**An Introduction to Proteogenomics**

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**Supplementary Lecture – 9**
**Mass spectrometry – Sample preparation and analysis – Part I**

Welcome to MOOC course on Introduction to Proteogenomics. Today I am going to talk about mass spectrometry based proteomics and various steps involved in doing mass spectrometry based proteomics. Let us look at this workflow for shotgun proteomics.

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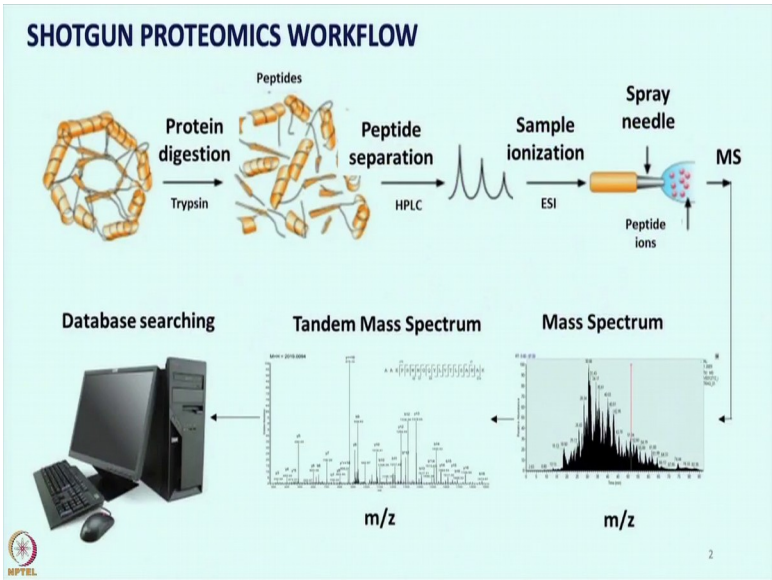


You have prepared the protein sample and now you want to analyze using mass spectrometry for protein identification or quantification. Very first step you would like to do the protein digestion using enzymes, like trypsin. Now, proteins have been digested and formed peptides. These peptides are separated using HPLC and then samples are ionized using ionization source like electrospray ionisation, from which these ions are now moving inside the mass analyzer and then you are generating these chromatogram based on the m/z values. You are using this information to search the databases, and then performing the protein identification, that is a nutshell of shotgun proteomics workflow.

Let us now go one by one. First you want to know that how much protein you have from the given sample. So, you need to perform the protein quantification. You can choose different

methods of protein quantification, but in the lab session we are going to show you Bradford quantification. Let us have this lab session.

So, now we have a bacterial lysate which we are going to quantify for how much protein is present inside the sample. So, for that what we are going to do? We are going to perform a Bradford assay, where I have taken different dilutions which is 0, 2, 4. Similarly, I had taken 6, 8 and 10 also, but I have I am just showing 3.

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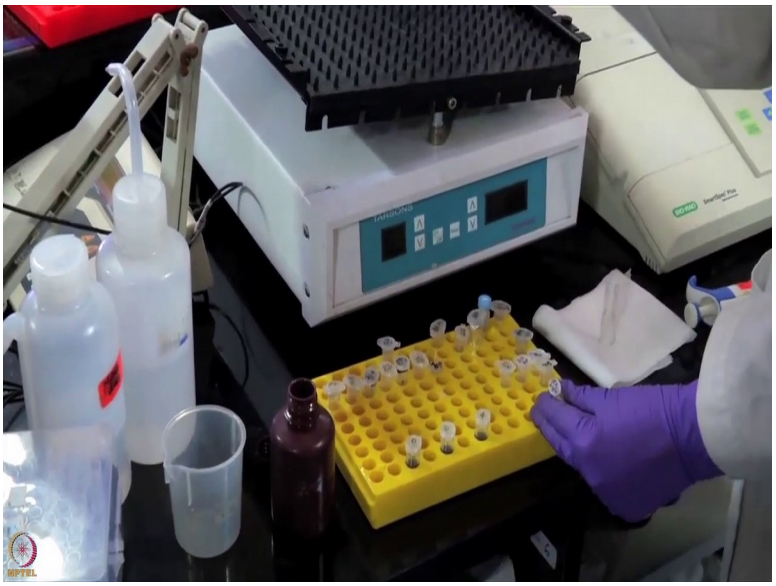

So, what we have to do? We have to add BSA which I have taken 2 mg per ml stock and I have added 1 micro litre, 2 micro litre, 3, 4 and 5 micro litre for different dilutions and then I had added 1 ml of Bradford reagent. So, this is 0 where I have not added any protein, I am just going to add Bradford reagent. Similarly, I will be adding means I have already added in different dilutions and then after adding we have to incubate it in the dark for 15 minutes. And after incubation then you have to take for 0. All these steps which I have done for standard I have also done it for the sample which I have taken again 2 micro litre and then similarly process with 1 ml of Bradford reagent and incubated for 15 minutes in the dark.

So, now we have to take the reading.

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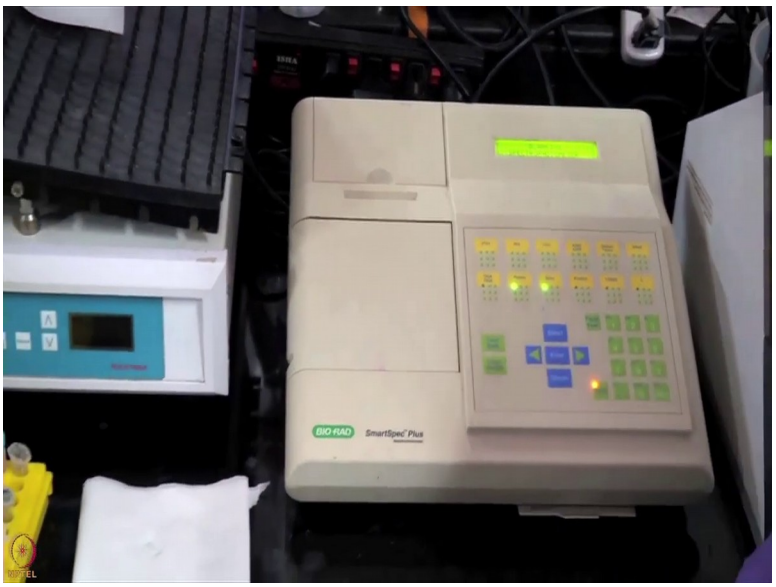


So, this is the photo spectrometer which we are going to use today. So, this is for the Bradford's assay that is why we are setting it at 595 nanometres. Now, it says to read the blank. So, we are going to use this cuvette, and here this portion as you can see there are two different faces, so we are going to use this face towards this arrow. Now, after placing it we will close it and press read blank, because this is the blank where no protein is present only Bradford's reagent is there. Similarly, we are going to take the reading for 2, 4, 6, 8 and 10 also, followed by sample.

Now, I will show you, I have no I am not going to show all the steps, now I am going to show you how you can plot the graph of by using these standards and then you can calculate the value of the unknown protein which you want to calculate.

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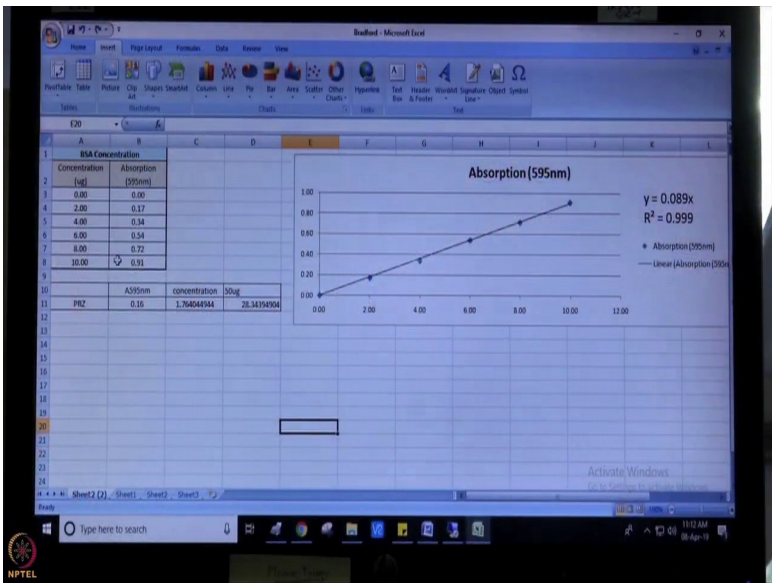


So, we have already made a table for the readings for this as this is 0 microgram where no BSA was added, only Bradford reagent was there. Here this is 2 micro litre, 2 microgram per micro litre, 4 microgram per micro litre, 6, 8, and 10. Similarly, for these, these are the following readings. And then what we have done? We have plotted a graph by using these values and we can see that the R square that is regression coefficient is equal to 0.99 which is reliable. Now, we will take the absorbance of the unknown sample which is approximately 0.16. Now, we will plot, we will put this value in this equation which is shown here on the right hand side. Now, what is this? This is our value for y axis. After substituting the value of y we will be able to find the value of x which we have got here as the concentration of the protein.

Now, you have quantified your protein and you are ready for the next steps. But before we proceed for the next steps, let us quickly recap what we are going to do in today's workflow of mass spectrometry based proteomics. I will try to give you again the recap of how a mass spec look, how we are going to run the samples and their visualization and finally, data analysis and interpretation.

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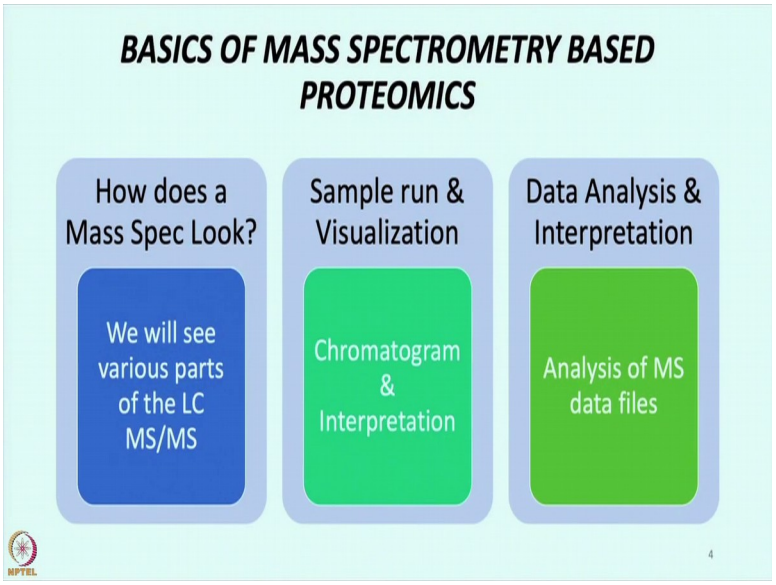


Let us now walk through the steps involved in a mass spectrometry based proteomic experiment. First thing, you have done the protein quantification. It is also good idea to do the protein quality check using SDS PAGE gel. Here you will get an idea that how good the protein pattern is, if the protein is degraded or you are only having bias of a given protein only the high abundant proteins are being seen on the gel. I think that sample prep is not good. So, you need to first do a visualization on the SDS PAGE gel and make sure that you are able to represent all type of proteins on the gel from the low molecular weight to high molecular weight, they are all very well distributed on the given gel and then now these samples looks good, your quantification looks good now you are ready for the next step.

So, once you are ready for the proceeding for the proteomics mass spectrometry based workflow, then you would like to do the protein digestion. One could use either in gel digestion or in-solution digestion. In-solution digestion means you are directly proceeding from the proteins in the solution form and with various steps involving reduction, alkylation as well as the enzyme treatment using trypsin overnight, you can do the in solution digestion or else from the same gel pieces you can also do the protein identification using in-gel digestion method.

If in case you have a complex lysate sample, you can still run on a on a given gel very short gradient, need not to run the whole gel and then excise that whole gel piece put you know cut that into 3 or 4 pieces and now you can use that to do the protein identification for the entire

proteome from that given sample. So, you are not looking at the specific band in this case, but if the goal was to identify a given protein which you have purified or which you are more interested, then you can just simply cut a specific band or take a specific spot. So, in-gel digestion can be helpful in many of these steps.

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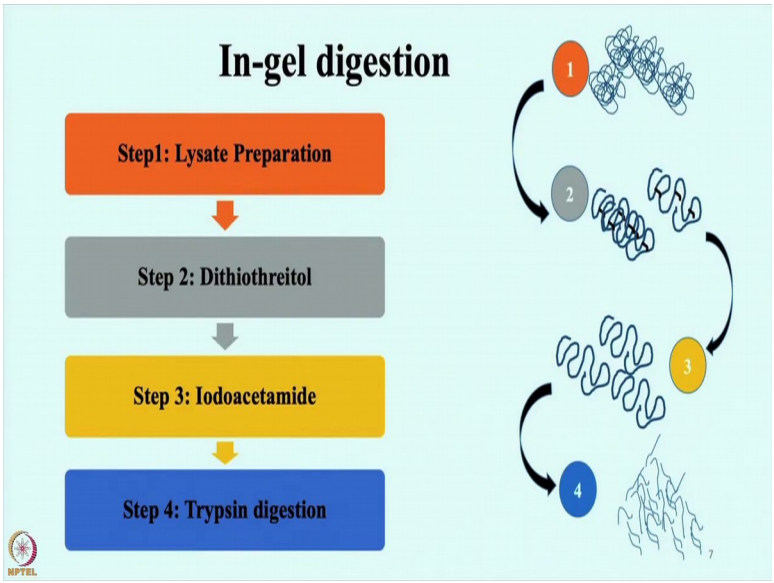


You will first do the lysate preparation, then do the reduction step using dithiothreitol. You do not want it to have the reoxidation step to again happen. So, you treat that with iodoacetamide or IAA and then proceed for the trypsin digestion, so that proteins can be chopped down to the peptides.

Let us now have the lab session where you will get more idea for the protocol used for doing in gel digestion.

So, now after running the SDS PAGE, now you have to stain and de stain the gel, so that the protein profile can be seen on the gel. So, for that we have already stained and destained the gel, you can see that this is the ladder and these are the different samples which we had run and here we want we are interested in all these 4 bands.

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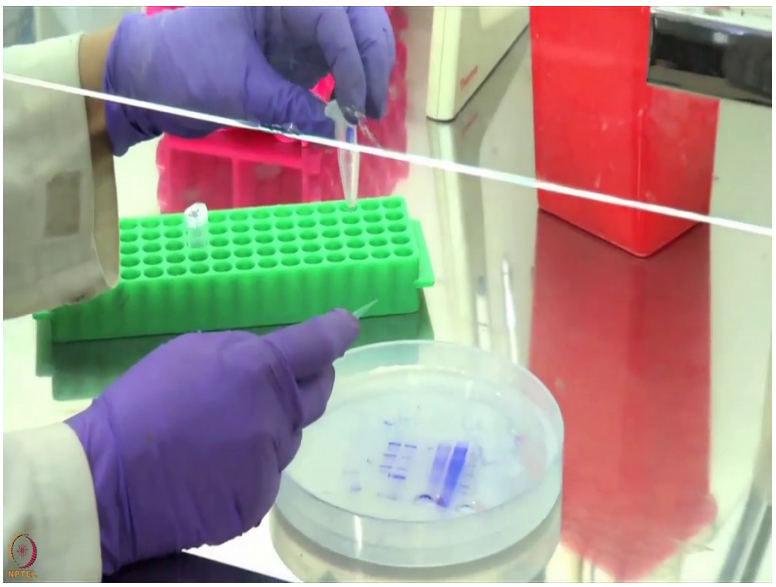

So, now, we have to excise the band and then we will I will show you how we can proceed with in gel digestion and then we can subject it to mass spectrometry to identify the protein which is present in the particular band.

Now, we have taken the gel here in the laminar airflow and we have taken the excision tubes in which we are going to cut the bands and keep the bands. So, now, let us see how to cut the bands. So, we are interested in this thick band. So, I am cutting this. Now taken the desired band. We can further chop the chop down this band and make it small into smaller pieces which I have already done and kept for you. So, you can see that we have to chop it fine, so that all the reagents which we are going to treat it with will be able to act properly on the gel pieces. So, for processing the band for in gel digestion by using trypsin we require these following solvents.

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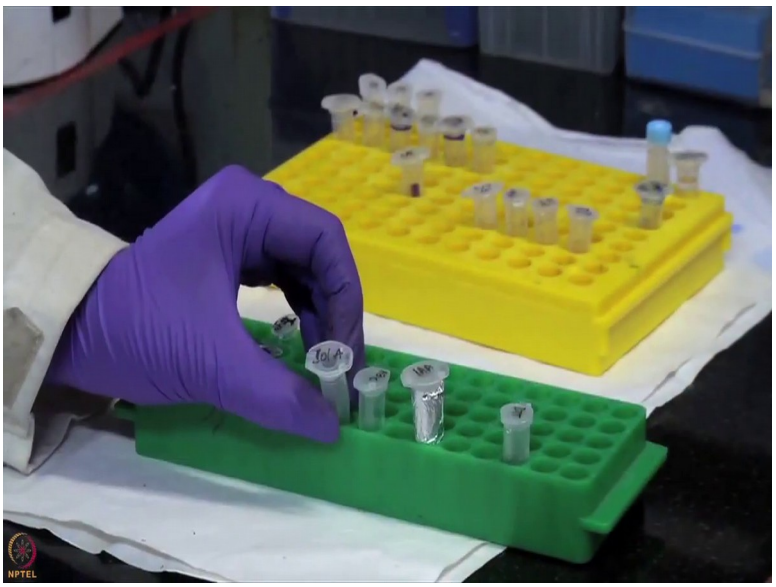

Solution a includes, acetonitrile and ammonium bicarbonate in 2 is to 1 ratio. Then ammonium bicarbonate, then IAA that is iodoacetamide and the last one is DTT. So, now, first of all we will add ABC to the excised band.

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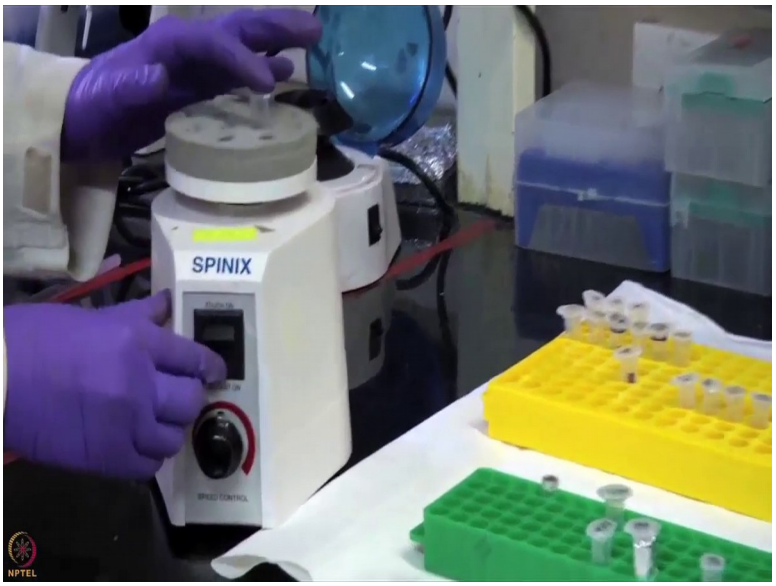


And now, we have to vortex it 10 minutes. But I am just going to show you the steps that is why I am not keeping it for 10 minutes.

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Then we have to give it a short spin. Then followed by this step you have to remove the ammonium bicarbonate solution and discard it. Then add solution A which is the mix of acetonitrile and ABC. Then again you have to vortex this gel piece for 10 minutes followed by a short spin. Again discard the solution, and follow this step for 3 to 4 times then after you have washed the gel pieces, then you have to reduce, then you have to add the reducing agent that is DTT, so that all the disulfide bonds can be reduced.

So, now, we are going to add DTT. So, this is the DTT. After adding DTT to the the gel pieces you have to incubate it at 37 degree Celsius for 1 hour, so that the DTT can penetrate and work on the gel pieces to reduce all the protein disulfide bonds. So, after the incubation you have to again discard the DTT, excess DTT which is there and give the wash by using ABC , and solution A. And after giving a wash by using ABC and solution A, you have to add iodoacetamide which is light sensitive, hence we have covered it with aluminium. After adding this we have to incubate it in the dark for half hour. So, after adding this IAA, you have to incubate it in the dark for 10 minutes. After incubation then you have to remove the IAA and again wash the gel pellets by using ABC and solution A.

Once you are done with washing steps then you have to add trypsin to the gel pieces in the dilution form whichever is appropriate for your experiment and then incubate this on the ice for half an hour. Incubation is done on the ice for, so that the trypsin can penetrate inside the gel pieces and can reach the proteins to convert it into the peptides.

Now, once the incubation is over then you have to submerge the gel pieces by using ABC, so that the gel pieces are not dried up completely before the action of trypsin is done. So, we have submerged the pieces by using ABC and now we can incubate this at 37 degree Celsius for 16 hours, so that the trypsin which has already gone inside the gel pieces can start working at its optimal temperature. Once the incubation for trypsin digestion is over then you collect the supernatant part in a fresh vial , then you further extract by using different concentration of acetonitrile to extract the peptides from the gel pieces.

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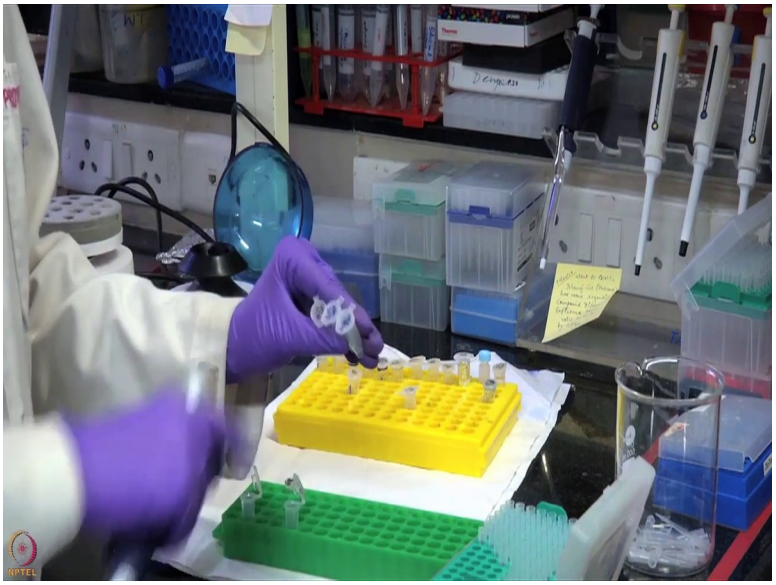

So, here I have used 40 percent, 80 percent and 100 percent ACN to extract the; to extract the peptides out of the gel. So, now, I am showing you the last step how we do this. So, you take 100 percent ACN, add to the gel pieces and again vertex it for 10 minutes. Once your vortex it for 10 minutes, then you have to again give it a short spin and then collect this solution as well to your peptides. Now, you have got the peptides in the fresh tube which you have taken, and now you have to dry this solution, so that you only have the dried peptides which you can store for a longer duration like 6 months or more, so that you can use it whenever it is required.

All right. So, now, you have done the in-gel digestion. Now, you are ready for proceeding for the mass spectrometry based experiment. But it is a good idea you should clean up your peptides and quantify them to make sure that you know, how much exact peptide you have before you go for loading in the mass spectrometer. You should not overload the peptide

because otherwise it is going to clog the column. You should make sure the sample is clean with the salts removed, otherwise again it will affect the column, it may block the column or you should not you know have the quantification errors because if you need to compare two samples you should load equal amount.

So, you know a general recommendation is that you could have if you have a clean sample peptide you can load almost 500 nano gram to 1 microgram that will give you decent proteome coverage. Of course, sometimes people go even 2 or 5 microgram, but that will be on the higher side that may affect your column as well. So, to do the peptide quantification you will reconstitute your sample which is peptide in the dried form.

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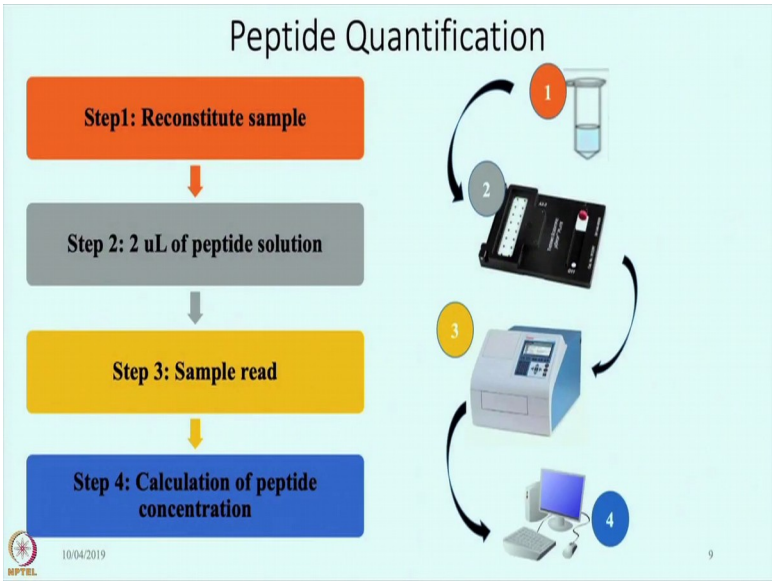


Now, you will take two microliter of peptide in the solution, do the absorbance reading and then do the calculation of peptide concentration using a very simple as scopes method. Let us now have a lab session where you will be exposed to do the peptide quantification.

So, till now you have seen that we have extracted the peptides out of gel pieces and we have dried the peptides. So, now, we have the dried peptides with us by which we have dried using speed vac, and now we have to reconstitute the dried peptides by using 0.1 percent formic acid which I am taking approximately 20 micro litre and then you have to reconstitute it by doing, by performing vortex. The amount of reconstitute, the amount of FA to use for reconstitution depends on the concentration of peptides you had loaded on the gel and you

have collected after extraction. So, once this is reconstituted now we have to go for peptide quantification.

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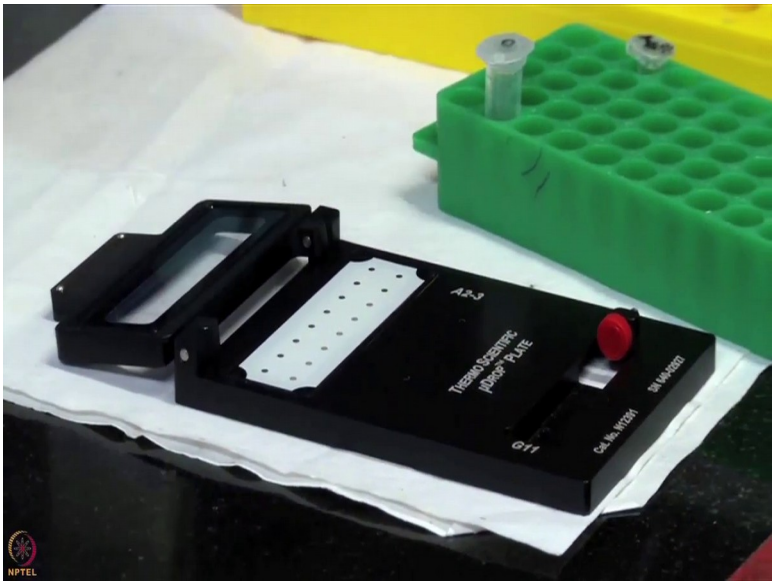


So, now on the very first well you have to load only formic acid which you had used for reconstituting your peptides. So, I am loading 2 micro litre of formic acid, followed by reconstituted peptides in formic acid as the test. Now, we have loaded the sample on this. Now, will go, now we can cover this. Now, we can go to the instrument which is used to read this plate. After spotting this on the, after spotting the peptides on the plate now we are going to use this machine which is known as multi scan go for reading the peptide concentration.

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So, now we are using this particular software which is known as Scanit software 4.1 for micro plate reader.

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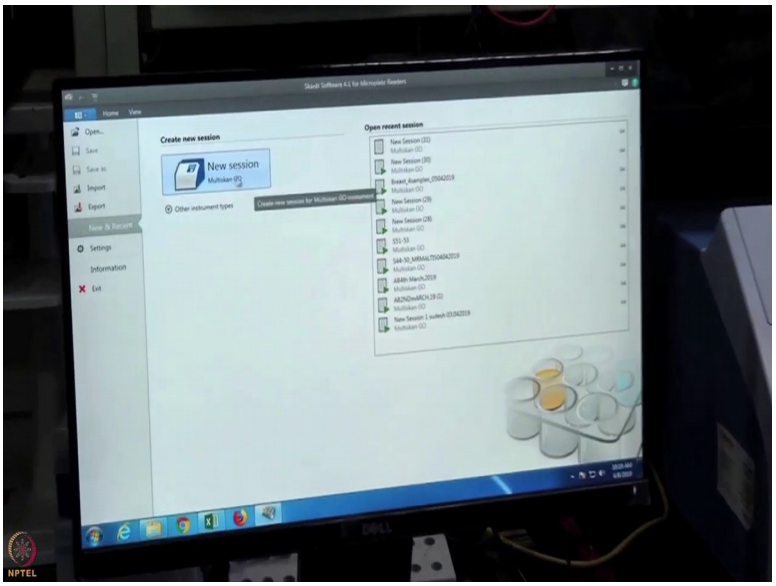


So, we will go on the new session.

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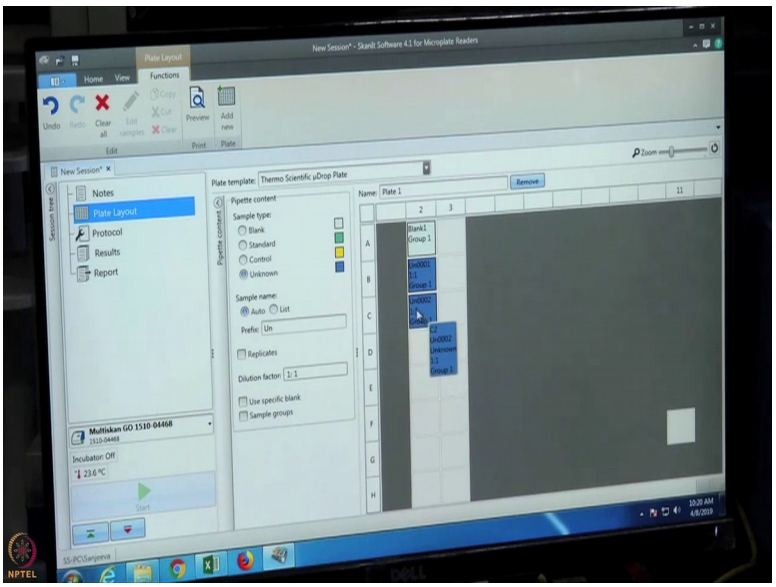


Now, we have to design the plate template and the one which we are using is micro drop plate, hence we will select this. Now, we know first is the blank which is containing only 0.1 percent FA. So, will mark it as blank and the second is our unknown sample which we have marked it as unknown. Now, we have taken it in duplicate that is why we are again marking the other one as unknown.

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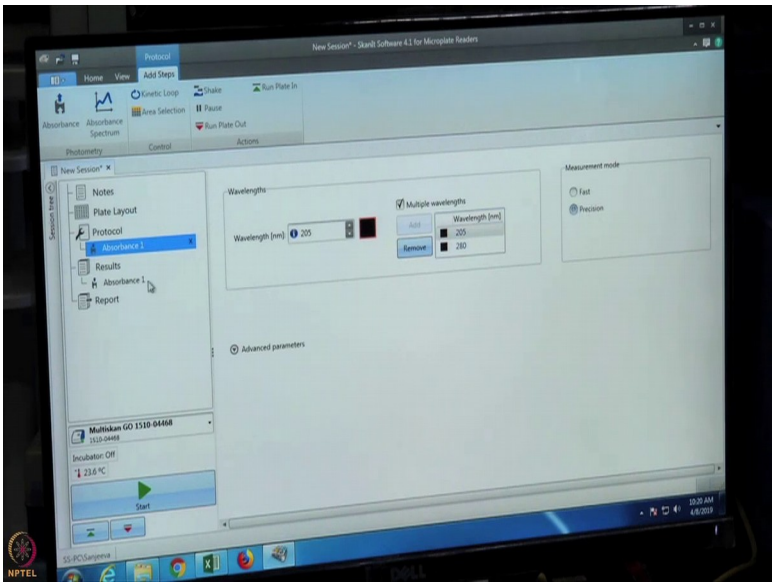


Now, we will go for the protocols. Click on the protocol we can select different absorbance. So, we are selecting two different absorbance that is 280, we have added this now 205. Now,

we are going to measure the peptide concentration by using these two different wavelengths and we will go for a precision method.

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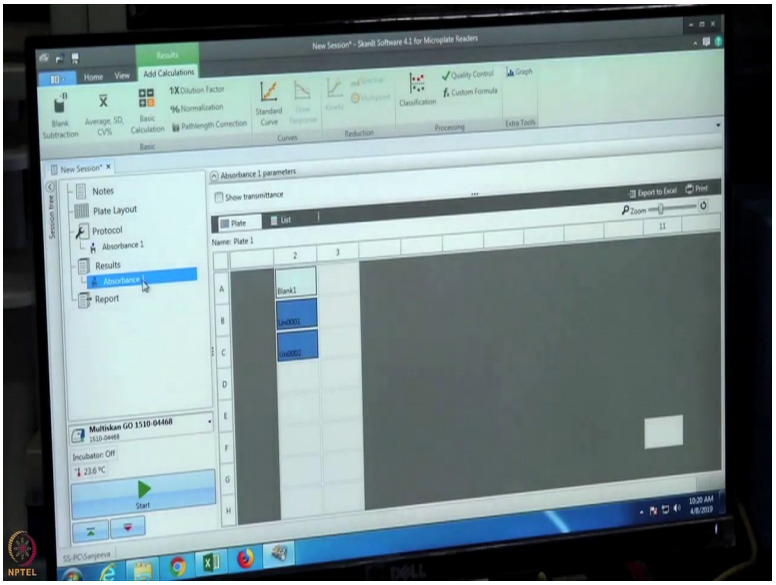


Now, here we have to go for blank subtraction, so that the software itself will go for a blank subtraction value and will only get the sorted values. Now, we will keep the plate inside the plate reader, so this is the button which we can use to bring the plate out. Now, here in this slot we can keep the plate in this position and then. Now, here we can start the read by clicking on the start. So, it takes some time.

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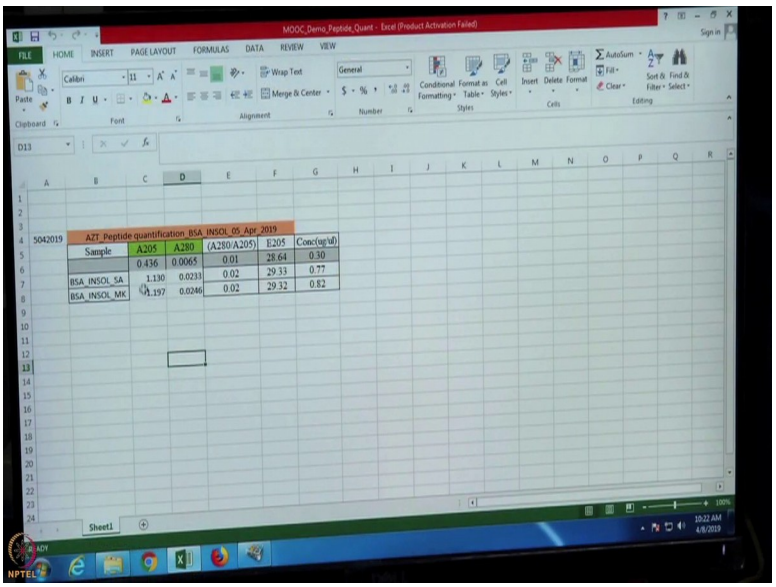

Now, we have already written the values of 205 which is blank subtracted and again , 280 which is again blank subtracted. By using Scopes method we have calculated the concentration of the protein which is present in the form of microgram per micro litre. So, this is how one can calculate the peptide concentration which we have started from the very beginning by digesting the peptide using in-gel digestion, desalting it and then we had quantified the peptides. So, now, by using this concentration of peptide we can inject 500 nanogram to 1 microgram of the peptides in the mass spectrometry.

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