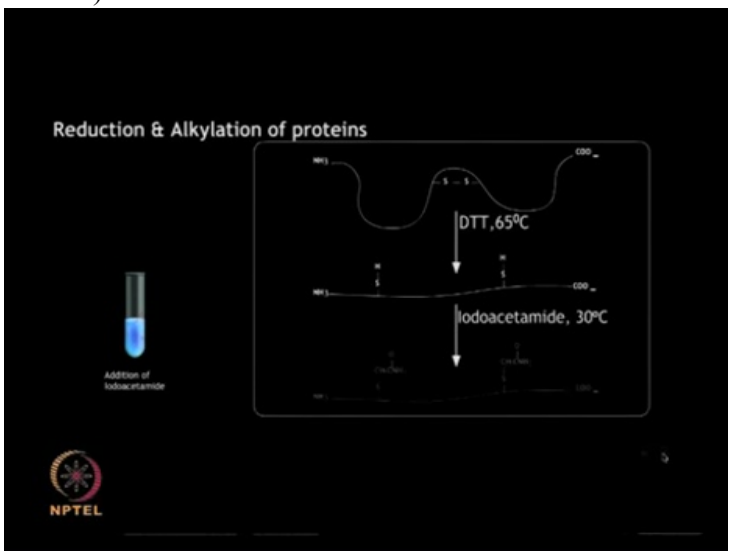

This is followed by treatment with IodoAcetAmide IAA which alkylates the sulfhydryl group and thereby prevents the reformation of disulfide bonds.

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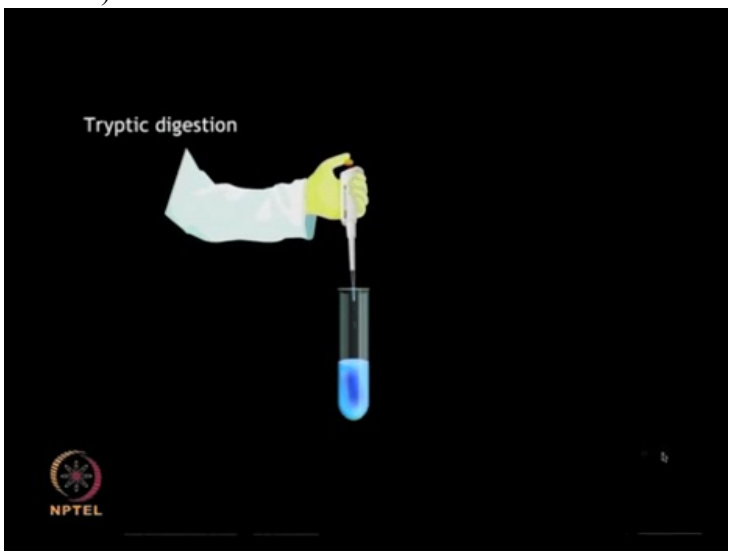

After cleavage of the disulfide bonds…

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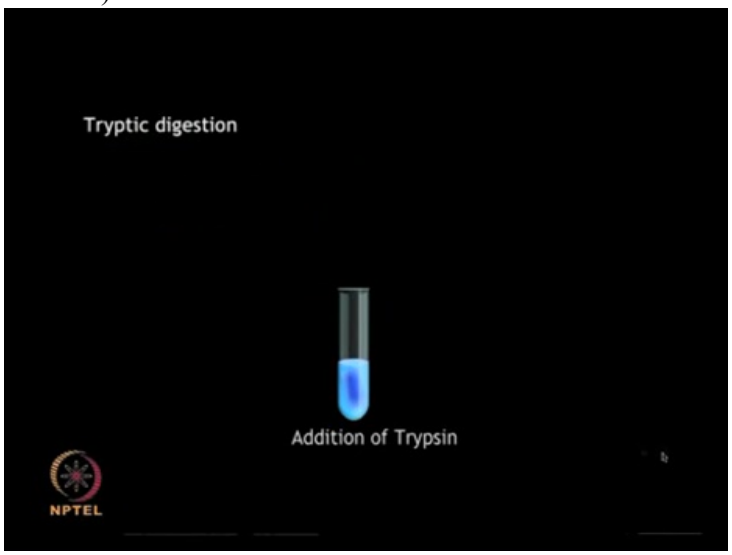

…the protein is treated with a proteolytic enzyme The most commonly used enzymes include Trypsin.

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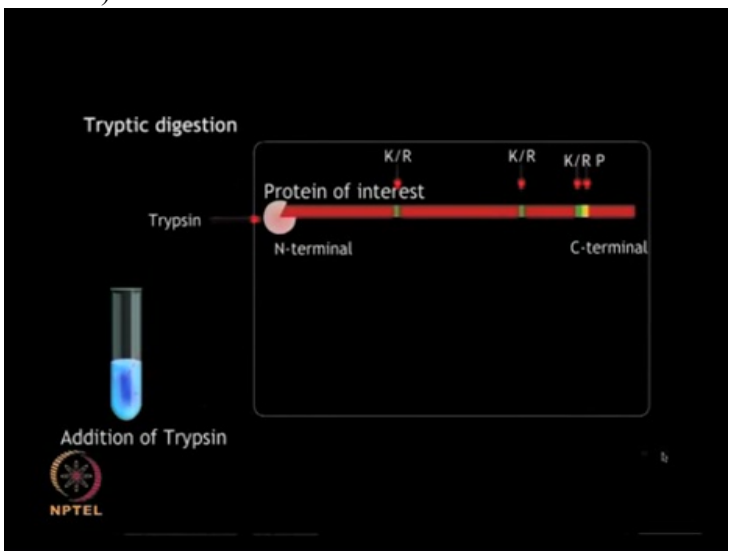

Trypsin cleaves the protein at specific residues…

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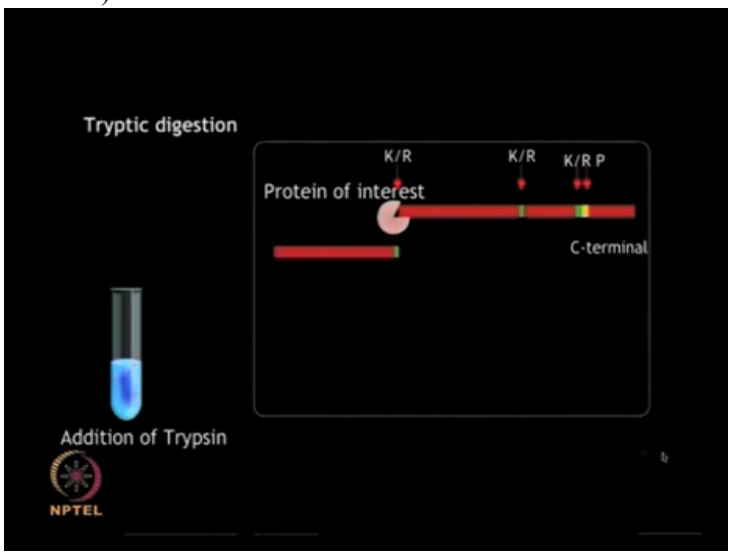

…arginine and lysine …

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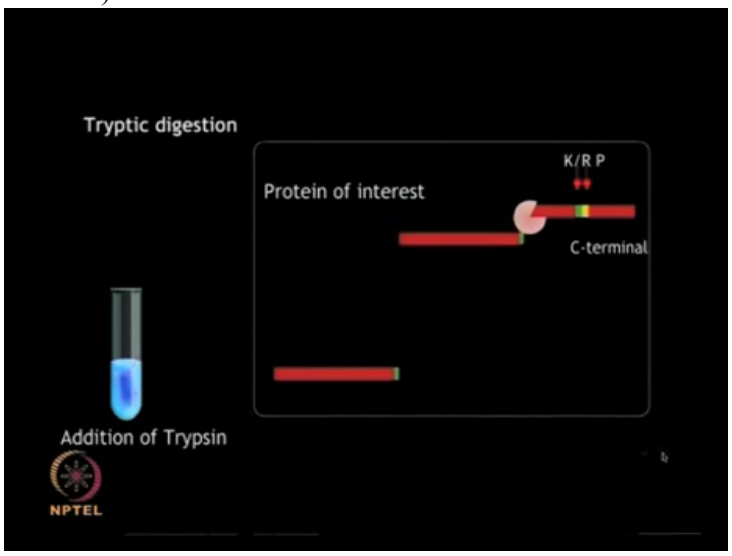

… and generates a smaller peptide fragments. This trypted digest is used for …

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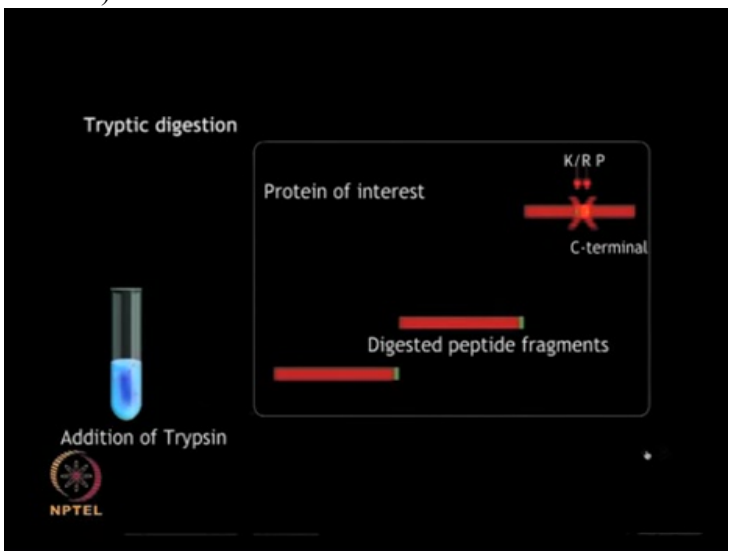

… further purification and analysis.

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

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Clean the glass plate and place the gel on the plate.

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Excise the…

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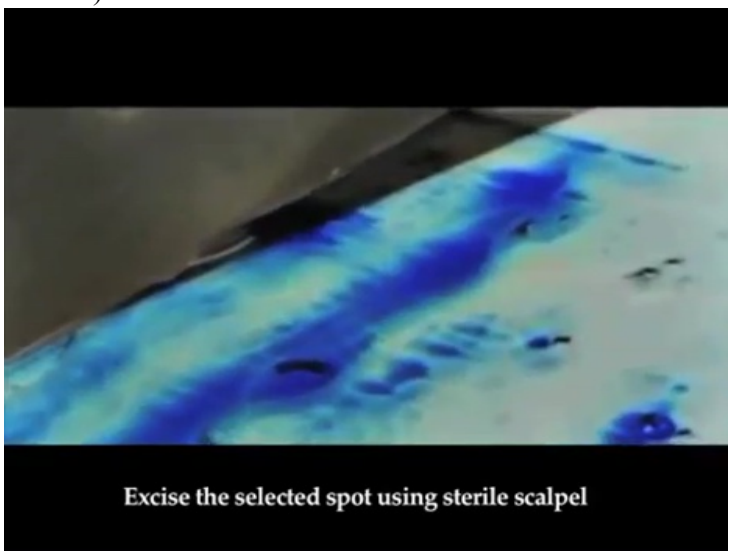

… selected spot using sterile scalpel.

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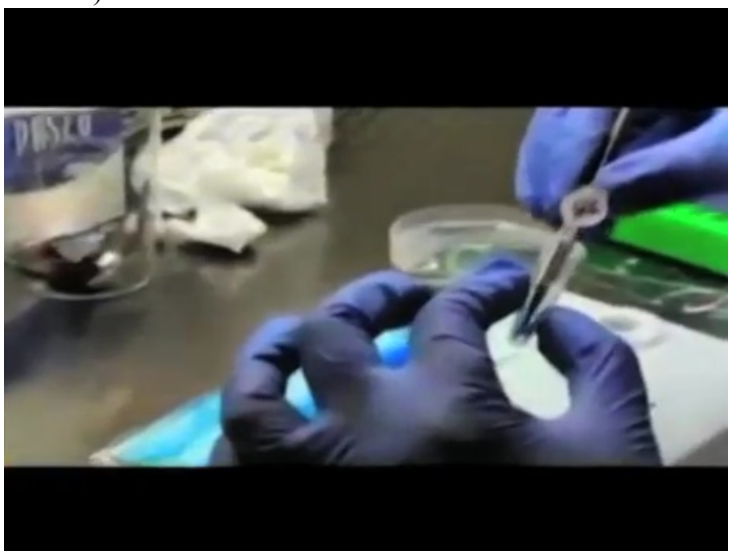

Place the excised spot in the fresh Eppendorf tube.

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Clean the glass plate again.

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Place the excised spot on the clean glass plate.

Place the excised spot on the clean glass plate

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…slice the big gel pieces…

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…into small pieces and use them for in-gel digestion.

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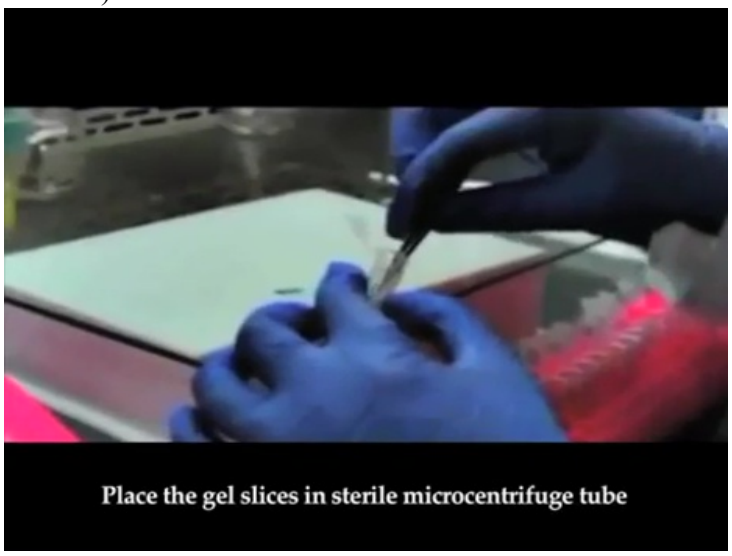

Now place the excised gel …

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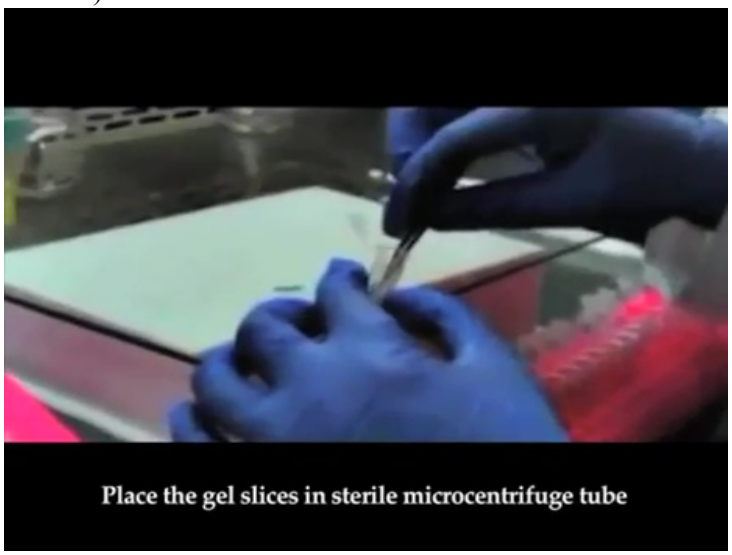

… in the clean, sterile Eppendorf tube.

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Add the …

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… stain removal solution

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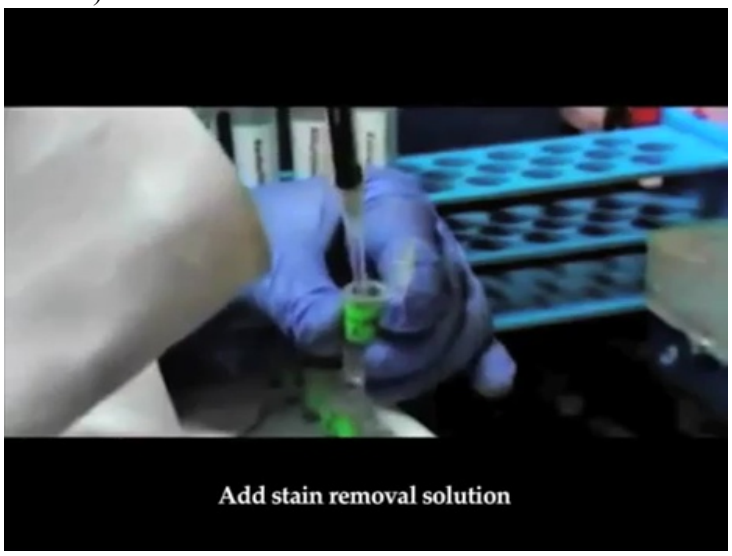

…to the spot.

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Keep it on the shaker for 30 minutes at room temperature.

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After 30 minutes discard the solution.

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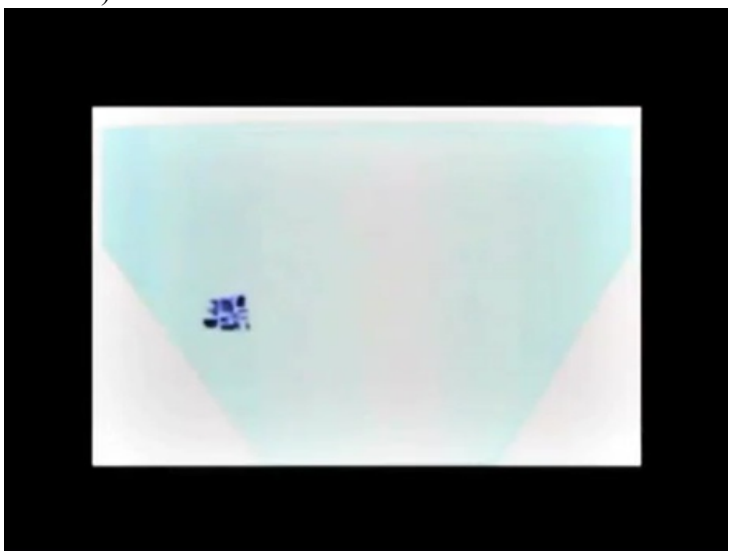

During the treatment…

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…de-staining solution acts on the gel pieces causing it to swell

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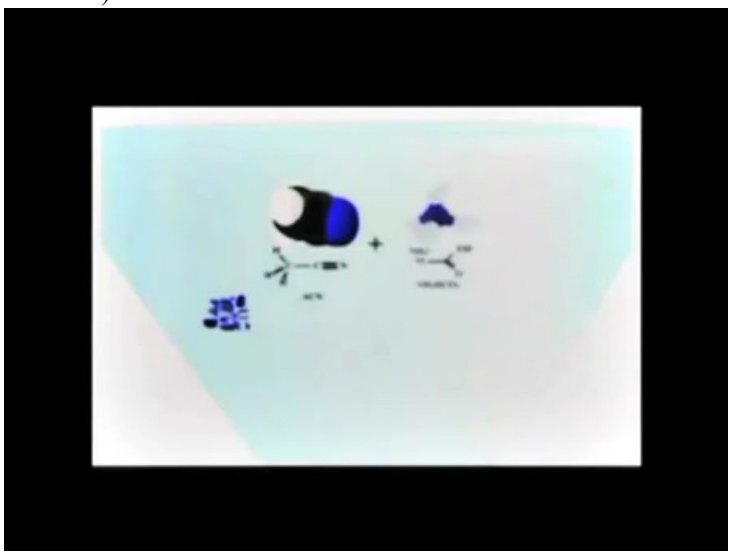

…and de-stain …

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…by reducing the interactions

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… between protein and dye

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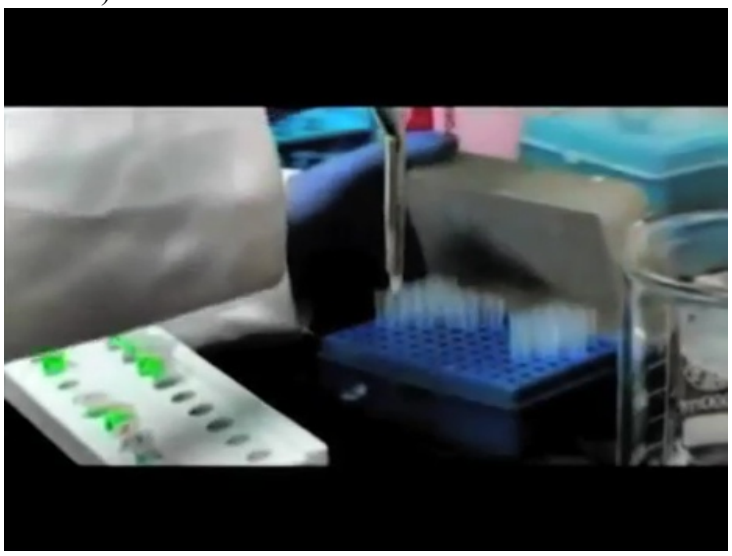

Add…

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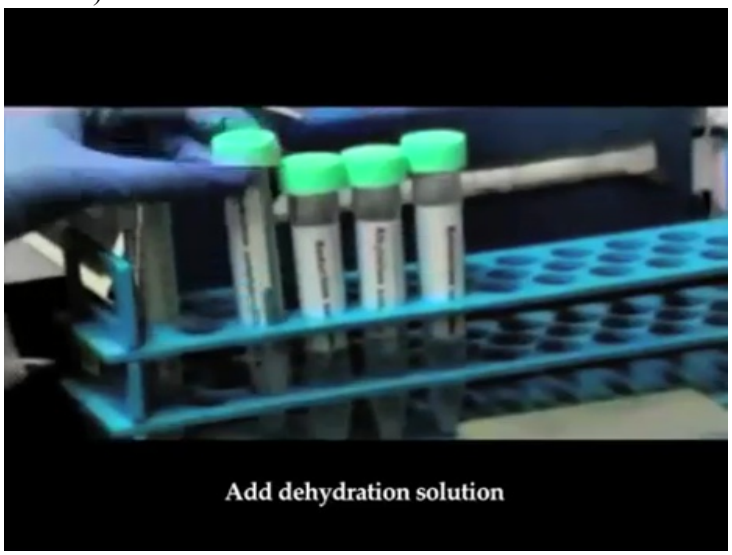

… dehydration solution …

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… to the gel pieces.

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Rotate for 30 minutes at room temperature.

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After 30 minutes, discard the solution.

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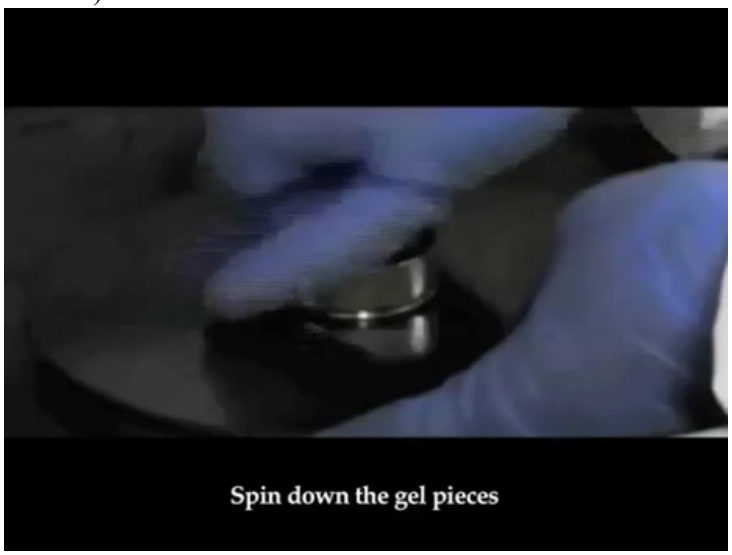

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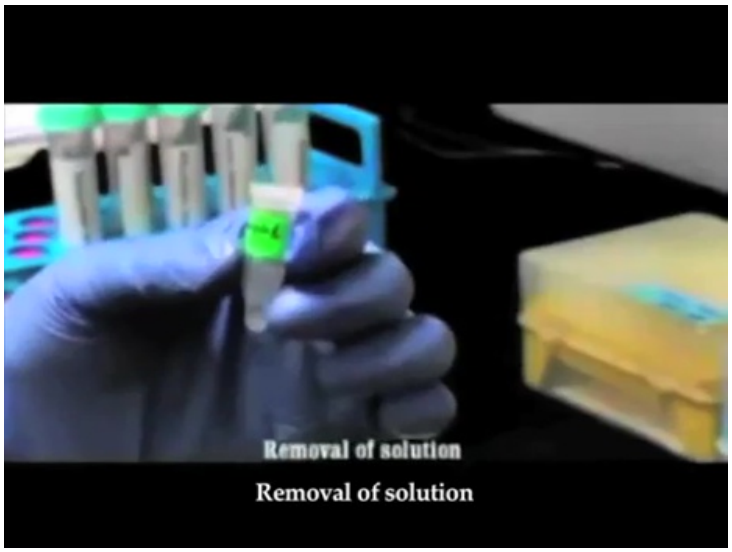

After 30 minutes, remove the solution.

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Repeat the step twice to ensure …

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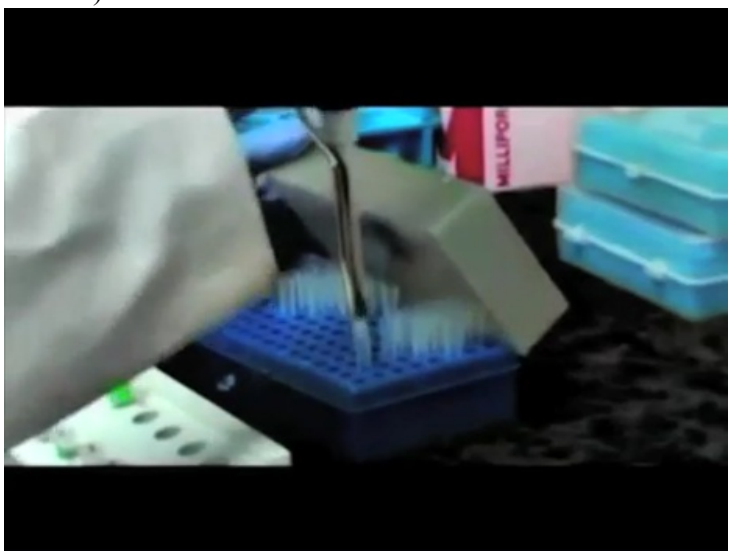

…complete removal of stain from the gel.

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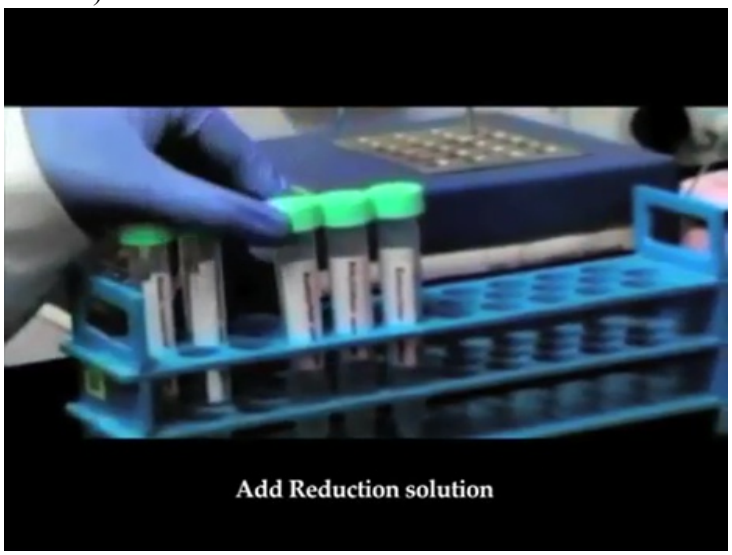


Add reduction solution

(Refer Slide Time 15:37)

…to the gel pieces…

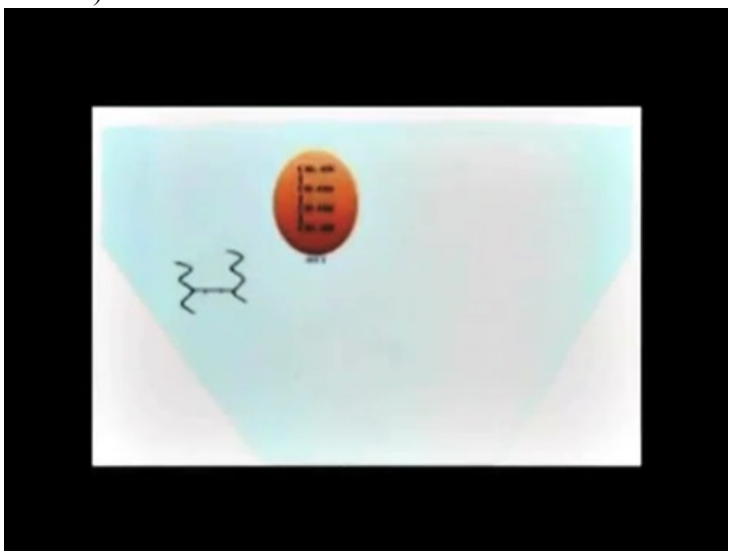
(Refer Slide Time 15:50)

…. and place it at 56 degrees Centigrade for 60 minutes.

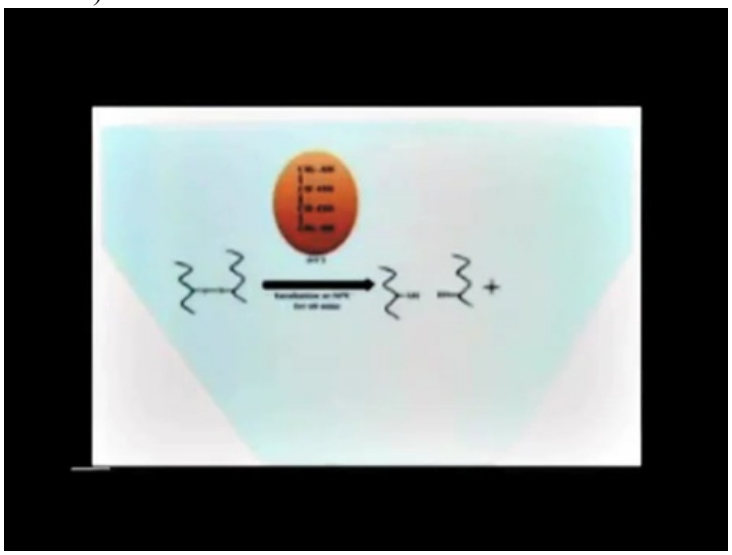
(Refer Slide Time 16:01)

Here, we will see…
(Refer Slide Time 16:02)

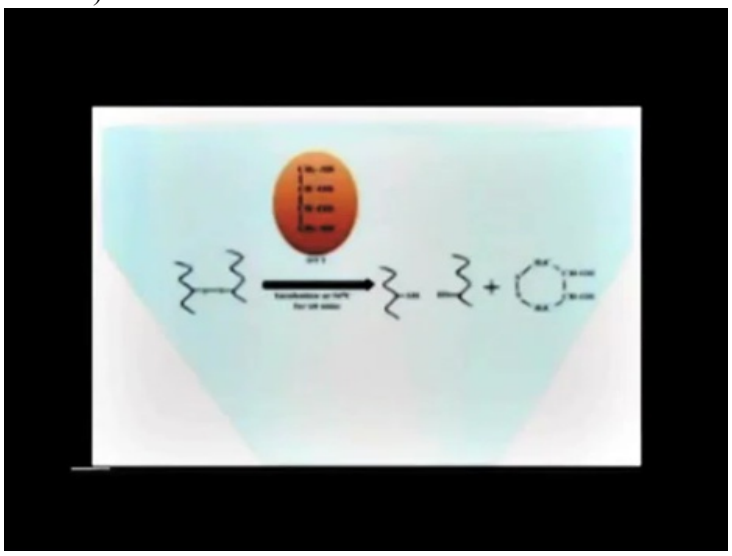
(Refer Slide Time 16:03)



(Refer Slide Time 16:16)



(Refer Slide Time 16:17)



…how reduction solution with DTT breaks the disulfide bonds.

(Refer Slide Time 16:20)

(Refer Slide Time 16:24)

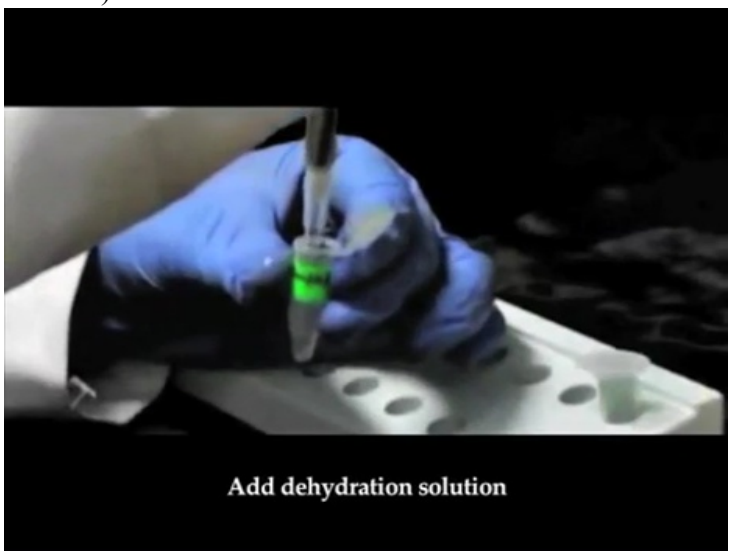
Discard the solution

(Refer Slide Time 16:31)

(Refer Slide Time 16:34)

Wash the gel pieces with dehydration solution and after that …

(Refer Slide Time 16:41)



… add dehydration solution and discard the solution …

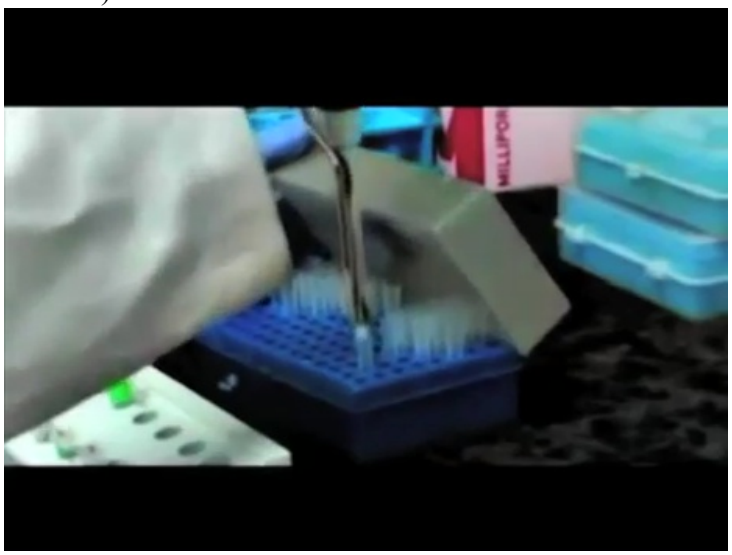
(Refer Slide Time 16:49)

… after vortexing.

(Refer Slide Time 16:55)

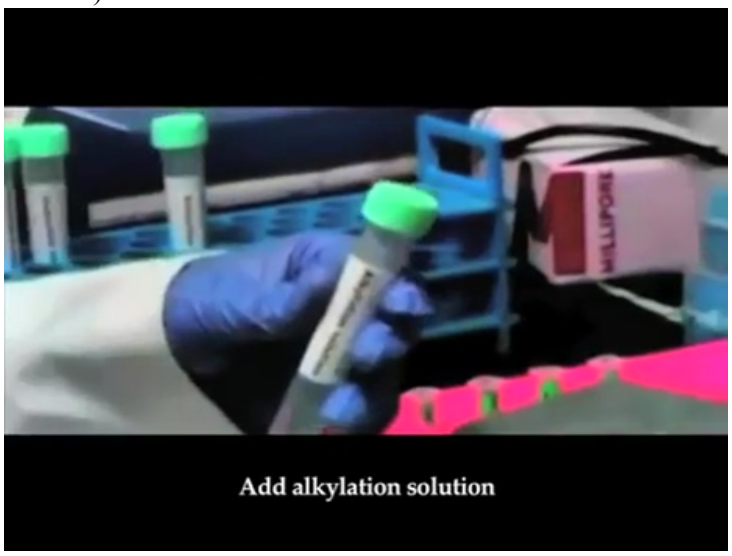
(Refer Slide Time 17:01)

(Refer Slide Time 17:06)



To the same gel pieces…

(Refer Slide Time 17:13)



…add alkylation solution.

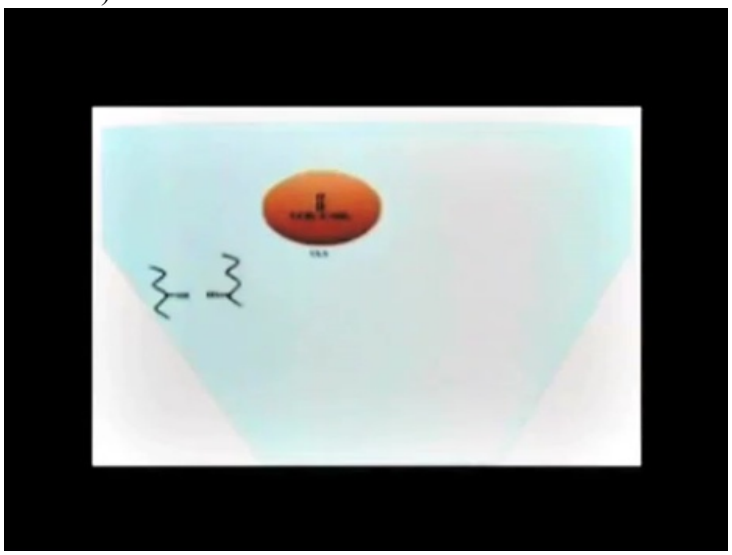
(Refer Slide Time 17:33)

And keep in dark for 20 minutes at room temperature.

(Refer Slide Time 17:48)

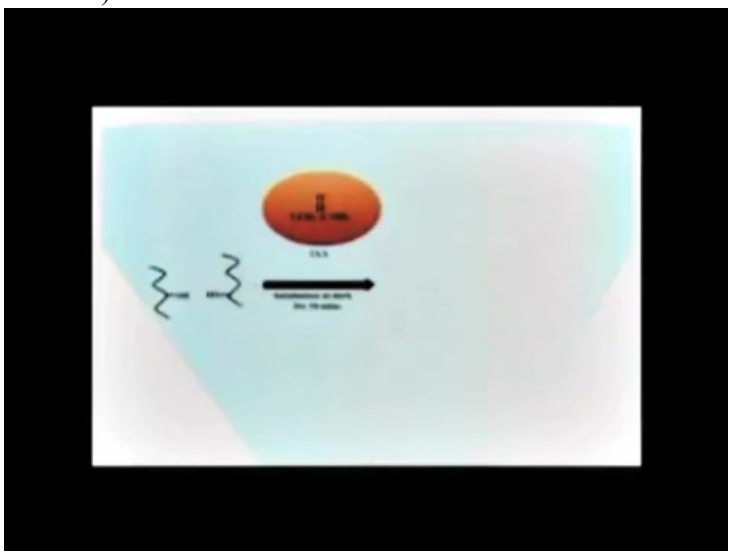
Alkylation solution having IAA reacts …

(Refer Slide Time 17:49)



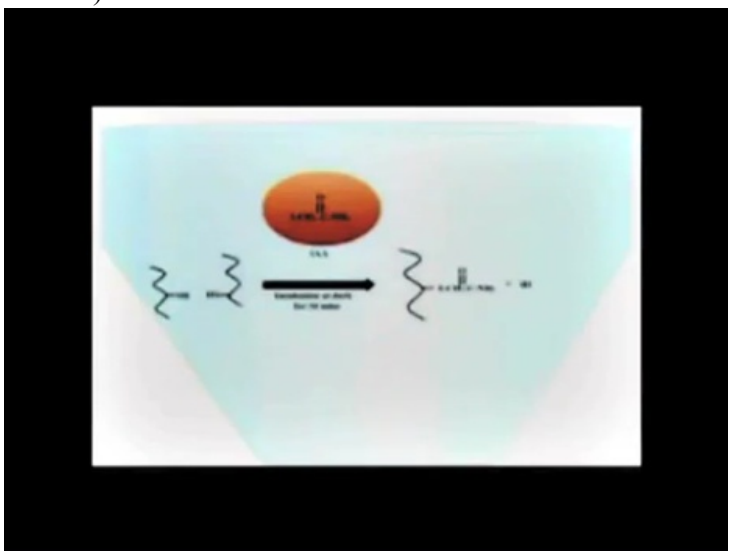
…with free sulfhydryl group and prevents the …

(Refer Slide Time 17:51)



…reformation of …

(Refer Slide Time 17:54)



disulfide bonds.

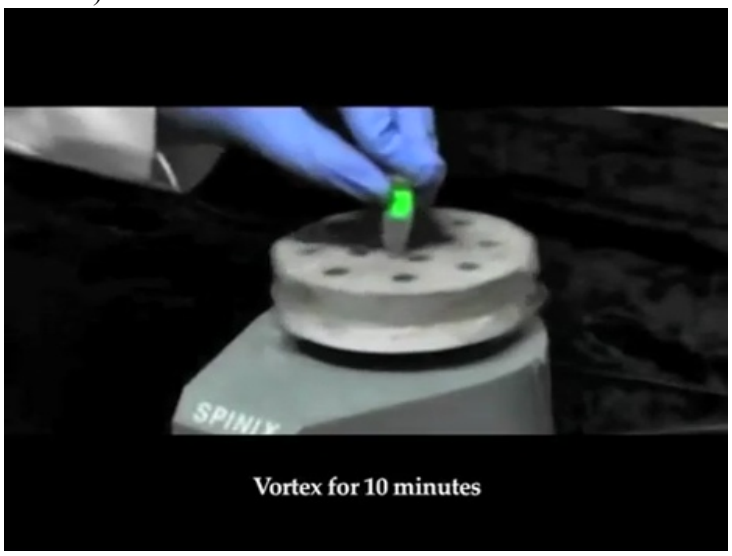
(Refer Slide Time 18:08)

Remove the alkylation solution.

(Refer Slide Time 18:18)

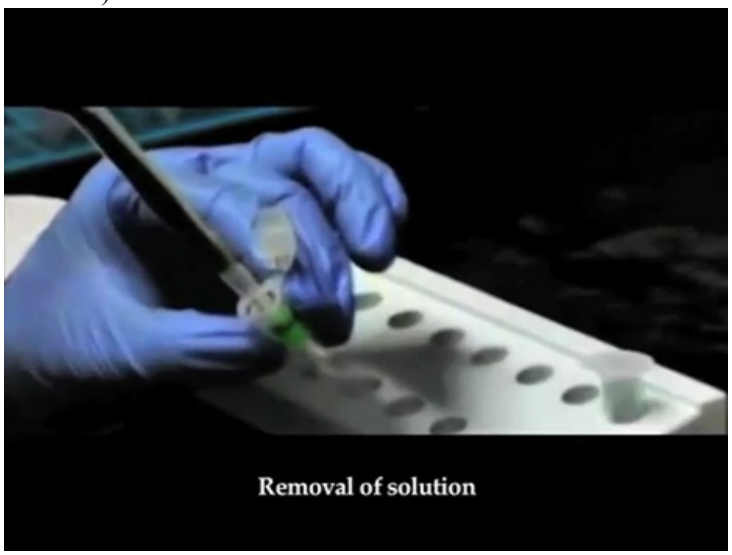
Add dehydration solution

(Refer Slide Time 18:28)



Vortex it for 10 minutes to wash the gel pieces.

(Refer Slide Time 18:35)

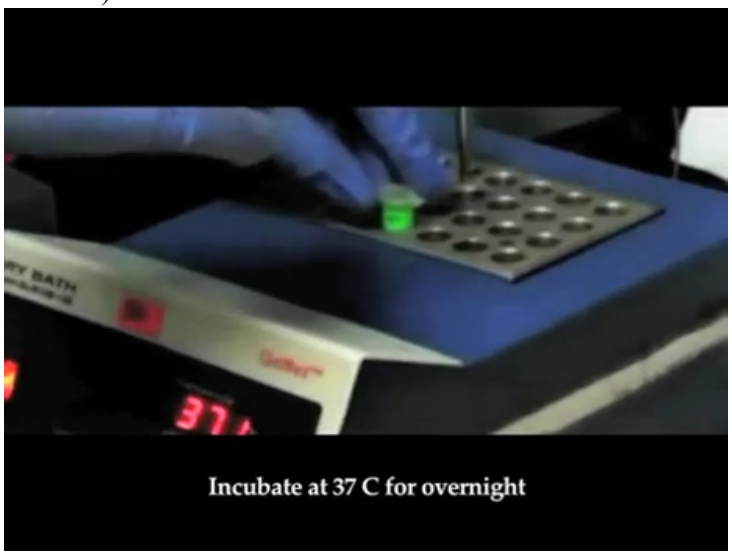


Discard the solution and dry the gel pieces completely.

(Refer Slide Time 18:53)

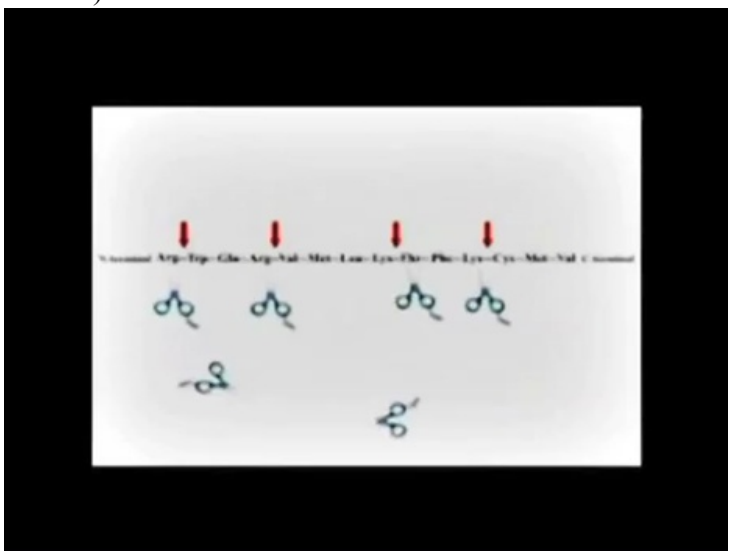
Trypsin solution was prepared and added to the dried gel pieces.

(Refer Slide Time 19:11)



Keep it overnight at 37 degrees Centigrade in dry bath.

(Refer Slide Time 19:23)



Trypsin is a proteolytic enzyme which cleaves the protein…

(Refer Slide Time 19:24)

… at the carboxyl terminal of lysine or arginine and leads to the formation of small peptides …

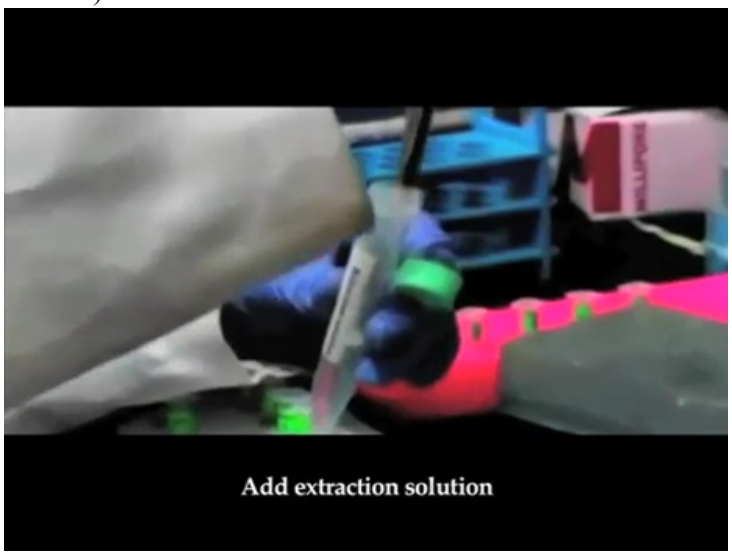
(Refer Slide Time 19:29)

… of 8 to 35 Daltons amino acid length.

(Refer Slide Time 19:32)

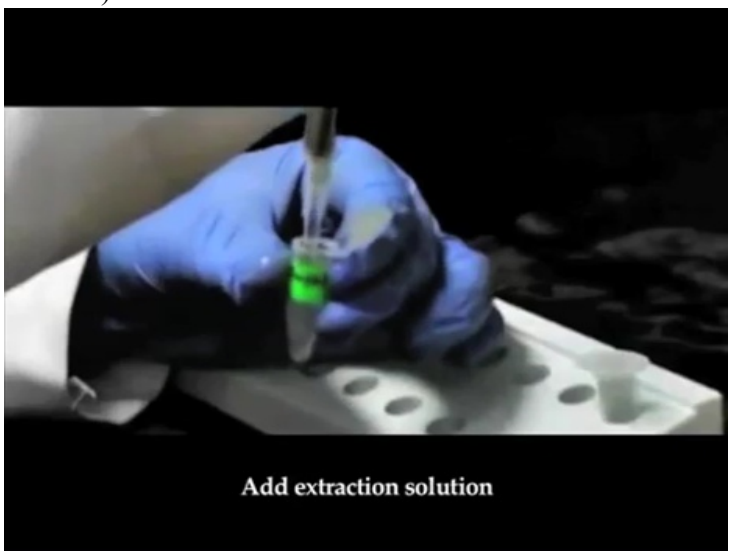
Trypsin activity was stopped by keeping the reaction mixture in ice.

(Refer Slide Time 19:48)



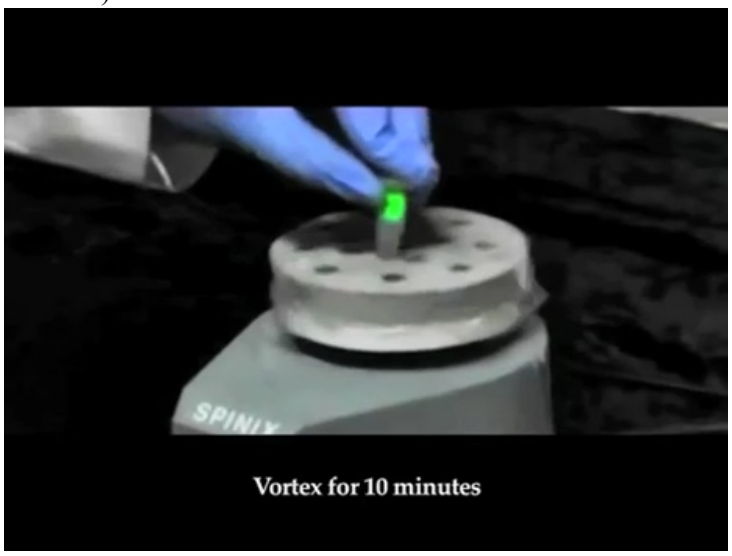
Extraction solution having acetonitrile in 0.1% Trifluoroacetic acid was added …

(Refer Slide Time 19:55)



…to the gel pieces.

(Refer Slide Time 20:02)



Vortex vigorously for 10 minutes.

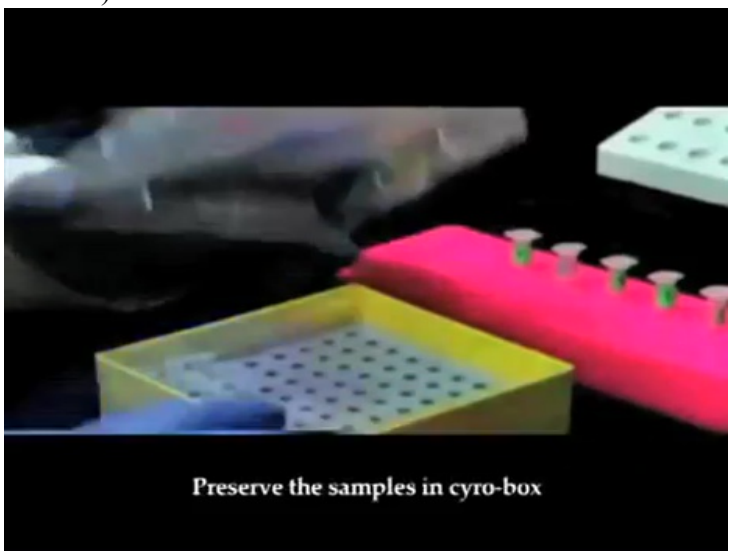
(Refer Slide Time 20:09)

The supernatant was collected and …

(Refer Slide Time 20:19)

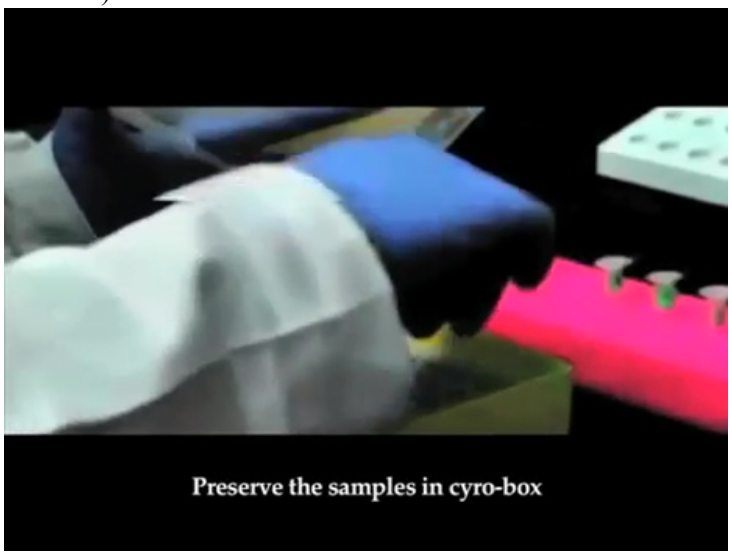
…extraction process was repeated twice.

(Refer Slide Time 20:45)



Preserve ….

(Refer Slide Time 20:50)

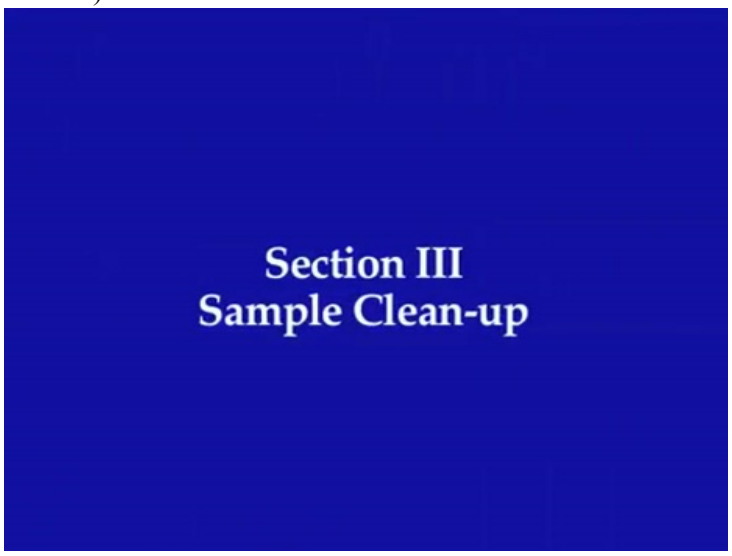


…the samples ….

(Refer Slide Time 21:07)

… in -20 degrees Centigrade till further use.

(Refer Slide Time 21:09)

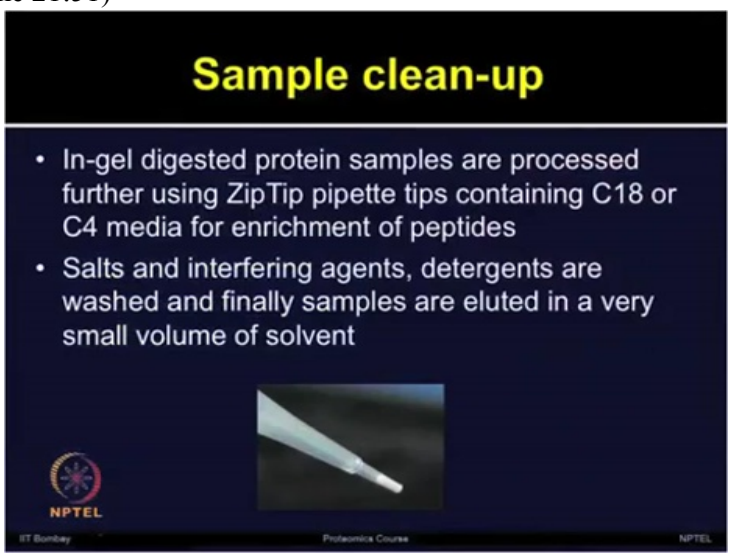


So after watching this video now you are very clear of the in-gel digestion process, how various steps are important to perform these experiments.

Now once we have done the in-gel digestion you can directly use these tryptic digest for further mass spectrometric analysis but often recommended, that in between you add one more step which is sample cleaning.

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.

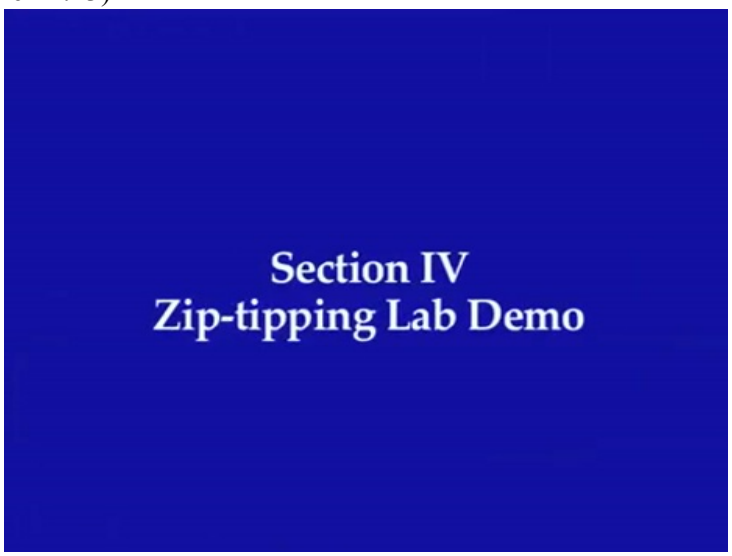
(Refer Slide Time 21:51)



So it is recommended that one should use the cleanup step in between. So the in-gel digested protein samples 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 22:23)



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

(Refer Slide Time 22:43)

The ZipTips can be incorporated into high throughput robotic devices or multi-channel pipettes for the high throughput application.

Let me show this video for the sample cleanup by using ZipTips.

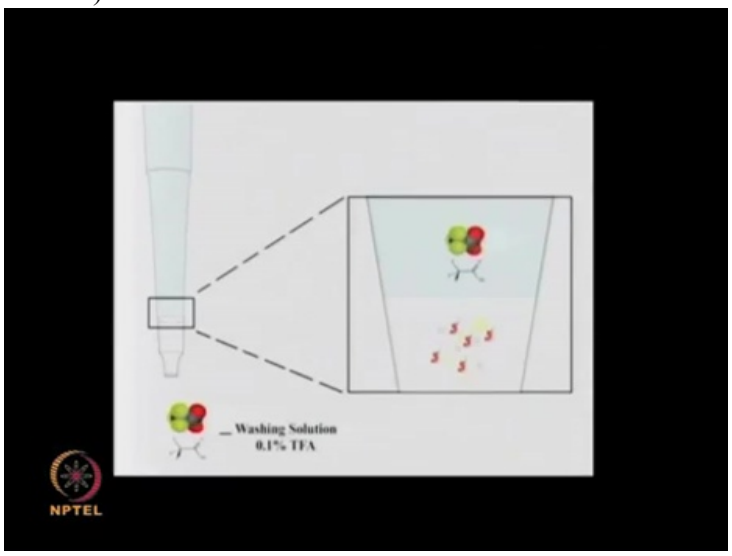
(Refer Slide Time 23:00)

In-gel digested protein samples …

(Refer Slide Time 23:02)

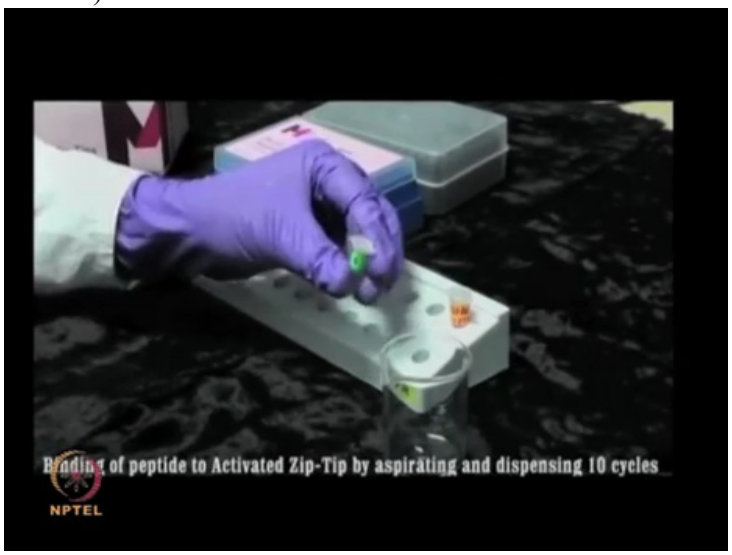
…can be further processed by using ZipTip.

(Refer Slide Time 23:08)



This animation shows the washing solution of 0.1% PFA passing through the ZipTip column.

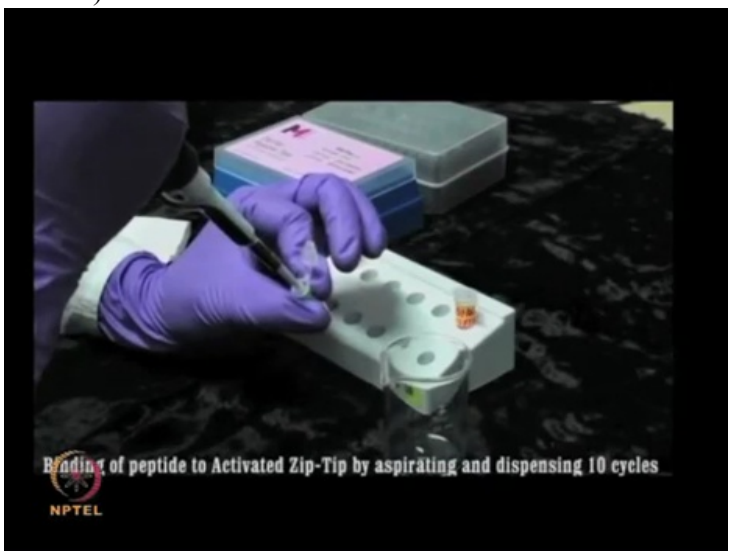
(Refer Slide Time 23:18)



Now load the sample of your interest on to the ZipTip by pipetting 5 to 10 micro liters of samples.

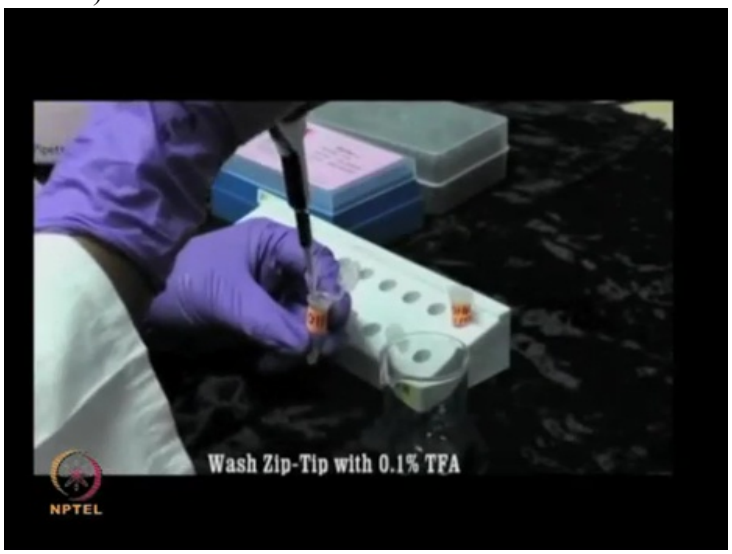
And this step has to be repeated 10 to 15 times.

(Refer Slide Time 23:46)



You can do the binding of peptides to this activated ZipTip by aspirating and dispensing 10 cycles.

(Refer Slide Time 23:54)



So samples are passed ….

(Refer Slide Time 23:57)

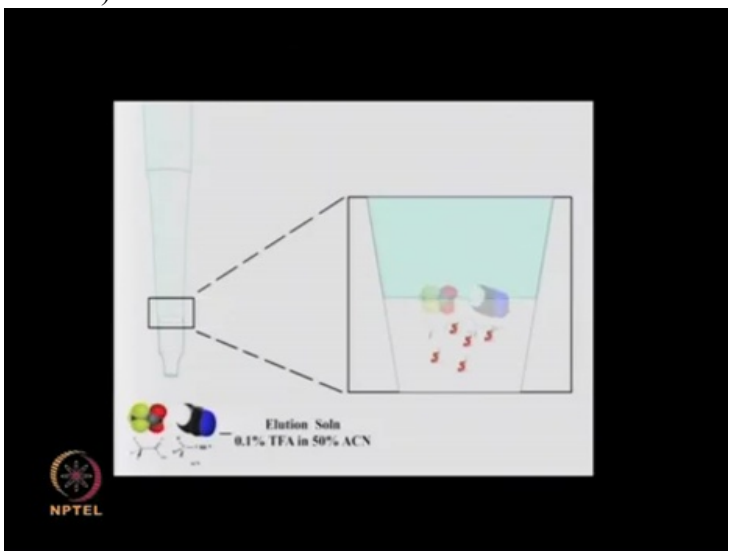
… through the activated ZipTip where there are captured in particular bed of chromatographic media. Now wash this C18 tip thrice with 10 micro liter …

(Refer Slide Time 24:17)

… of 0.1% TFA to remove the salt and other interfering components

So these steps ensure that salts and detergents are washed and finally samples can be eluted in very small volume of solvent.

(Refer Slide Time 24:44)



Now elute the sample from the ZipTip with 10 micro liter of 50 to 70% acetonitrile in 0.1% of Trifluoroacetic acid

(Refer Slide Time 25:03)

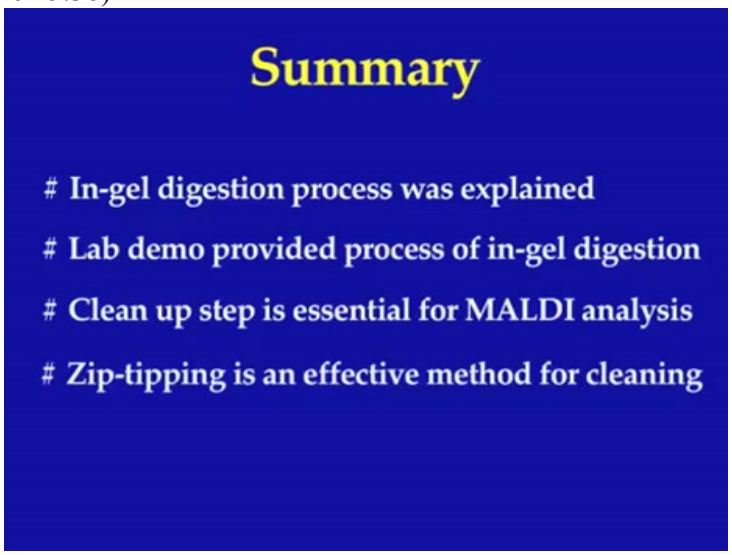
After the elution is done, then you can keep these process samples in cryoboxes …

(Refer Slide Time 25:17)

… and store in -20 degree Centigrade freezers.

In-gel digested samples which have been processed using ZipTip can be further analyzed by using mass spectrometer.

(Refer Slide Time 25:38)



(Refer Slide Time 25:41)