Mass Spectrometry Based Proteomics
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Mod 03 Lecture Number 15

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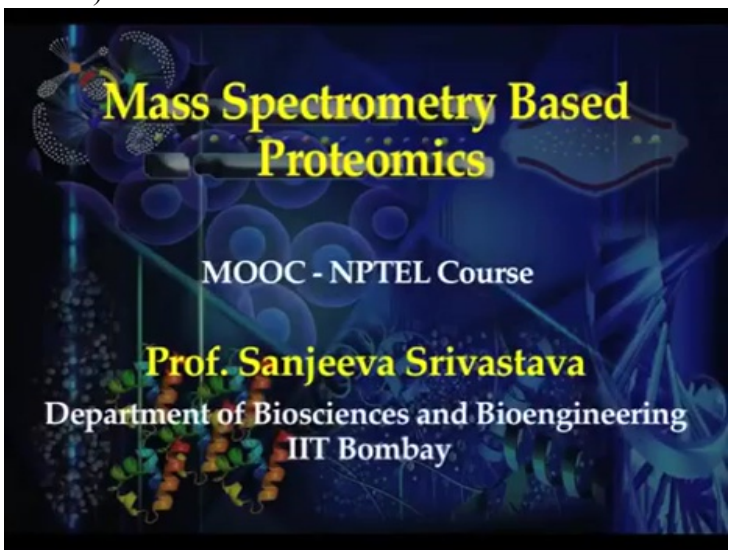

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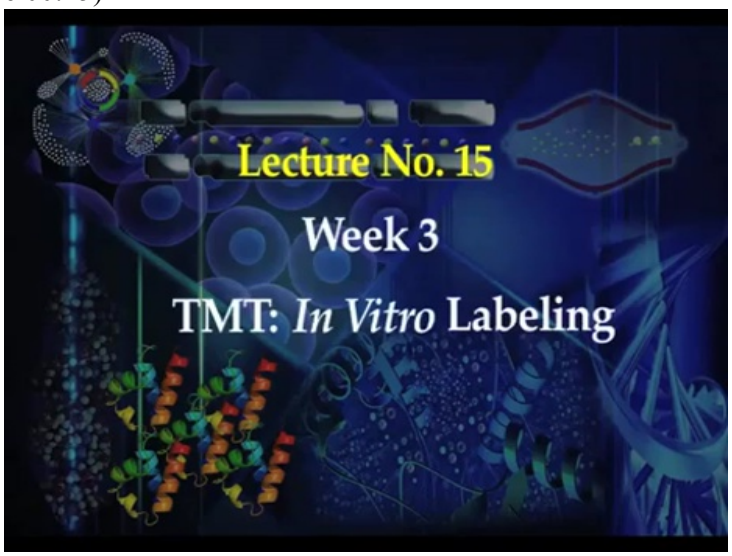

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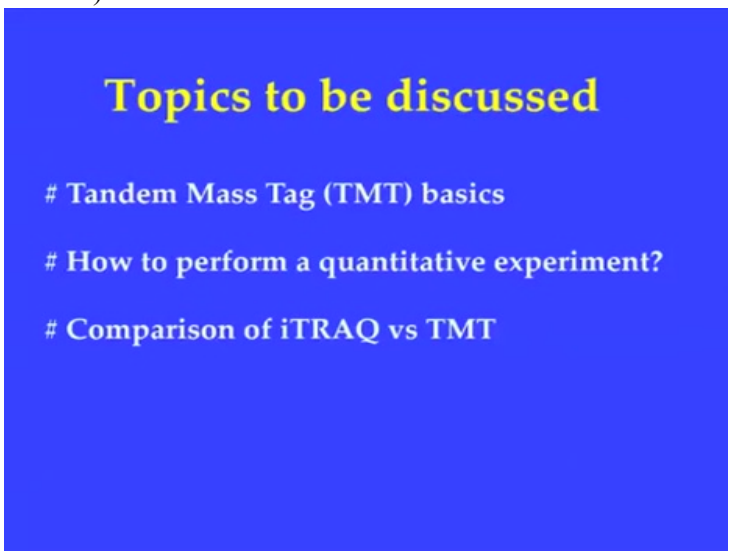

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Tandem Mass Tag or TMT, this method is similar to the iTRAQ which we just discussed. TMT is also MS/MS based quantitative technique

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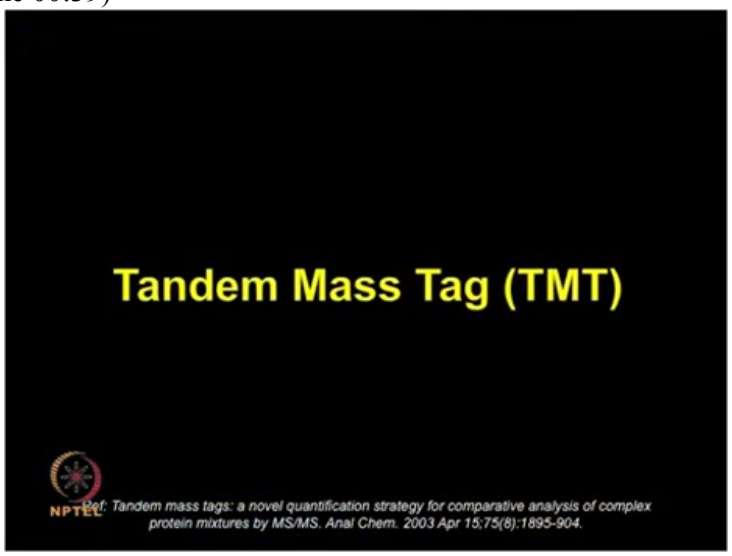

…which uses the isotopomer labels referred as Tandem Mass Tags It also provides the accurate quantification of peptides and proteins.

The Tandem Mass Tags have been developed by the Proteome Sciences and currently commercialized by Thermo Fisher. I have given you the reference for the original study on Tandem Mass Tag in this slide.

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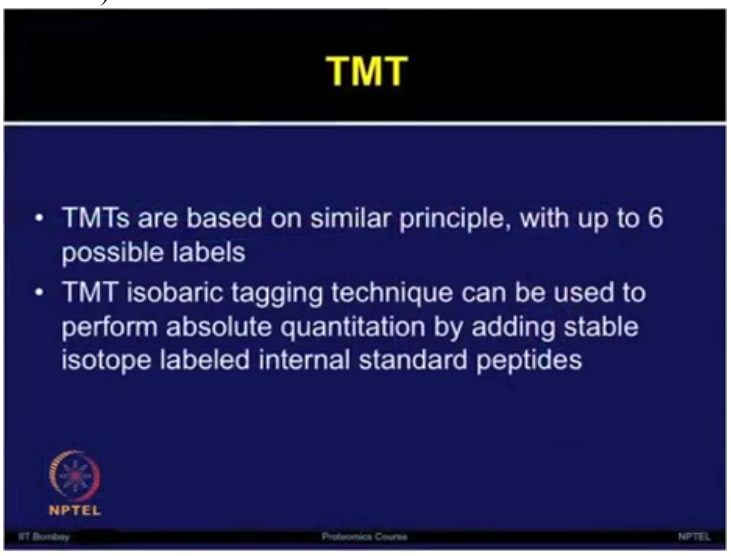

So these Tandem Mass Tags, they are based on the similar principle of iTRAQ. Here the possibility for multiplexing is up to 6 possible labels.

The TMT isobaring technique can be used to perform absolute quantification by adding stable isotope-labeled internal standard peptides. It can be done by comparing the peptides from a target protein to a known amount of labeled standard peptide spiked into sample. In that way the absolute quantification can be obtained.

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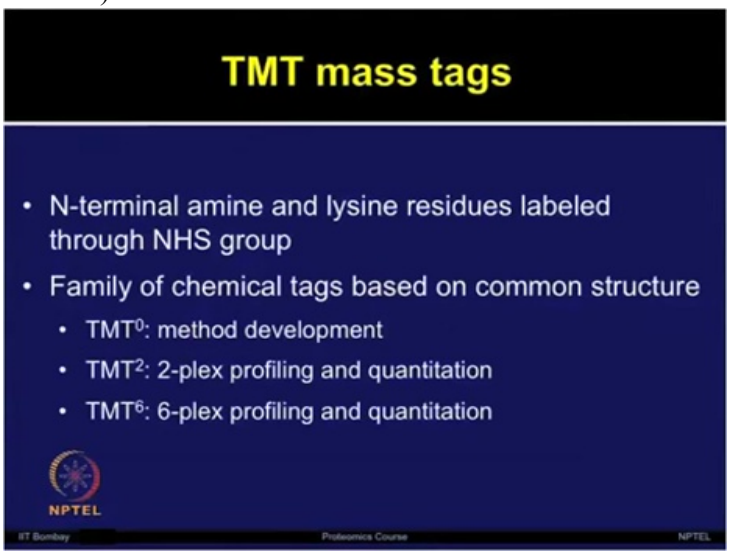

The N-terminal amine and lysine residues are labeled though NHS group. There are family of chemical tags which are based on common structure. The series of TMT tags available TMT0, TMT 2-plex and TMT 6-plex.

So these TMTs are an innovative set of isobaric mass tags for labeling the proteins and peptides at amine functions and mixing of up to 6 different protein samples are possible.

While duplex and 6-plex labels TMT differ by the number of isotopic substitutions, TMT0 is non-isotopically substituted structure that has been produced for only method development.

During the MS/MS analysis, the TMT tags give rise to 6 reporter ions from 126 to 131 Dalton, therefore it allows for the relative quantitation. In TMT 6-plex, each tag adds a mass of 229 Daltons per labeled amine to the protein. The TMT duplex and TMT0 share the TMT complex structures.

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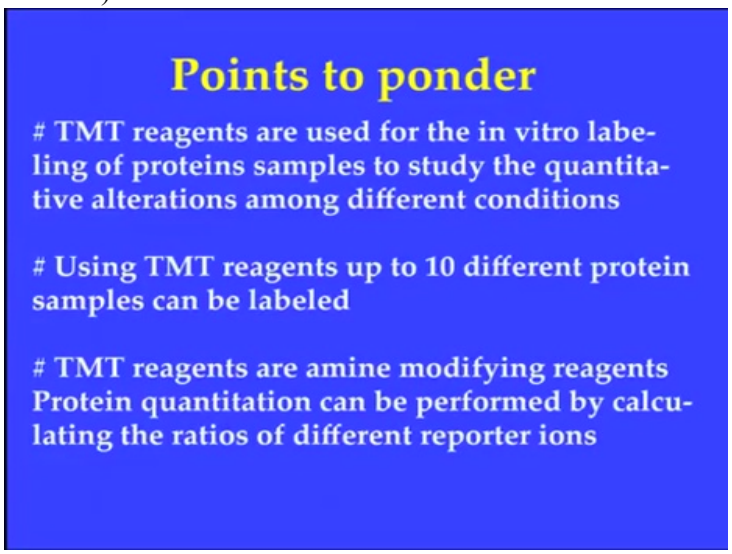

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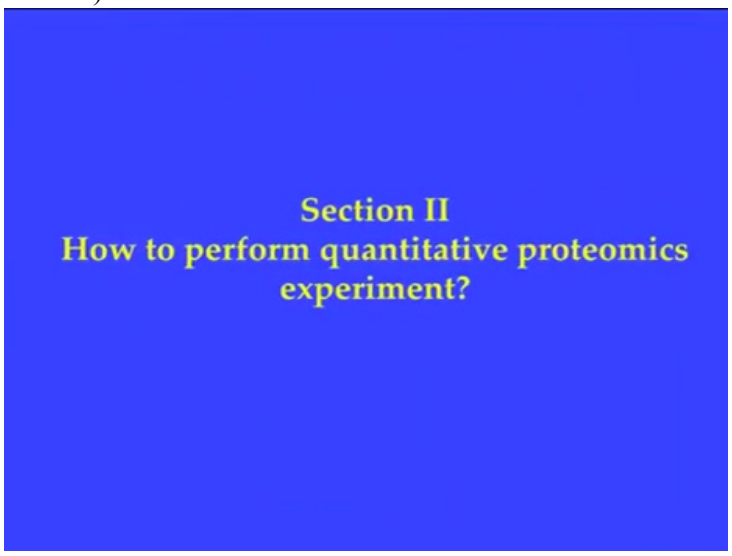

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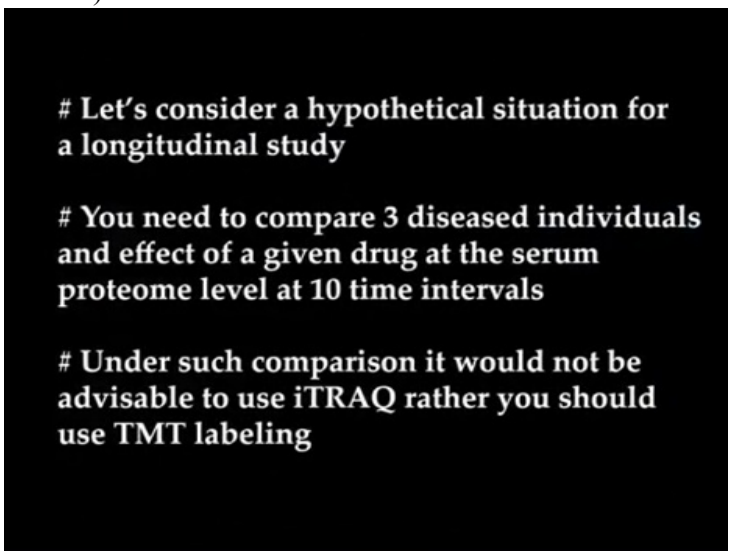

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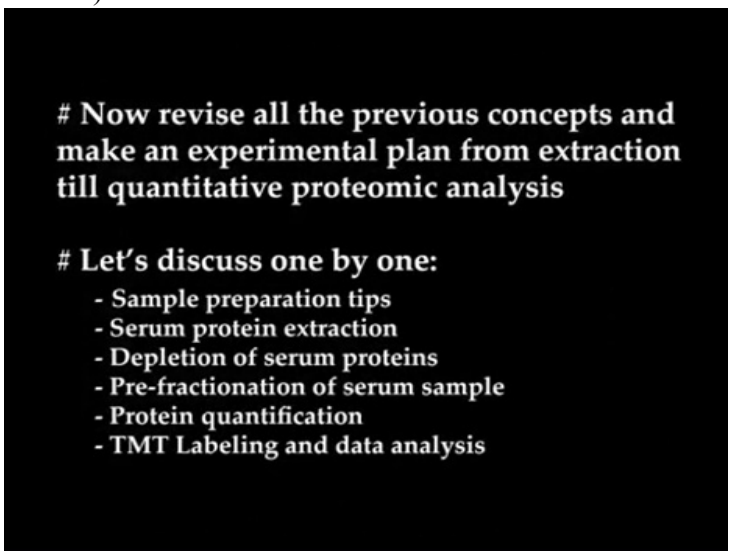

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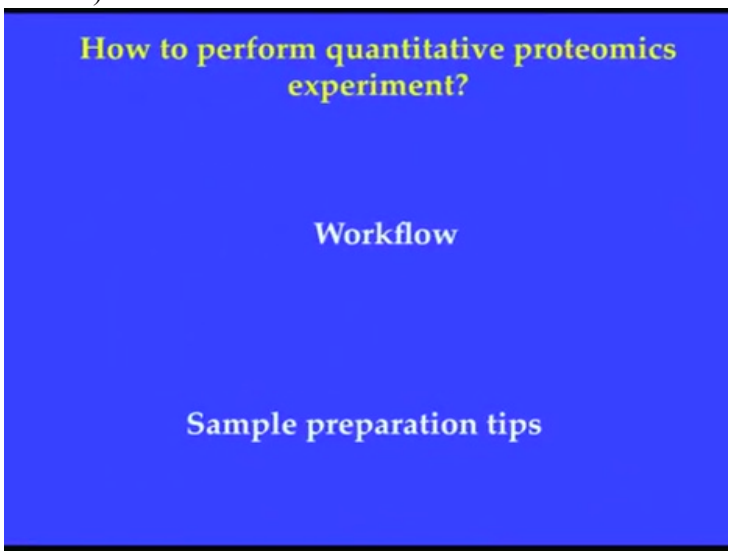

Proteome is very complex. Whether you want to perform your proteomic analysis for the whole organism

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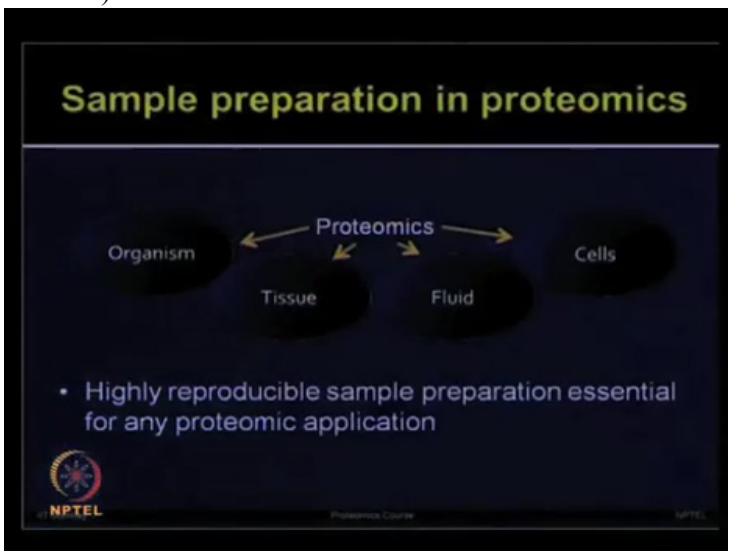

it means you want to know all the proteins present in a given organism or in a tissue or in body fluids or in different types of cells.

Proteomics can be global or it can be very targeted or expression based. So, very highly reproducible samples are very important for performing comparative proteomic analysis.

If you want to know the difference in your sample as compared to the controls, you need to ensure that your sample preparation is very reproducible. If you introduce some artifacts to begin with, then obviously you are not going to identify reproducible biological changes.

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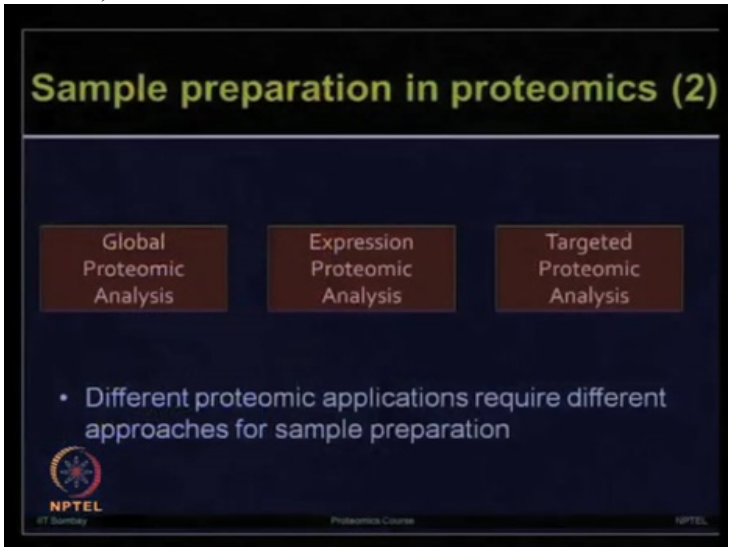

So let me give you three different terminologies here for proteomic analysis; one is global proteome analysis, expression proteome analysis and targeted proteome analysis.

When I am talking about global proteome analysis, it means your aim is to characterize all the proteins present in the given sample.

Expression proteome analysis, it means you are mainly interested to look for those changes which are due to any chemical or your treatment, those are induced; either going up or down, the protein amount is changing.

So the protein expression analysis, that is most commonly used for various types of clinical and different studies.

Targeted proteomic analysis; if you are very focused for a given organelle or a given sample type often you would like to know what is happening in that particular proteome, for example mitochondrial proteome.

So, one need to try different types of strategies when thinking about performing a sample preparation; What is your objective; whether you want to do global profiling or you want to do expression profiling? In either case you need to extract all your proteins present in that particular target sample.

Now when you are looking at targeted proteome analysis, you just want to pre-fractionate your sample in such a way that only that particular component is isolated and all the proteins from that organelle or cell is being extracted. So, different types of strategies need to be used to perform these types of proteomic analysis.

Now all of this sample processing involves solubilisation, denaturation, reduction and treatment of sample proteins.

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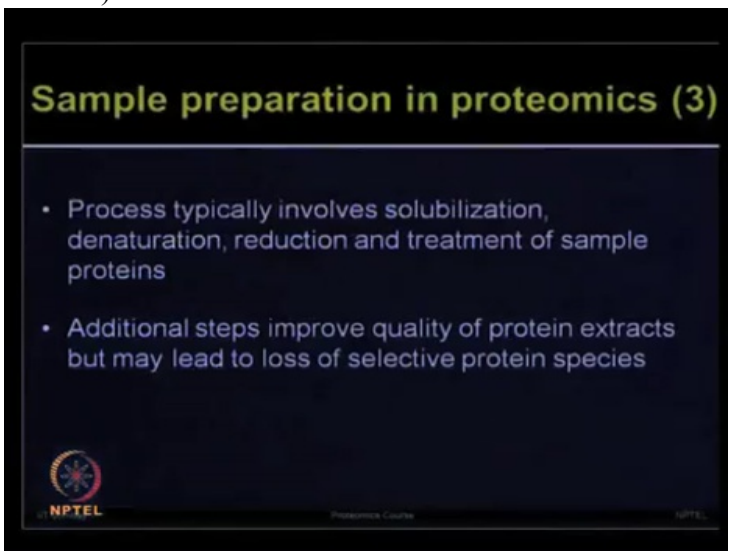

But you need to involve additional steps depending upon the type of samples and your type of objectives so that the protein quality, the protein extract can be improved. And while you are doing this you have to be very cautious that, when you are performing various steps and sequential types of extractions you may also lose a small fraction of the proteins.

So, one has to be careful when adding various additional steps during the sample preparation. Now protein extraction protocols, they need to ensure that most, if not all the proteins, in a cell or organelle are extracted.

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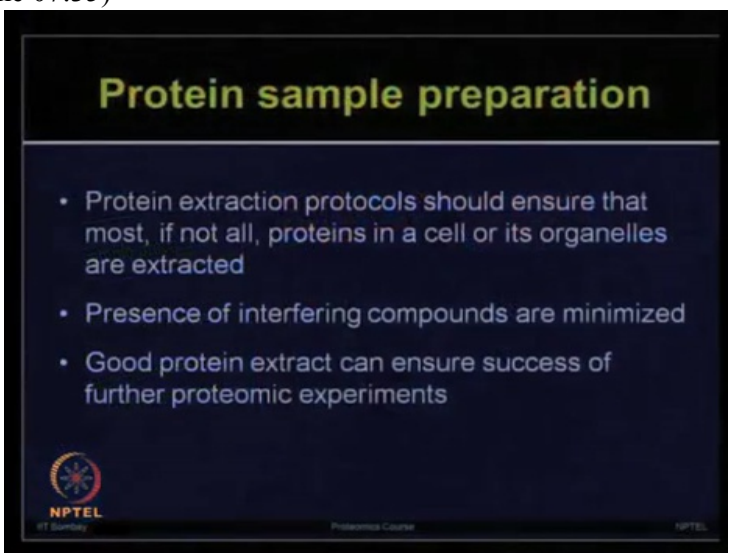

The presence of interfering compounds should be minimized. So if you have optimized a very good protein extraction procedure that should ensure that you have a very wide

proteome coverage and that is ultimately going to determine the success of your proteomic experiment.

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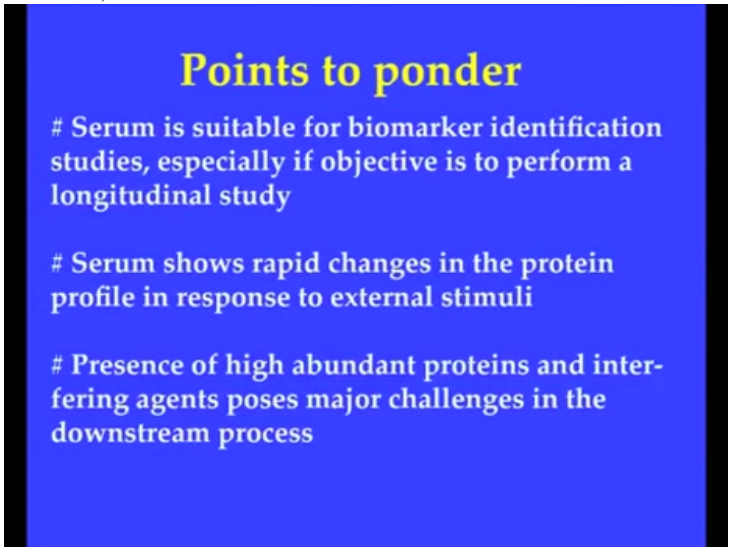

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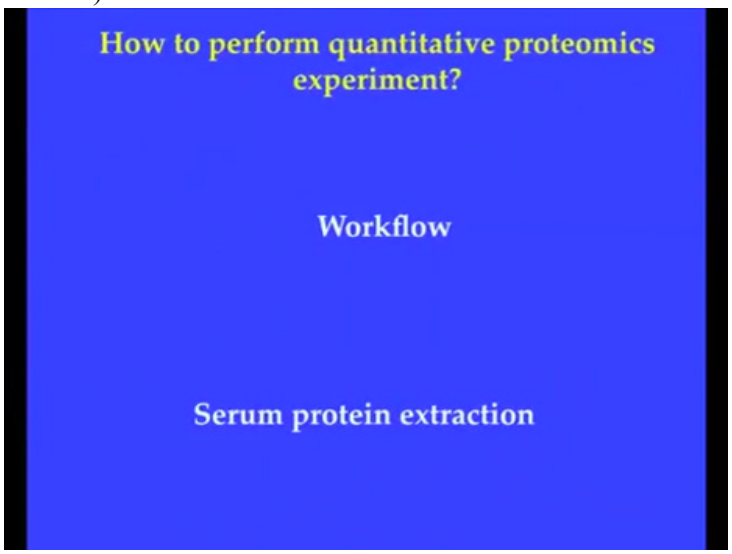

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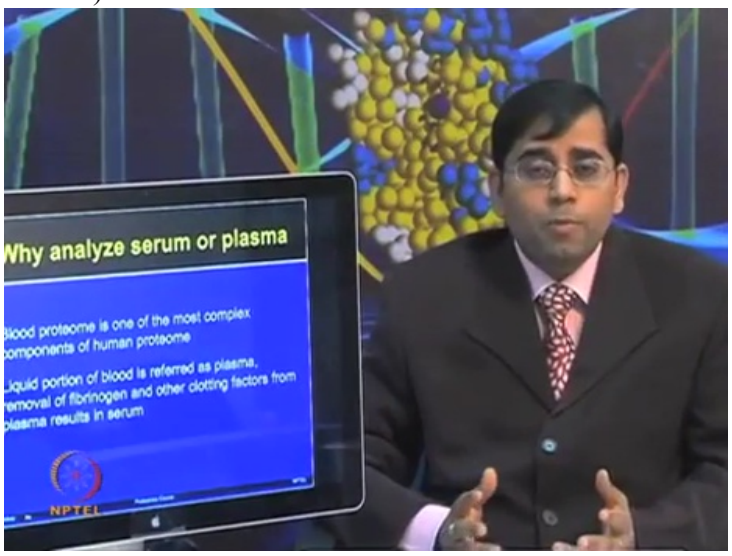

You may ask why to analyze serum or plasma for any proteomic applications.

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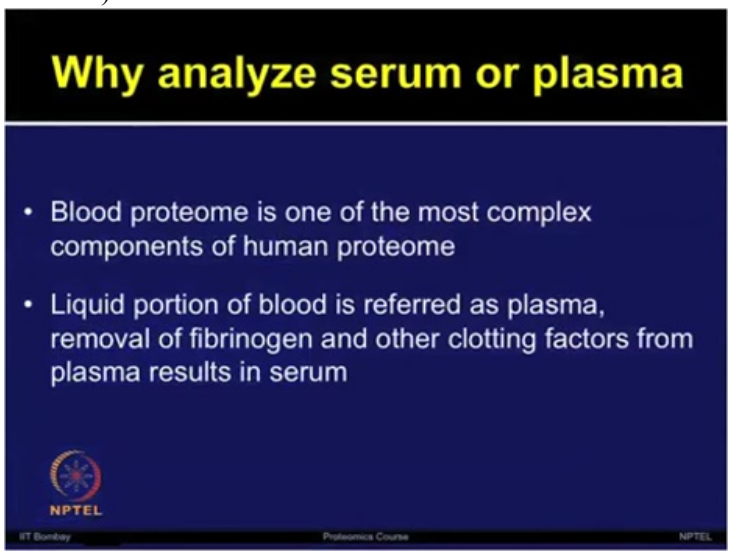

As you know, blood proteome is one of the most complicated components of the human proteome. The liquid portion of the blood is referred to as plasma and the removal of fibrinogen as well as other clotting factors from the plasma results into serum.

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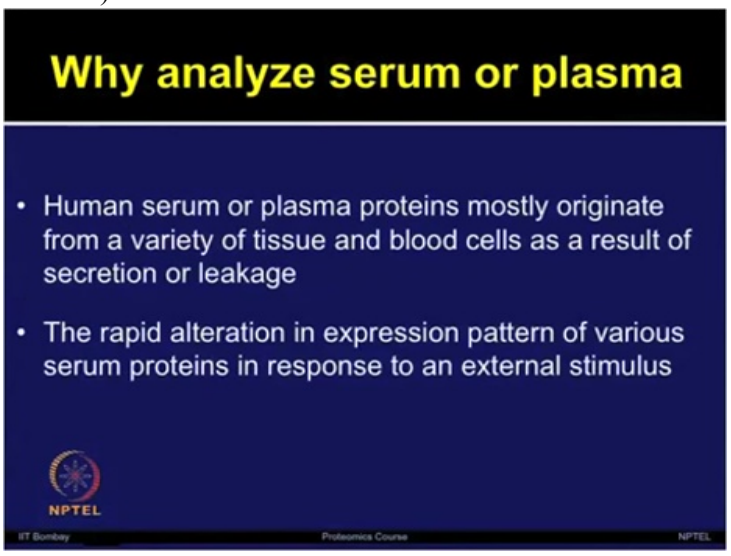

So human serum or plasma proteins, they mostly originate from a variety of tissue and blood cells as a result of secretion or leakage from the neighboring tissue or the blood cells.

The rapid alteration in the expression pattern of various serum proteins due to response of a disease condition or an external stimulus is true reflection of physiological conditions occurring in an individual.

So to get the feel about all the physiological changes happening in a patient due to a disease, people analyze serum or plasma proteome very oftenly.

Because blood removal for various types of tests are being performed in clinics, so blood is very easily accessible sample and performing serum or plasma proteome becomes very easy as compared to dissecting out tissue for further analysis.

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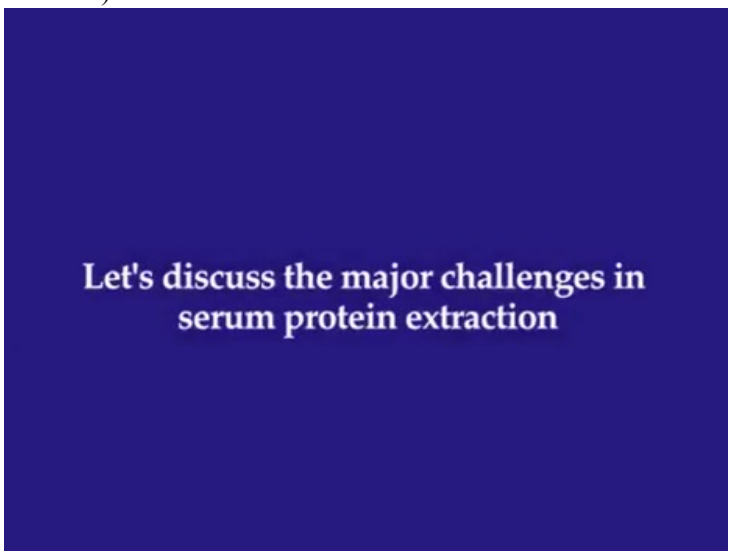

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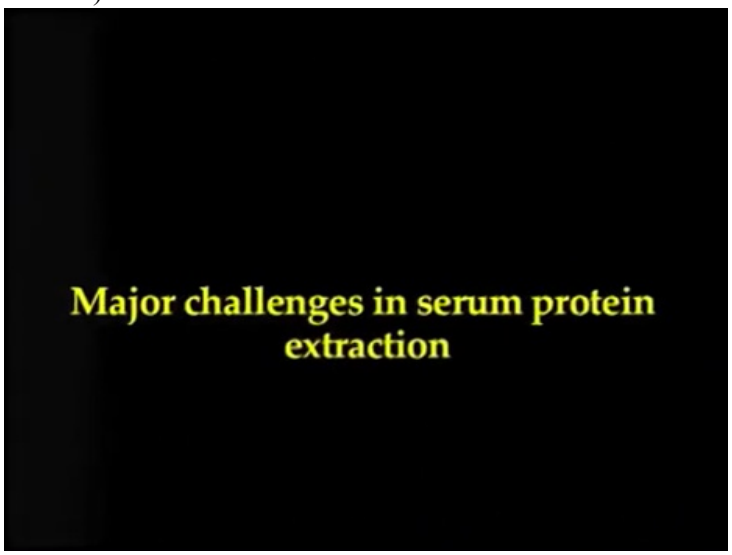

Although sample removal is easy but sample analysis, the serum or plasma proteome analysis is not so easy. There are major challenges in serum or plasma proteome analysis.

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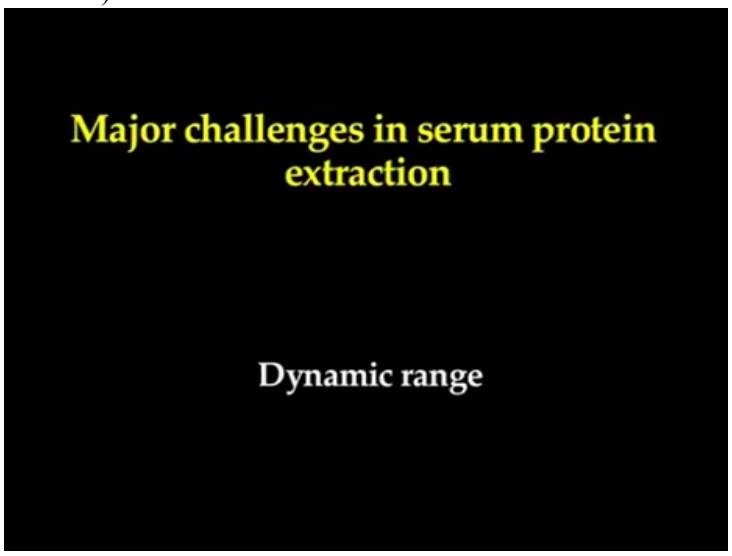

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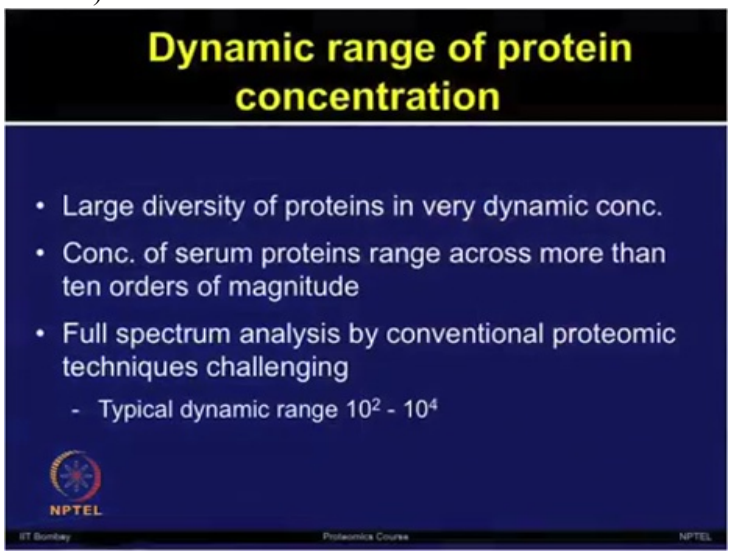

Let's talk about some of these challenges point-wise.

The first point, the dynamic range of the proteins concentration; in serum there is a large diversity of proteins which provides a very dynamic environment of almost 10 to the power 10 magnitude.

So the concentration of serum proteins ranges more than 10 orders of magnitude. If you want to obtain the full spectrum of serum or plasma by applying any of the conventional proteomic techniques, it is very challenging because the typical dynamic range for any of these

techniques, any these platforms will be much smaller between 10 to the power 2 to 10 to the power 4.

So how to capture all the dynamic events which are happening in the serum if the dynamic events in the serum are very large and your detection techniques are not able to capture the whole dynamic range?

So to avoid these issues people try to remove some of the abundant proteins from very complex serum proteome so that overall, the dynamic range can be reduced and minimized.

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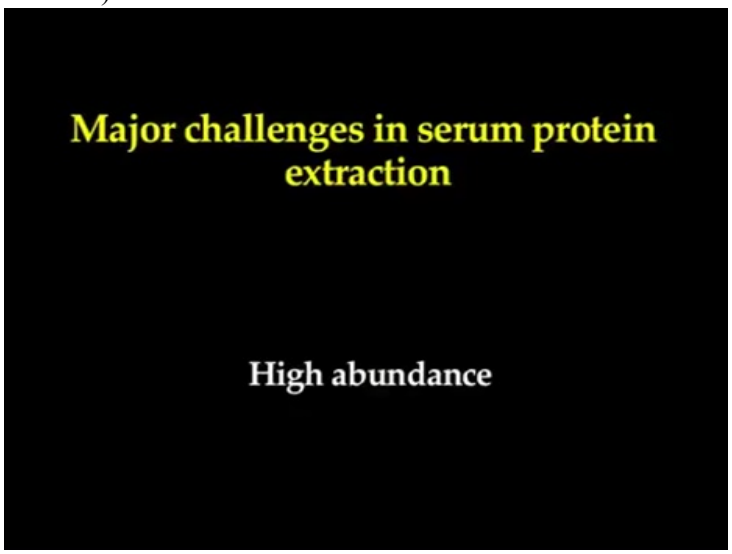

So second point, the high abundance proteins; there are different high abundant proteins which are present in serum and plasma which makes its analysis very, very complicated.

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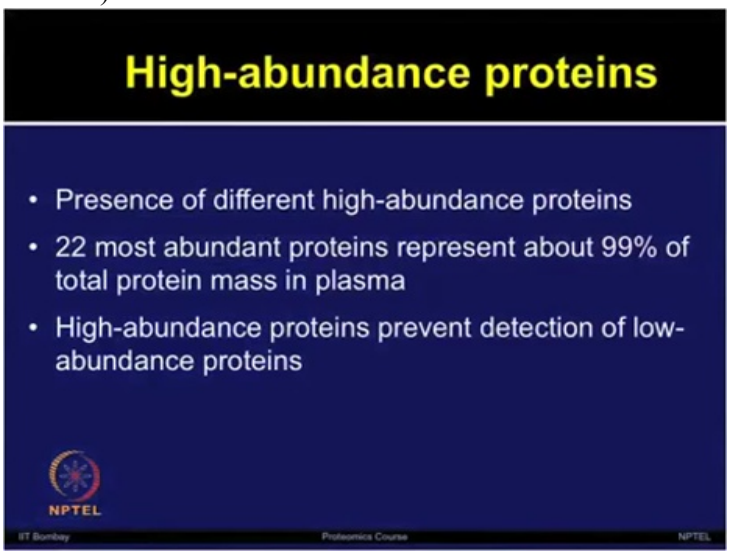

There are almost 22 highly abundant proteins present in serum, which represent about 99% of total protein mass of serum or plasma. These high abundance proteins prevent the detection of very low abundant proteins and often these low abundant proteins are target which you are looking for as a part of biomarker discovery.

So, how to get rid of the high abundant proteins? I will describe some of the strategies which can be used to remove high abundant proteins in the next couple of slides. But first let's talk about which are the other challenging factors for the serum or plasma analysis.

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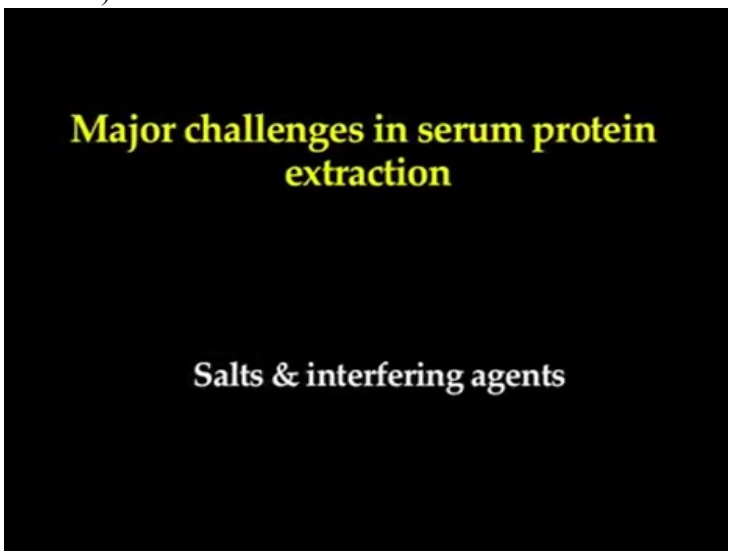

The third point,

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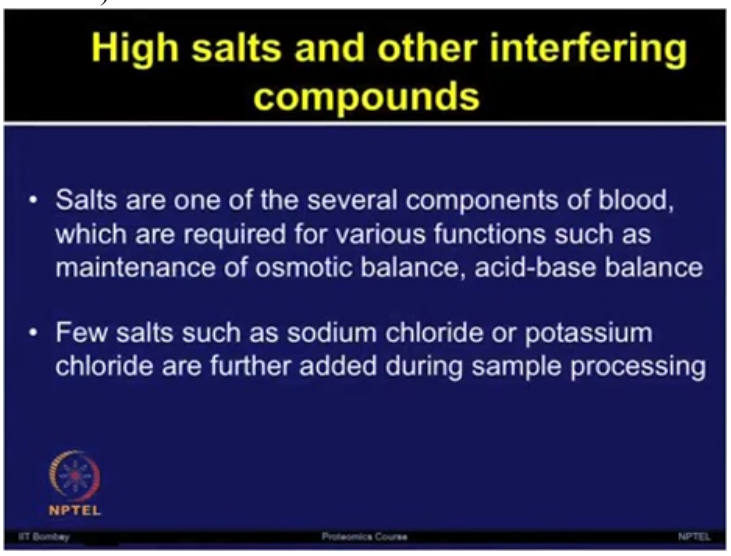

the presence of high salt and other interfering compounds.

As we have talked about different types of interfering compounds, so now you are familiar that salts are one of the several components which could be very much interfering during your proteomic applications.

Now salts are present in the blood which are required for various functions such as maintenance of osmotic balance, acid-base balance etc. Few salts such as sodium chloride or potassium chloride are also added when you are processing the serum sample.

During this whole sample manipulation, due to the intrinsic salt present in the blood as well as extrinsic salt added during the sample processing, the overall salt component becomes very high and that creates problems for various types of proteomic applications.

So, high salt and interfering compounds should be removed.

Now this presence of excessive salts, detergents and other contaminants can tremendously influence the electrophoretic separation of proteins. If your target technology is two-dimensional electrophoresis or other gel-based method you have to really ensure that salt is very low in the serum or plasma components.

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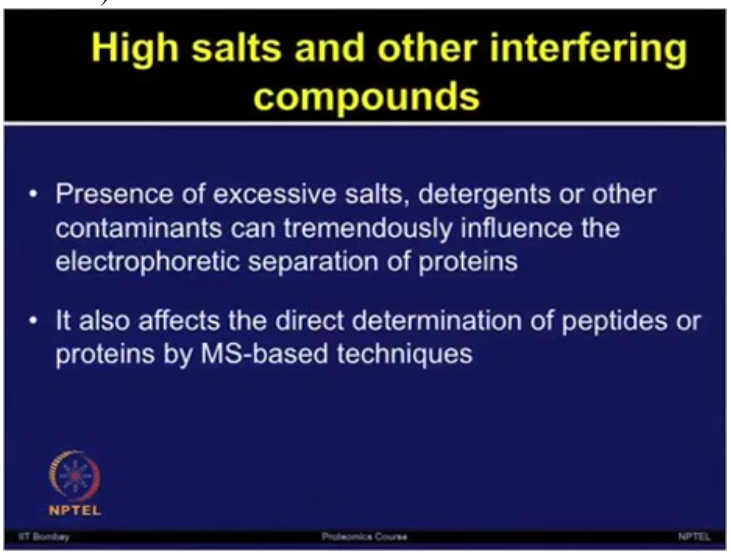

It also affects the direct determination of the proteins or peptides by mass spectrometry-based techniques. So regardless of whether you use gel-based or gel-free method, you have to ensure that the overall salt component is removed efficiently from the serum or plasma.

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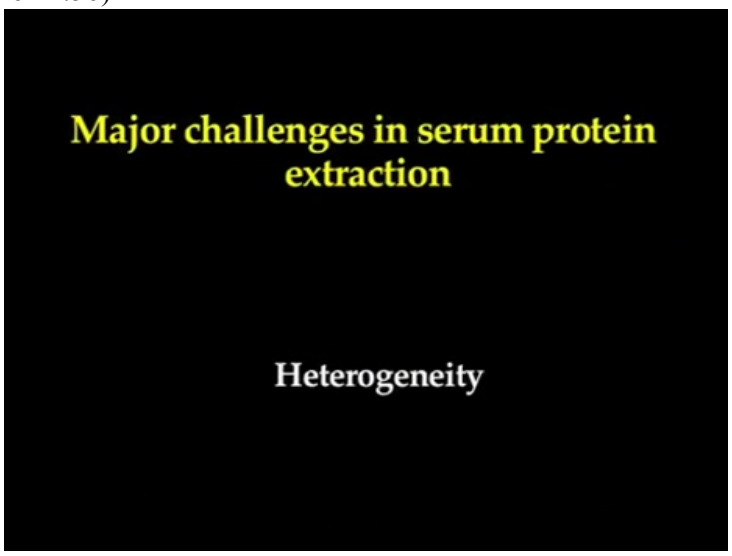

Now let's talk about the fourth point, other challenge. So variations among the individuals and lack of reproducibility; these are some of the very major issues in clinical studies, where you have inter- and intra-individual variations.

Intra-individual variations are obviously more expected but even within one person due to the diet, due to different type of medication, the serum or plasma proteins can be changed.

So how to avoid these inter- and intra-individual variations and if you refer to the previous lecture when we talked about how to minimize different types of these factors which are going to ensure the success for clinical studies.

Probably you will be able to keep an eye on different type of extrinsic and intrinsic influences which one should try to remove as much as one can during the clinical proteomic analysis.

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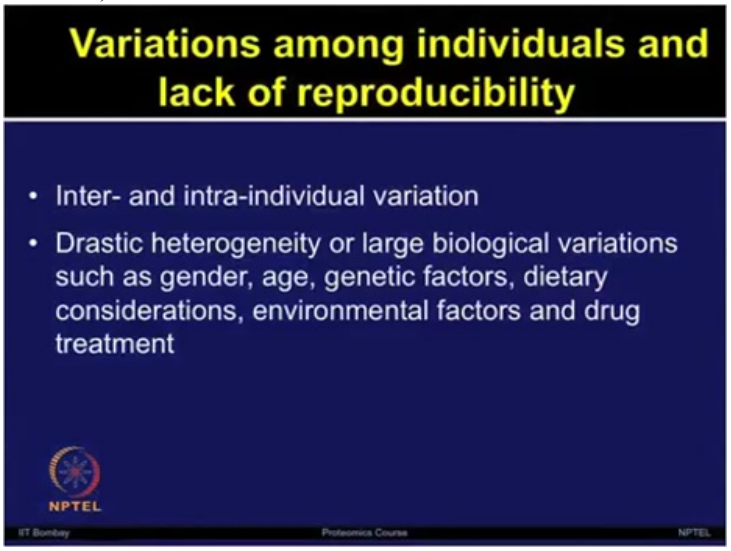

So the drastic heterogeneity or large biological variations such as gender, age, the genetic factors, dietary considerations, environmental factors and drug treatments are going to affect the reproducibility of your experiments.

So if you are careful in designing the experiments, one can at least minimize these variations and increase the reproducibility of the proteome analysis.

So now I am giving you an overview here for the serum sample preparation.

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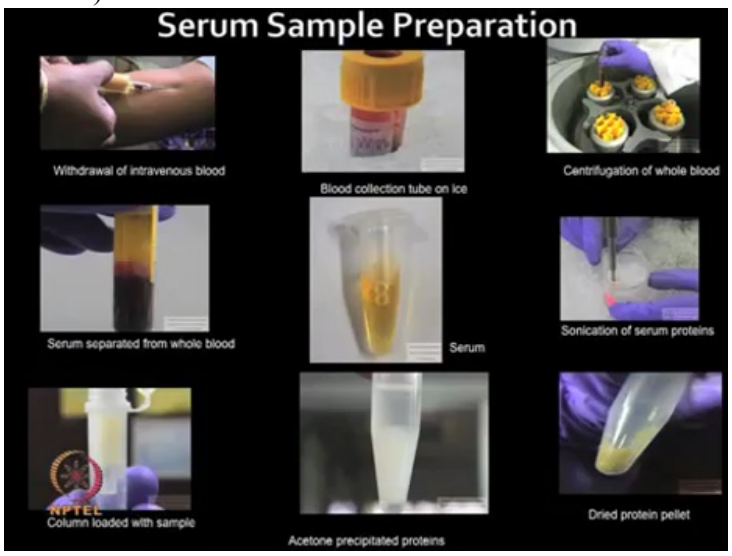

The various steps are shown in the images such as withdrawal of intravenous blood, blood collection in the tube, centrifugation step of whole blood, how to remove the serum from the whole blood, serum can be transferred in to fresh tubes.

Now for the serum proteome analysis different types of modifications can be performed such as sonication can be used for the disrupting the high abundant proteins and it also helps in better resolution.

Then depletion strategies to remove the abundant proteins, precipitation of these proteins by adding acetone and then drying out the protein pellet and reconstituting for the... in suitable buffer for the further proteomic applications.

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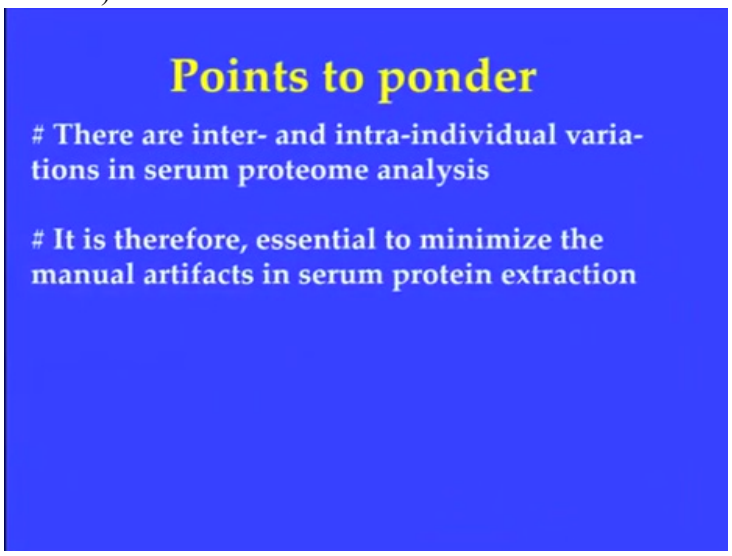

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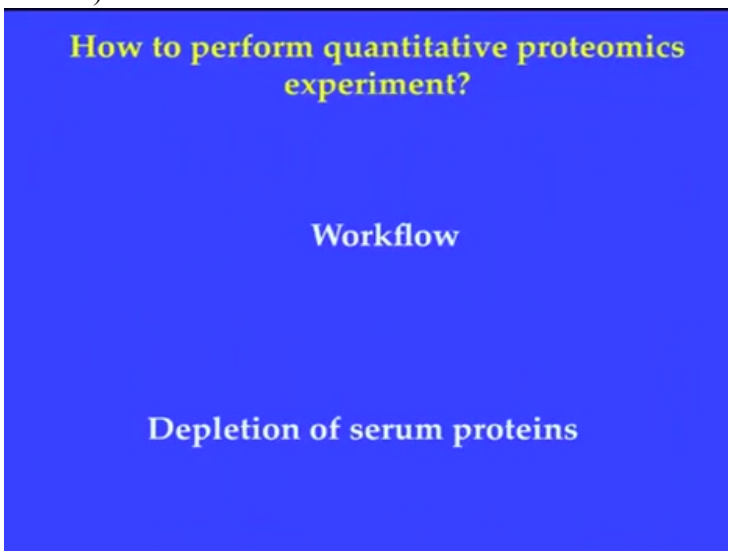

How to remove the high abundant proteins, which are present in the serum?

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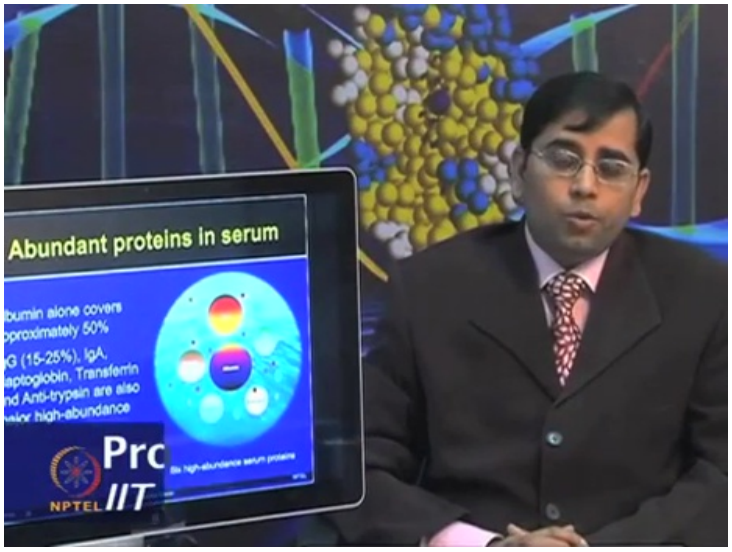

So, as we talked there are more than 20 abundant proteins which are present in the serum and albumin alone covers approximately about 50% of these abundant proteins,

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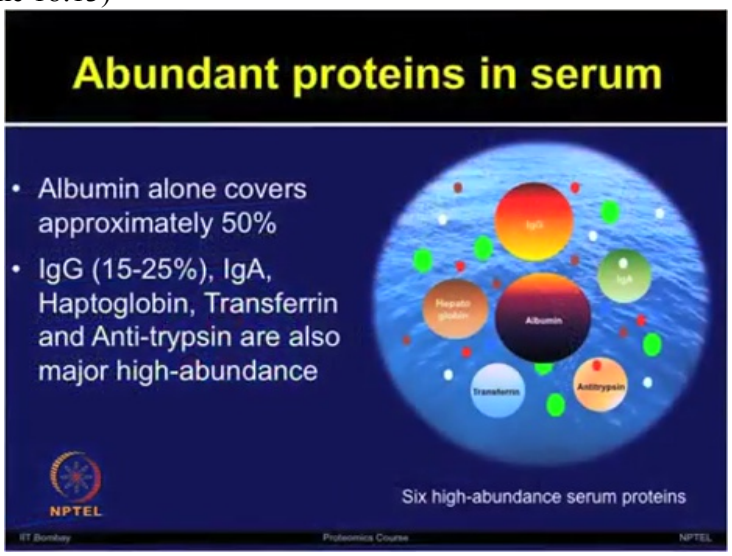

Immunoglobulin G contains 15-25% of the abundant proteins.

Then IgA, haptoglobin, transferrin and anti-trypsin, these are also major high abundant proteins. So in the diagram I have shown you the 6 high abundance serum proteins.

There are different ways people have tried to remove these abundant proteins; molecular weight cut-off and different types of chromatography methods have been used.

The affinity chromatography-based methods are highly efficient for the specific removal of these abundant proteins which are present in the serum.

Because there is always that fear with the molecular weight cut-off, whether along with albumin and other high molecular proteins, you will also get rid of your various high molecular weight proteins, which are non-abundant.

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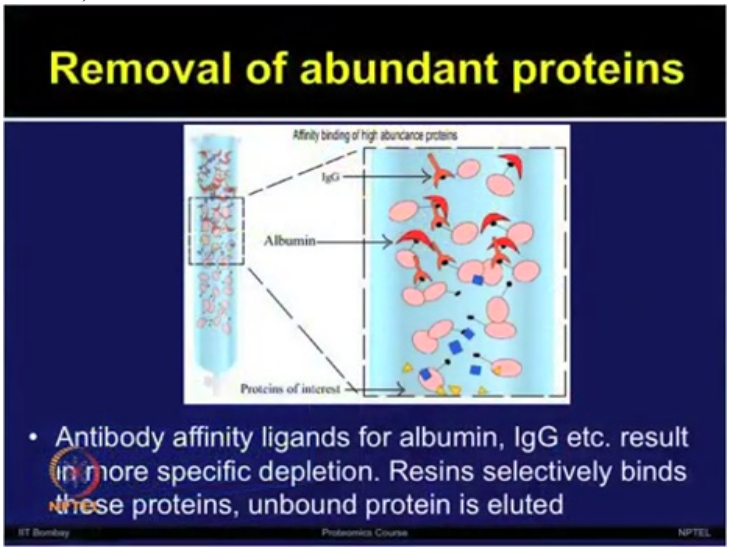

So, the affinity based methods ensure the specific targeting of albumin, IgG and other specific abundant proteins. The antibody affinity ligands are used for albumin, IgG and other abundant proteins and it results a very specific depletion.

These resins can selectively bind to these proteins and the unbound proteins can be eluted in suitable buffers.

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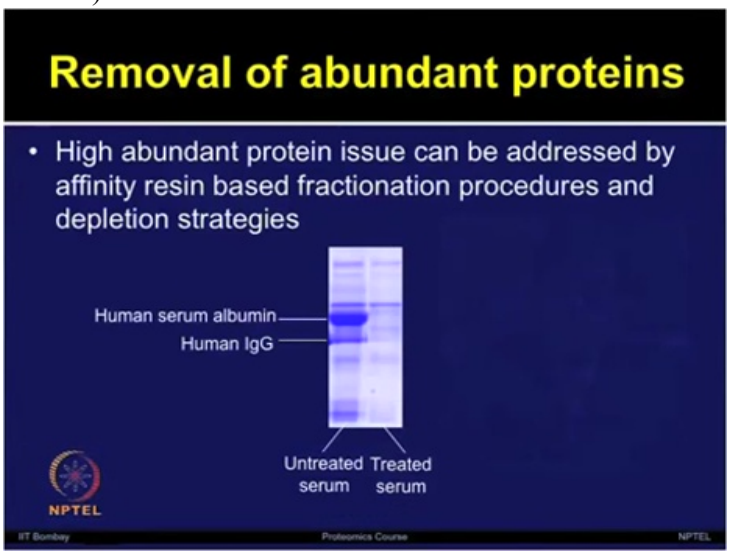

By applying the affinity resin-based fractionation method and different types of depletion strategies, one can get rid of high abundant proteins as shown in this image of SDS PAGE gel.

The left lane is loaded with untreated serum and the right lane is loaded with treated serum. As you can see, some of the very high abundant proteins such as albumin, IgG; those are efficiently removed, and which allowed some of the low abundant proteins to appear on the gel.

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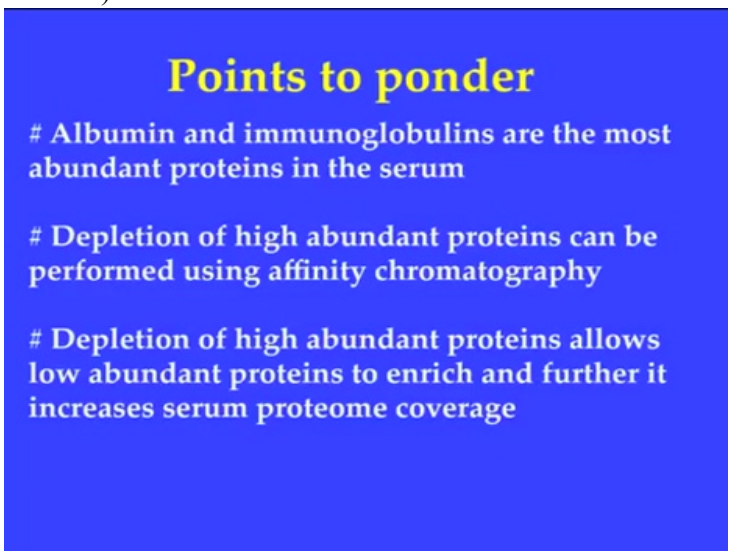

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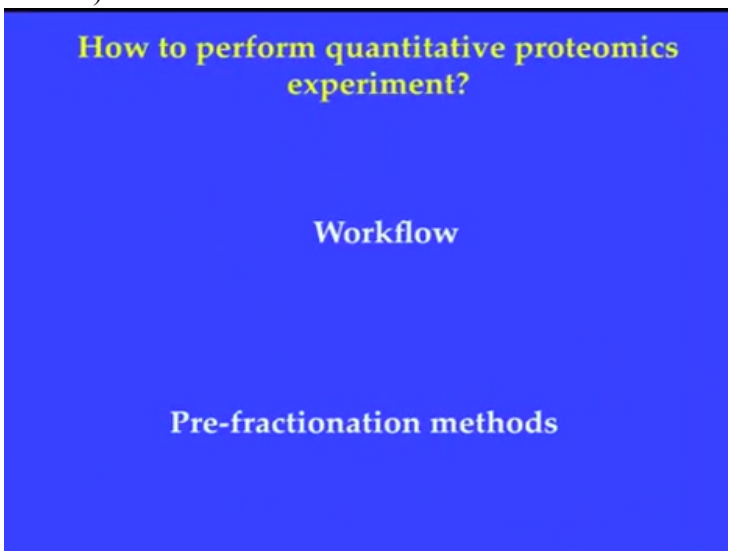

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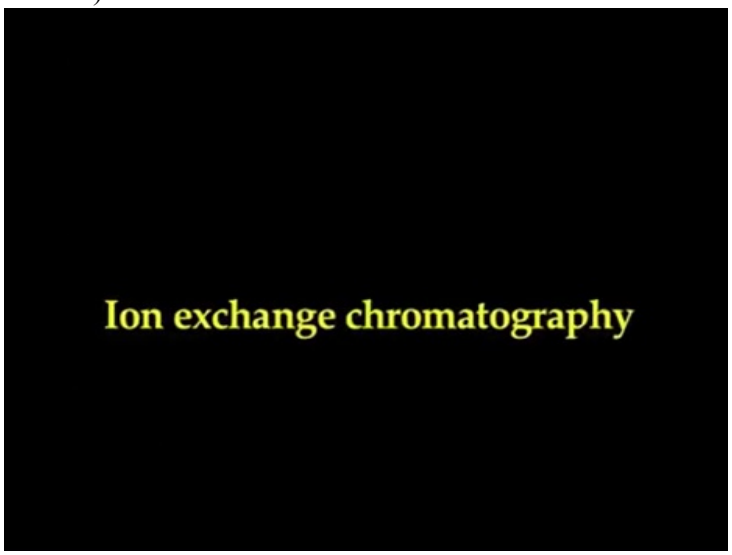

Ion exchange chromatography, this is one of the most versatile chromatographic separation method which relies on differences between number of charges and distribution of charged groups in defined pH and solvent conditions.

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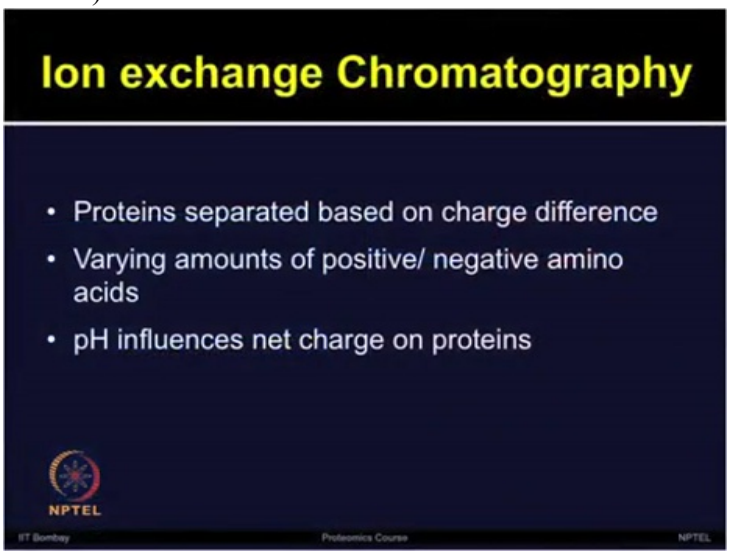

In ion exchange chromatography, the proteins are separated based on charge difference. Proteins with overall negative charge will interact with positive charges or vice versa. So by varying the amount of positive and negative amino acids and even pH can influence the net charge on proteins

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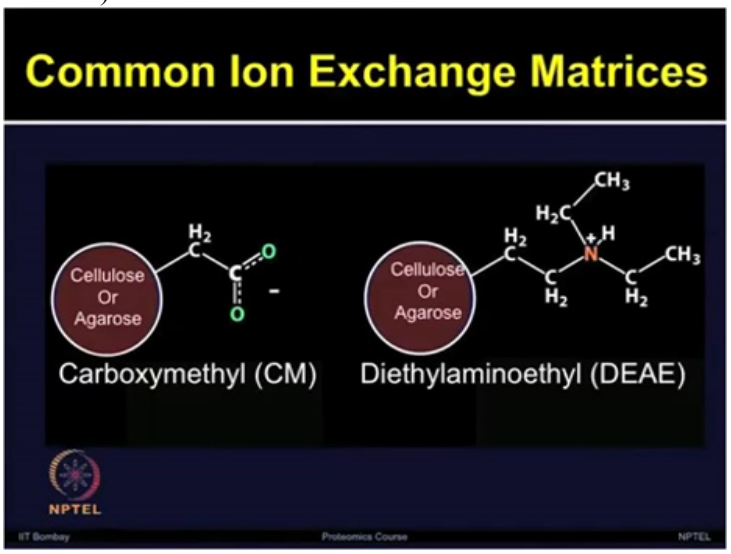

So in this slide some of the common ion exchange matrixes are shown, like CarboxyMethyl CM and DiEthylAminoEthyl DEAE.

So when a desired protein is positively charged, the cation exchange chromatography should be used. When a desired protein is negatively charged the anion exchange chromatography method should be used.

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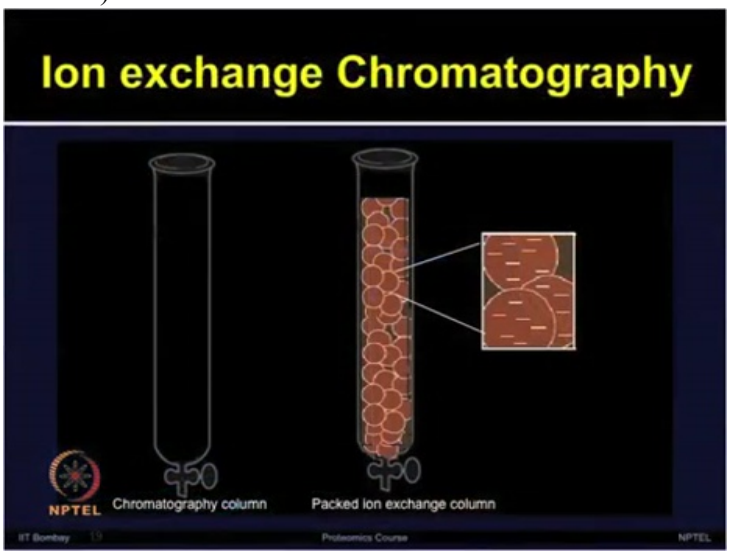

So in ion exchange chromatography, the column is packed with a resin whether it is cation or anion exchanger depending upon the charge of the protein that needs to be bound to the column and purified.

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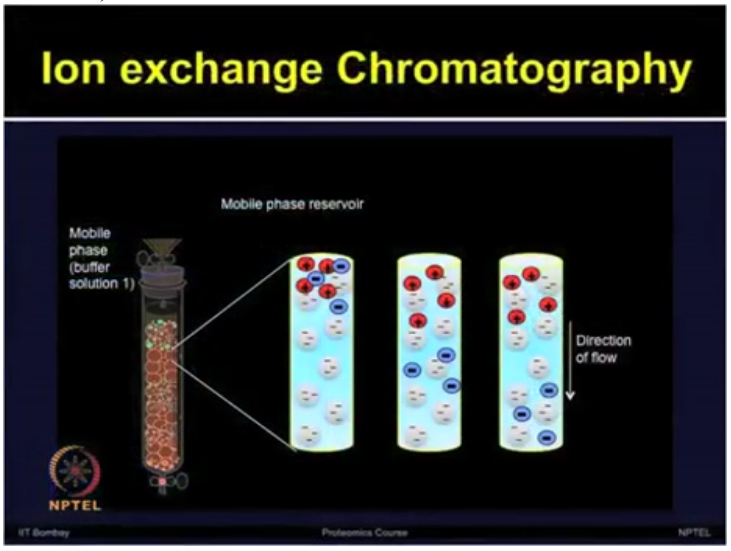

So proteins are adsorbed to the ion exchange column and then it can be desorbed by increasing the salt or altering the pH of the buffer which can change the charge on protein.

So various anionic buffers such as acetate and phosphate are used for cation exchange and cationic buffers such as Tris chloride or ethanolamine are used for the anion exchange

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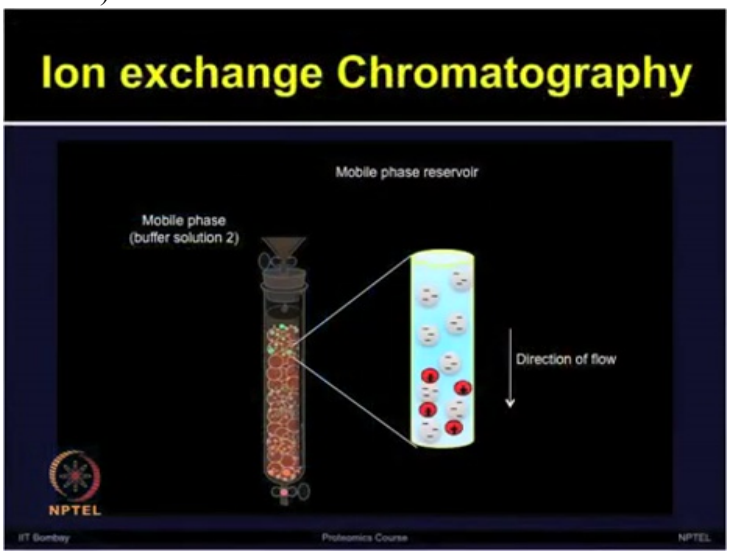

Now the buffer solution is changed so that the net pH of the protein of interest can be modified and it no longer binds to ion exchange resin.

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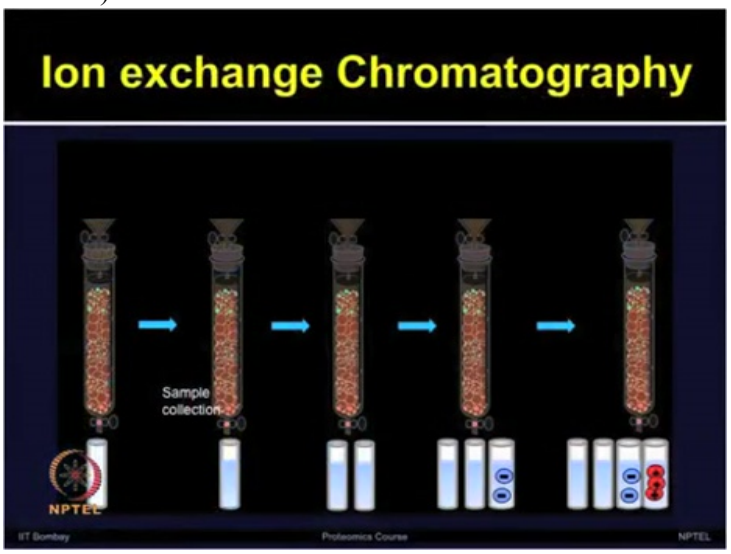

Therefore, the bound protein can eluted out as shown in this slide. So if you have negatively charged protein, which gets eluted first will be present in initial fractions, while the positively charged protein that bound to the column will be eluted in the later fractions or it will be vice versa.

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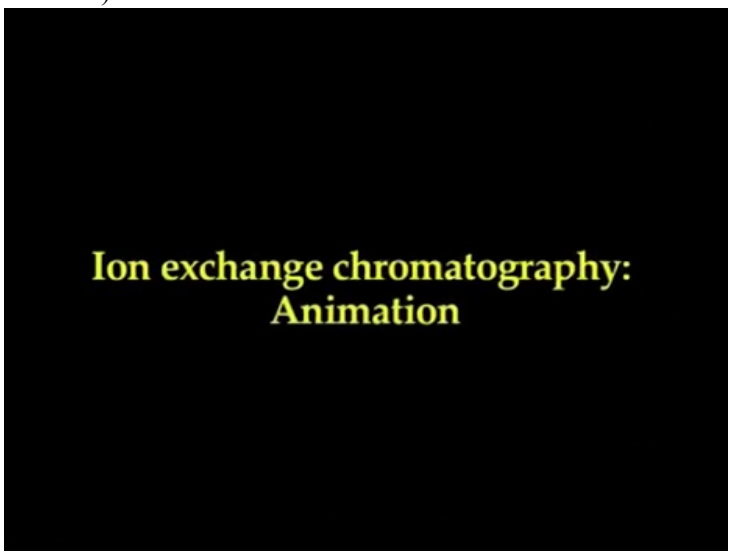

So let me describe how ion exchange chromatography works step by step in following animation.

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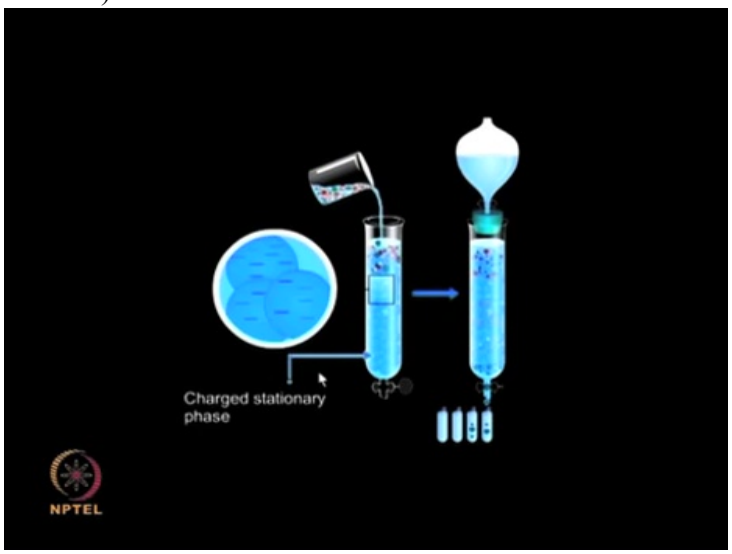

The charge stationary phase, the column stationary phase consists of a positively or negatively charged polymeric matrix which will bind molecules of opposite charge.

Commonly used ion exchangers include negatively charged carboxy-methyl cellulose or CM cellulose, which is a cation exchanger and positively charged DEAE cellulose which is an anion exchanger.

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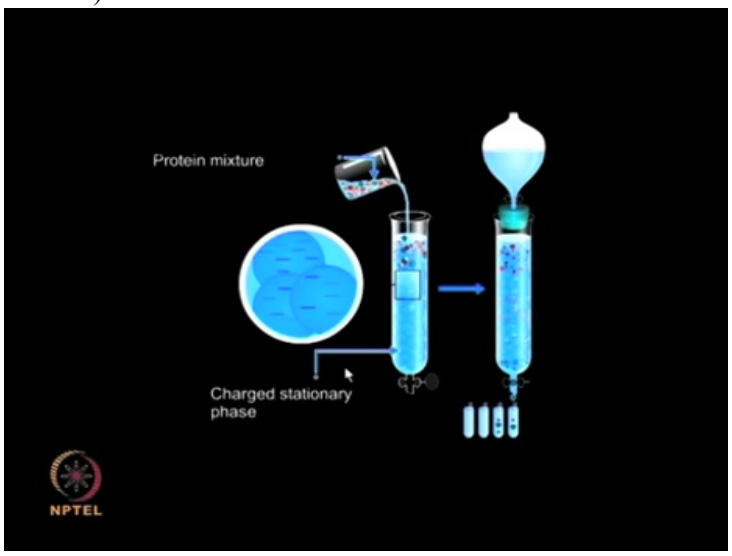

The protein mixture; unpurified protein mixture, which consist of proteins of different net charges are loaded on to the column. The proteins having charges opposite to that of stationary matrix will bind to it while remaining proteins will be eluted.

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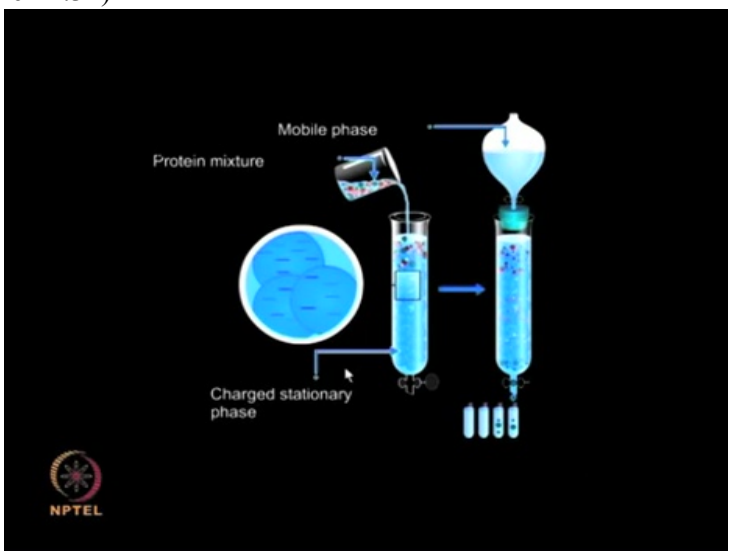

Mobile phase- the proteins are eluted out of the column by using suitable mobile phase and then samples are collected by using different sample fractions. The solution leaving the column can be collected in

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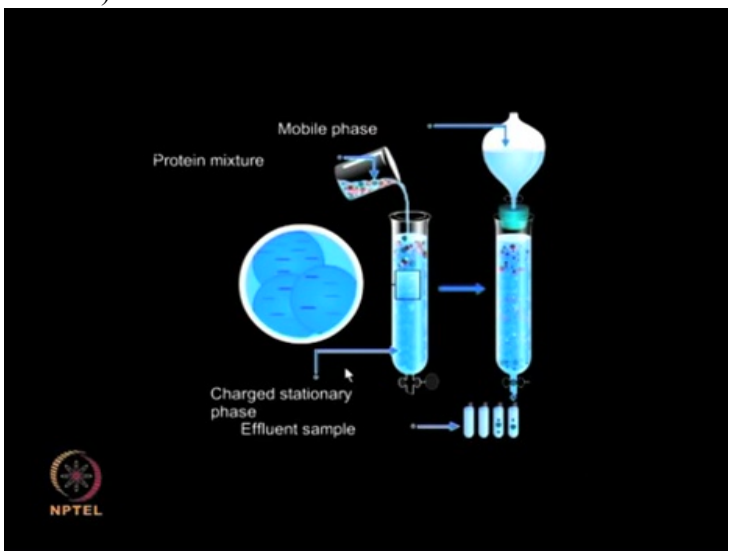

suitably sized fractions for further analysis. After giving you brief description of components, let me show you the process in animation

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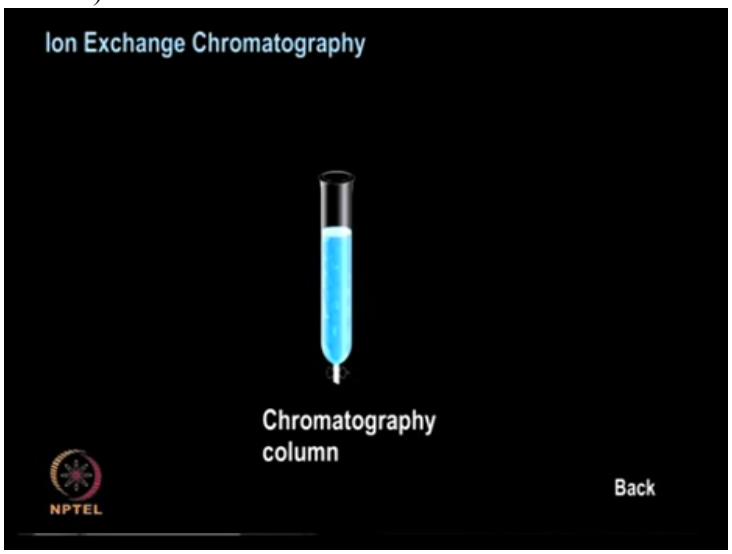

The column is packed with a suitable cation or anion exchange resin depending upon

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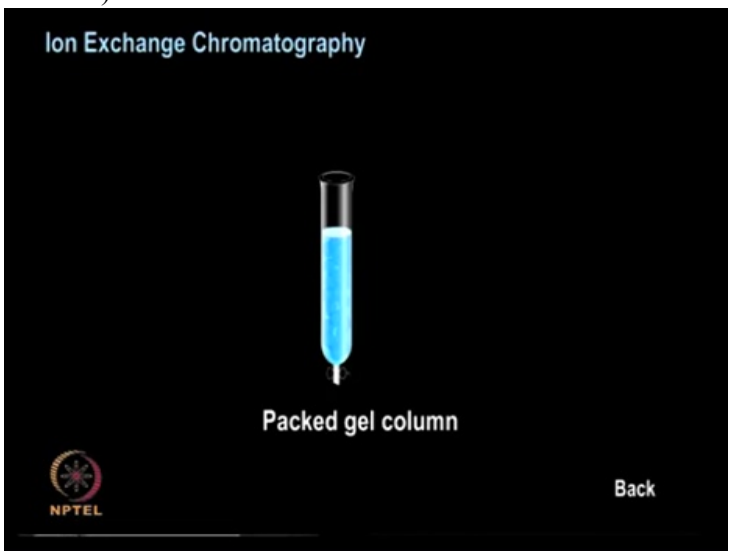

the charge of the protein

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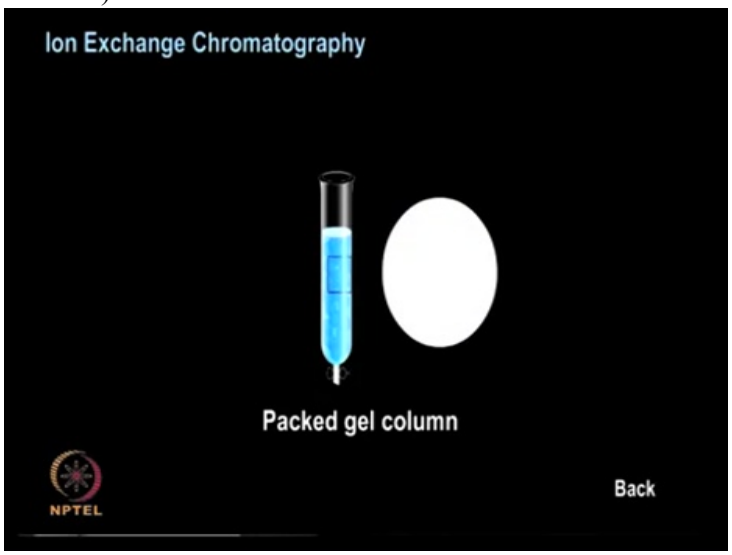

that needs to be bound to the column and purified. The anion exchange column is then loaded

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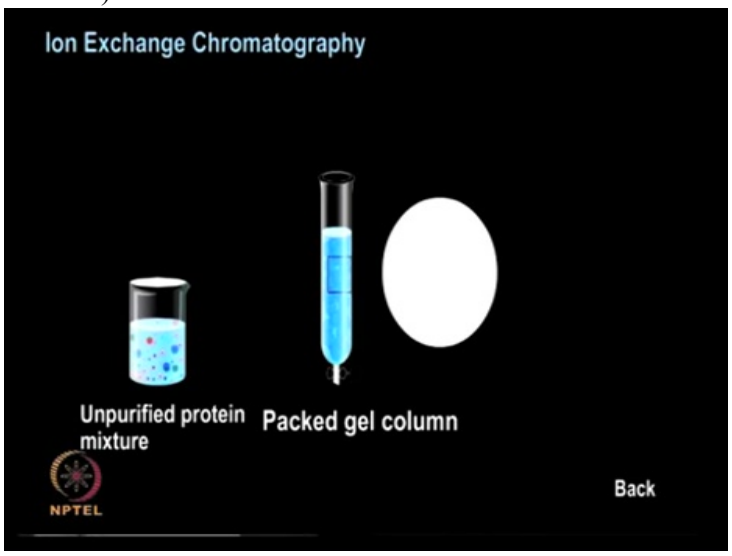

with the impure proteins mixture consisting of various positively and negatively charged proteins.

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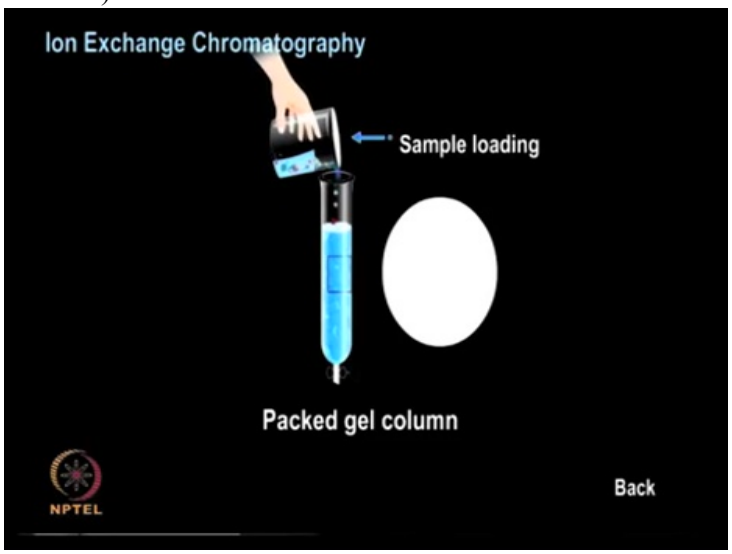

The column is eluted with a buffer solution of suitable pH such that negatively charged molecules are removed from the column

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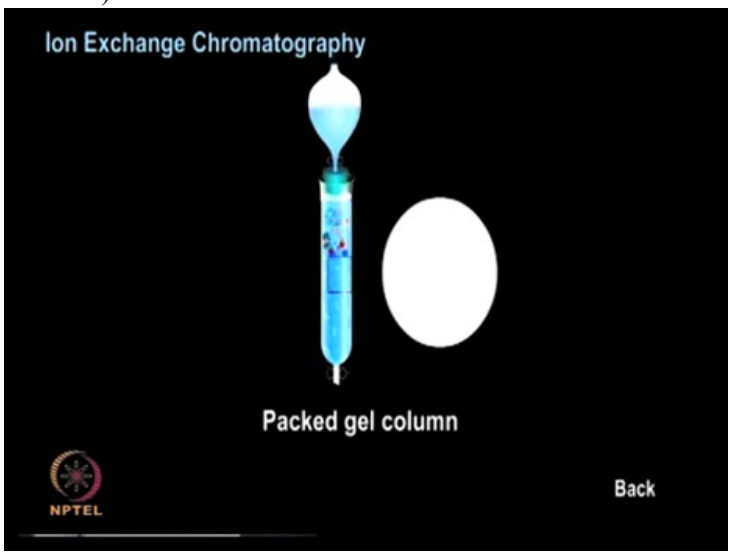

while the positively charged

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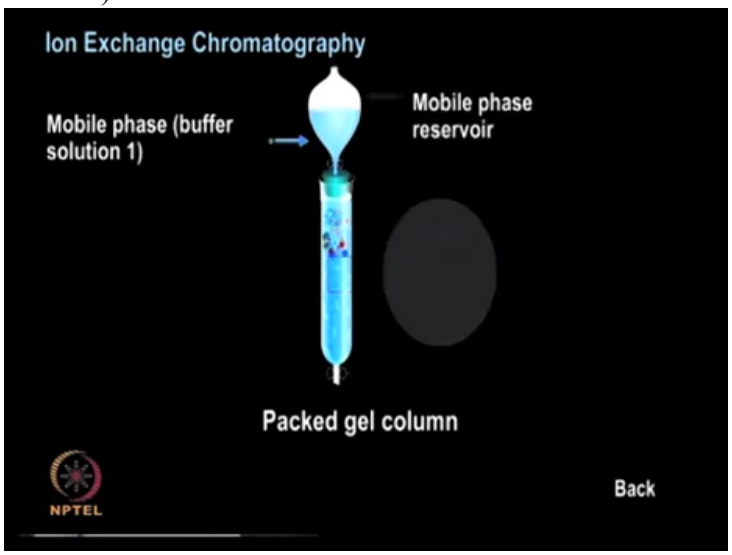

molecules remain

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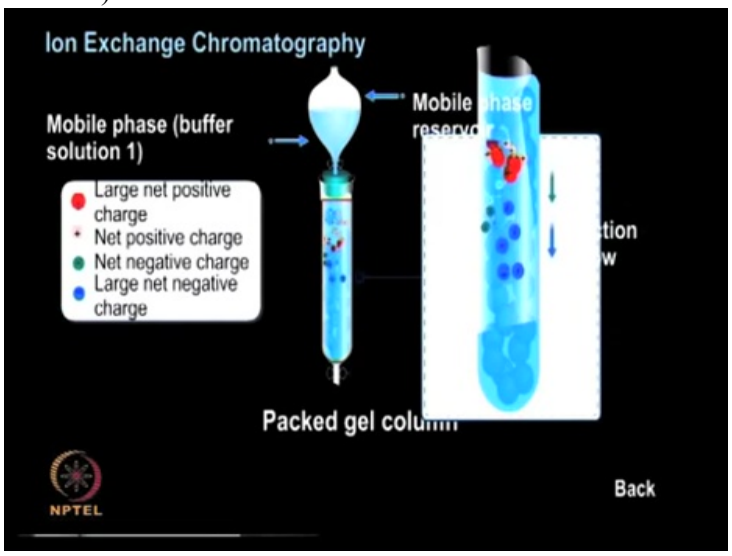

bound to the anion exchange resin. The buffer solution is then changed such that the net pH of the protein of interest is modified and no longer binds the ion exchange resin.

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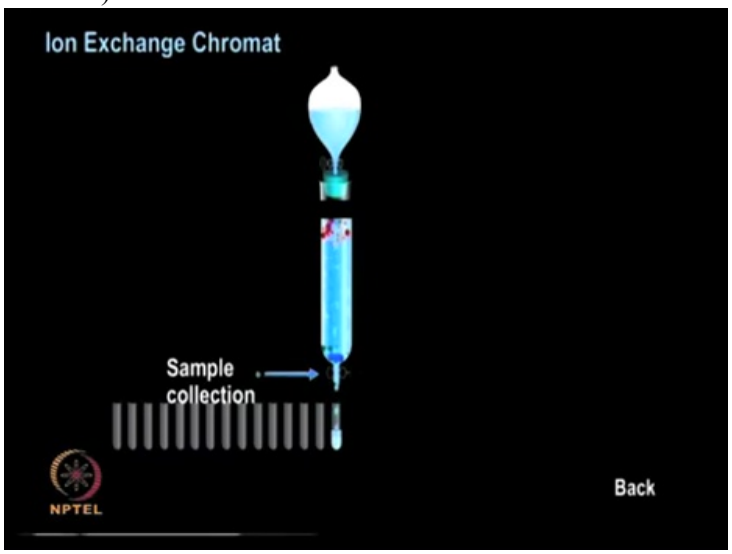

Therefore the bound protein also gets eluted out of the column in this manner. The fractions of appropriate size

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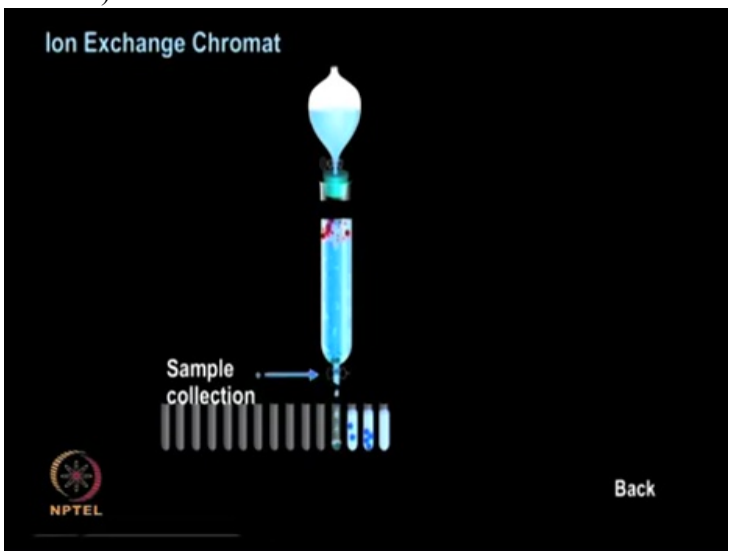

must be collected and

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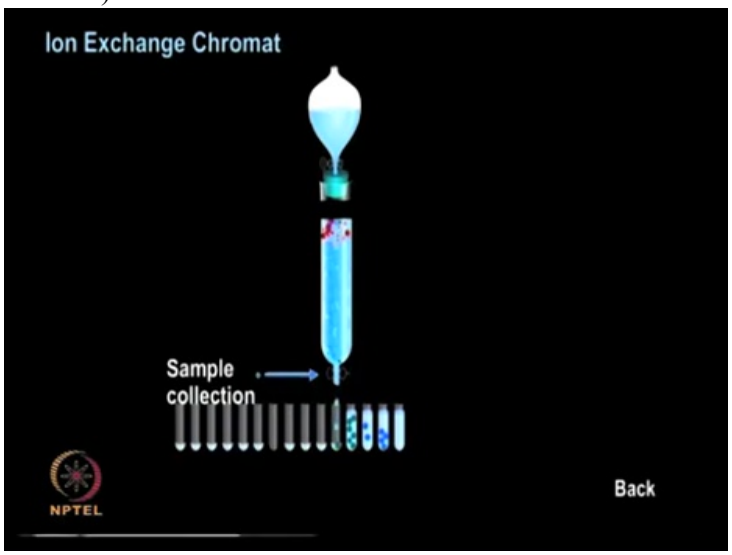

analyzed for their protein content. The negatively charged

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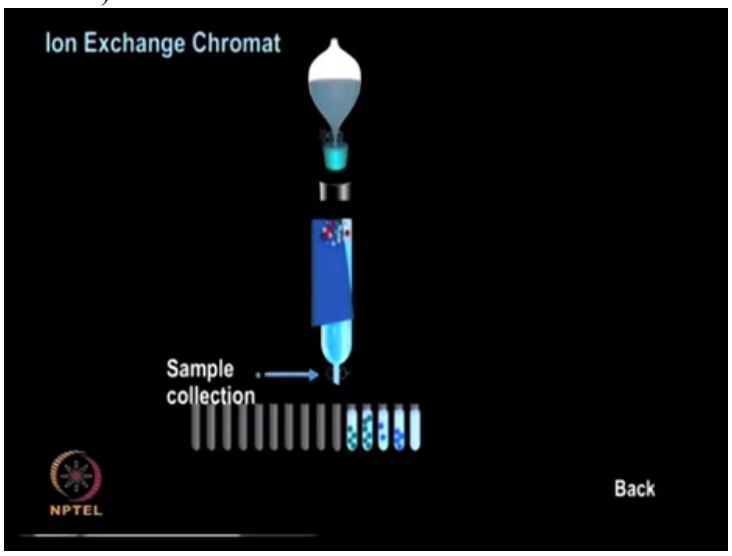

protein which gets eluted first will be present in the initial fractions while

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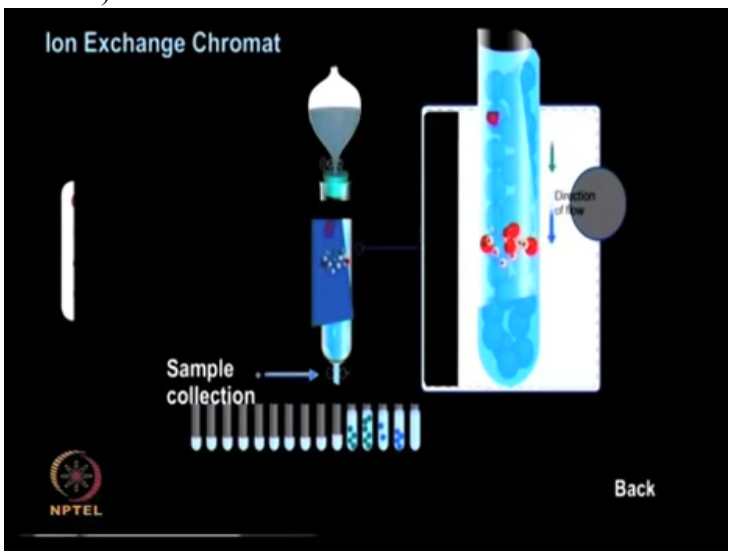

positively charged proteins which bound to the column

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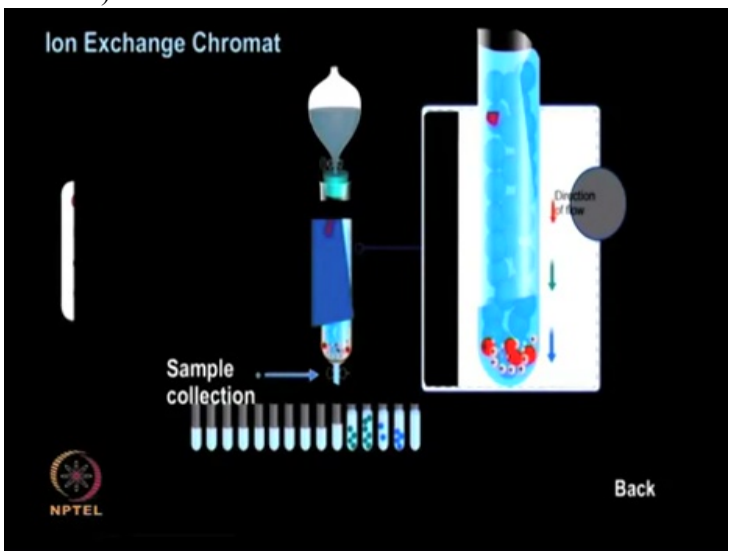

are eluted in later fractions. Once all the fractions are collected, then the protein content can be analyzed by using a spectrophotometer.

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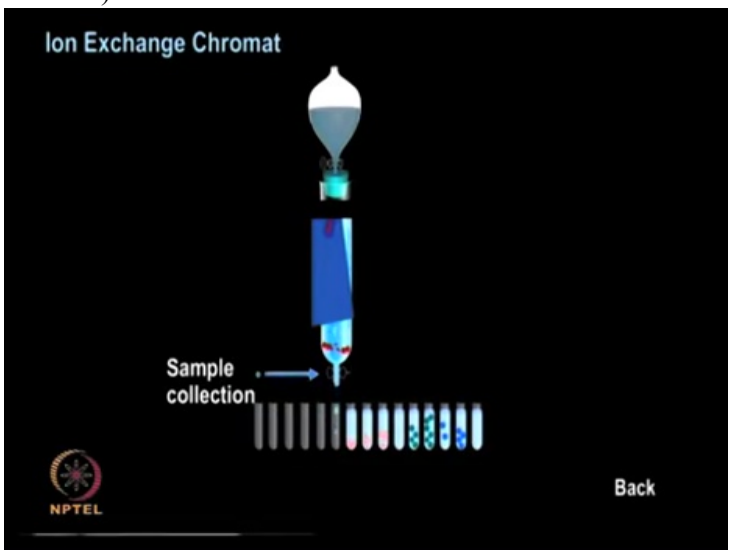

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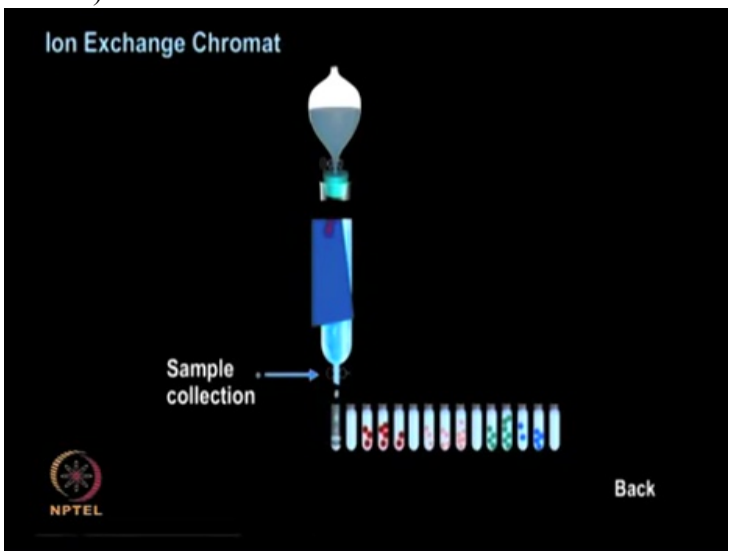

So analyze the contents, these fractions for their protein contents using a UV-visible spectrophotometer at 280 nano meters. A graph of eluant volume versus protein concentration can be plotted.

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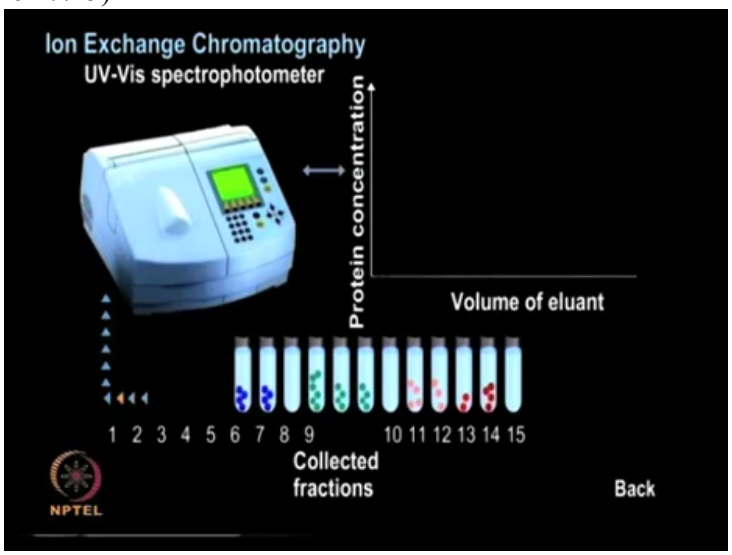

In this particular example negative charged large molecules coming first

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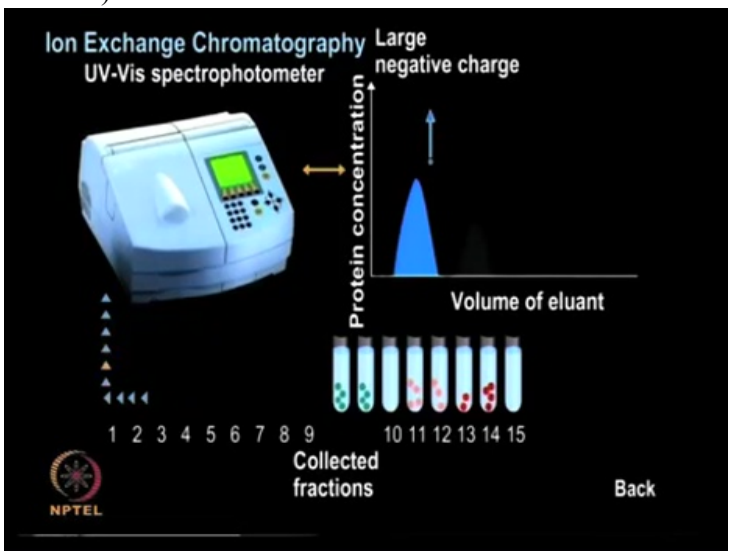

and then positively charged fractions are

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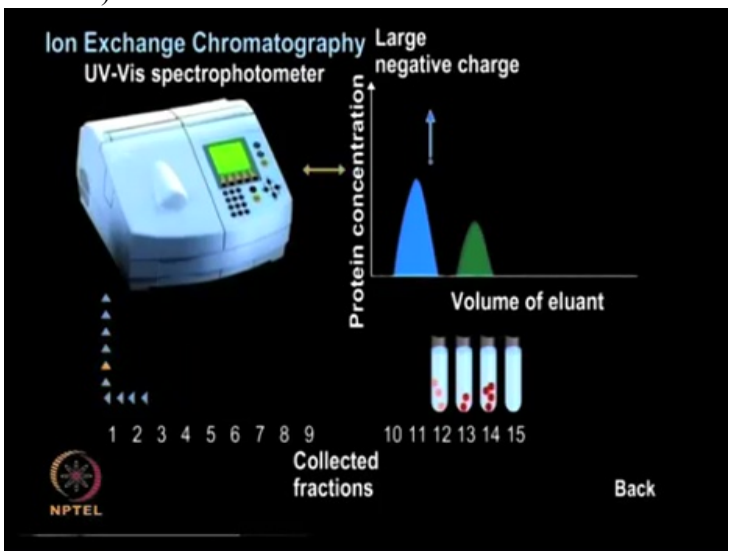

coming later.

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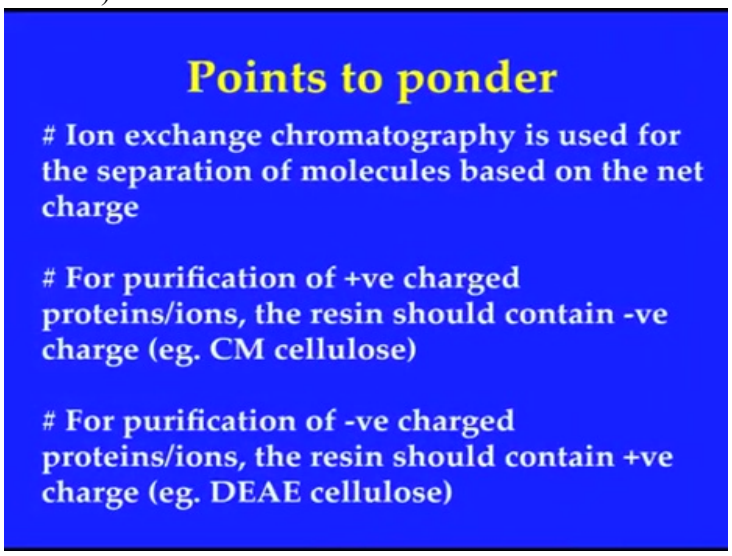

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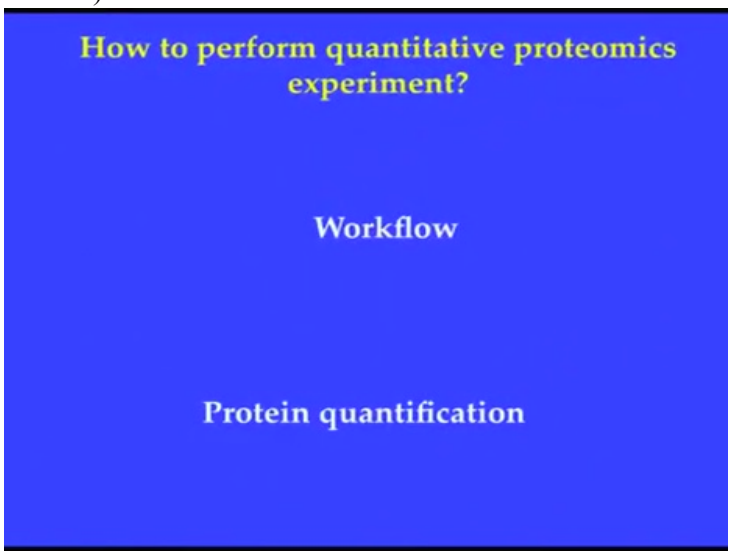

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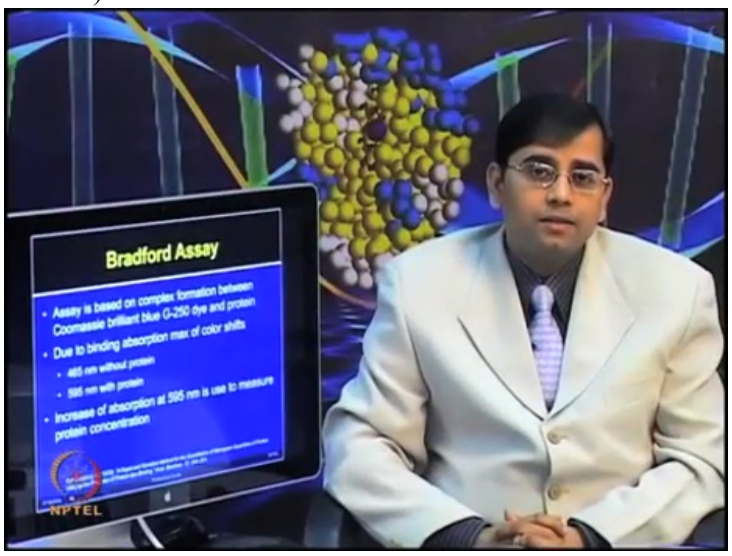

Bradford assay,

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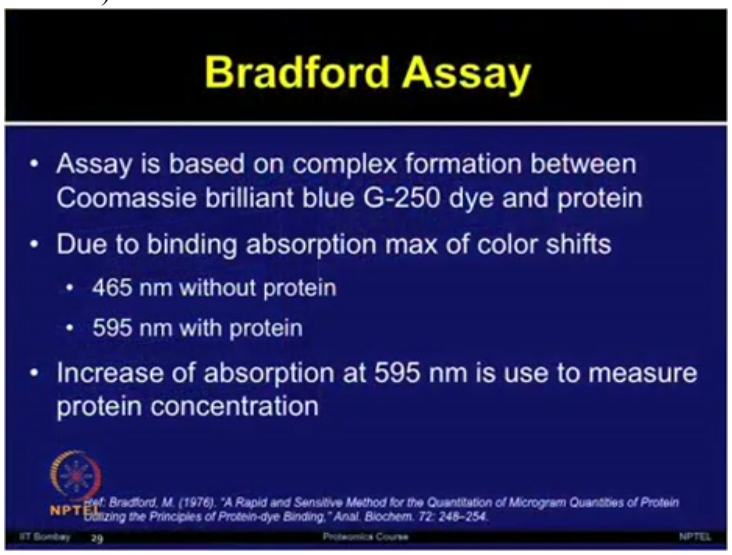

this assay is based on complex formation between Coomassie dye G-250 and protein. Due to the binding absorption max, the color shifts from 465 nano meter to 595 nano meter. This increase of absorption at 595 nano meter is used to measure protein concentration.

The Bradford assay has various advantages as compared to the Lowry or BCA method

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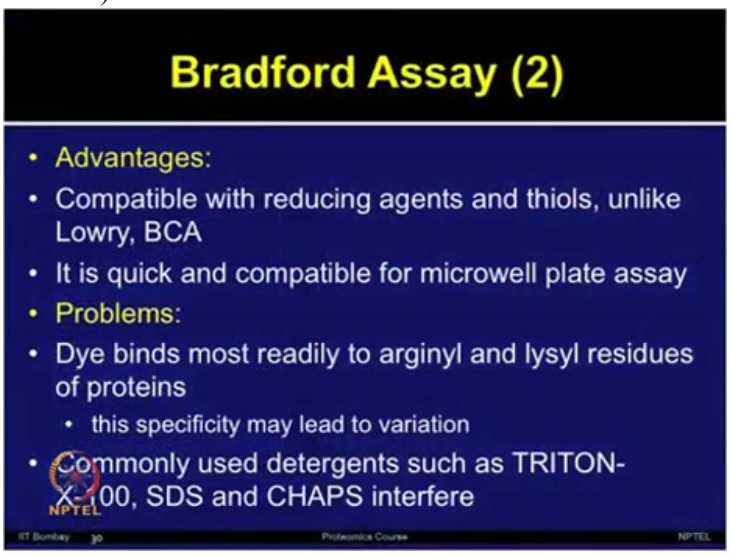

because it is compatible with reducing agents and thiols, which was not the case when we talked earlier about Lowry method and BCA method.

This method is also very quick and it is compatible for microwell plate assays which can be performed in the 96 well format.

But there are different problems with the Bradford assay as well, such as, the dye binds most readily to arginyl and lysyl residues of proteins and this specificity may lead to the variations.

Now there are various detergents such Triton-X100, SDS, and CHAP; they also interfere with the Bradford assay.

So researchers have come up with various types of modified Bradford assays for specific applications. Again you have to keep an eye; what is the component of your buffer and you need to ensure that it is compatible with the assay conditions or not.

So let us talk about Bradford assay in little bit more detail so that one can use that to determine protein concentration. So if you want to perform this assay, what are the requirements?

So, first of all you need one standard let's say

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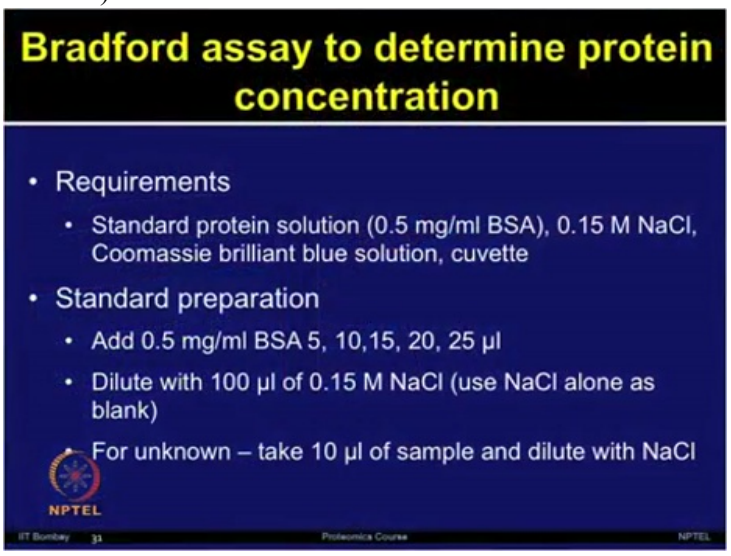

you can take BSA; you need some salt solution, Coomassie brilliant blue solution and cuvette. So for standard preparation you can take BSA and add different concentration of this; 5, 10, 15, 20, 25 micro liters or you can even go above that standard preparation series.

Then dilute this sample with 0.15 molar of sodium chloride and total volume make it to 100 micro liters.

One sample where there is no protein there; that can be used as the blank, which can be used to auto-zero the value in spectrophotometer; for as a blank and then further you can measure your samples.

For the unknown samples for which you want to determine the protein concentration, you can take 10 or 15 micro liter of the sample and dilute with the sodium chloride. Then same treatment can be performed for this whole experiment.

Now if your unknown is giving you absorbance higher than the your standard preparation whatever series you have made ; so it is better idea that you can try different dilutions of your unknown sample, because it has to fall within the standard curve of the standard dilutions what you have taken.

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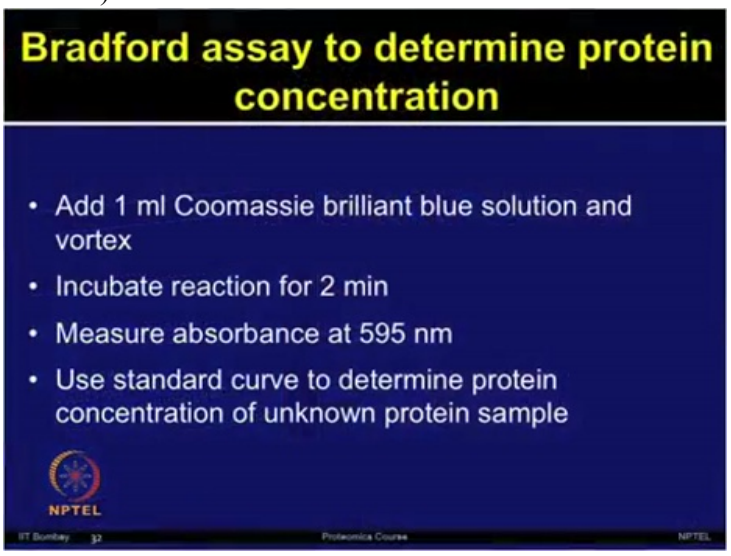

Now you can add 1 ml of Coomassie brilliant blue solution and vortex it.

After that one can incubate it for 2 minutes so that color can be developed and then it can be measured for absorbance at 595 nano meters.

Then, you can use standard curve to determine protein concentration of unknown protein sample. But often, once you plot your unknown protein sample you may realize your values for the protein sample are either too low or too high to be plotted in the standard curve. So, then you may need to adjust your unknown sample.

You may have to make dilution of it and use less volume or you may need to increase the volume of your sample so that it can fall within the range of the standard curve

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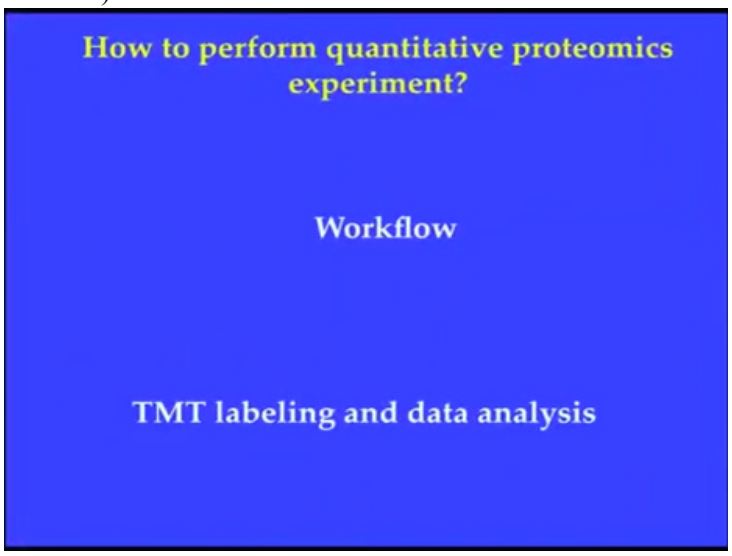

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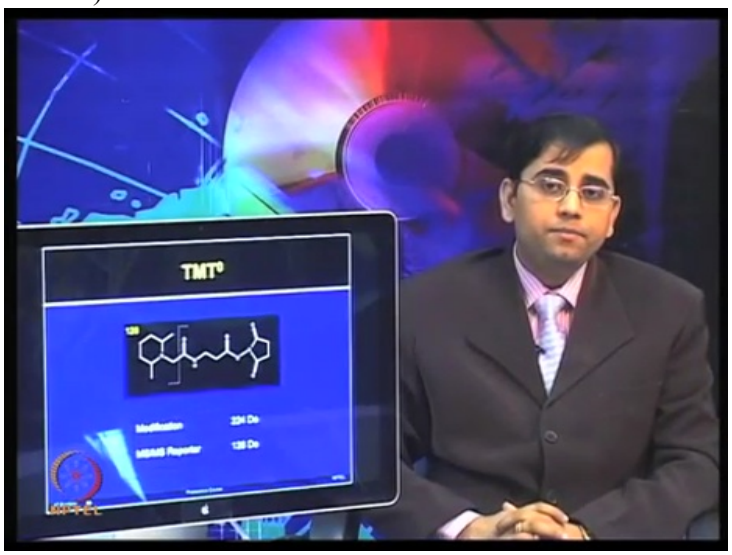

Let's look at TMT0 labeled structure in more detail.

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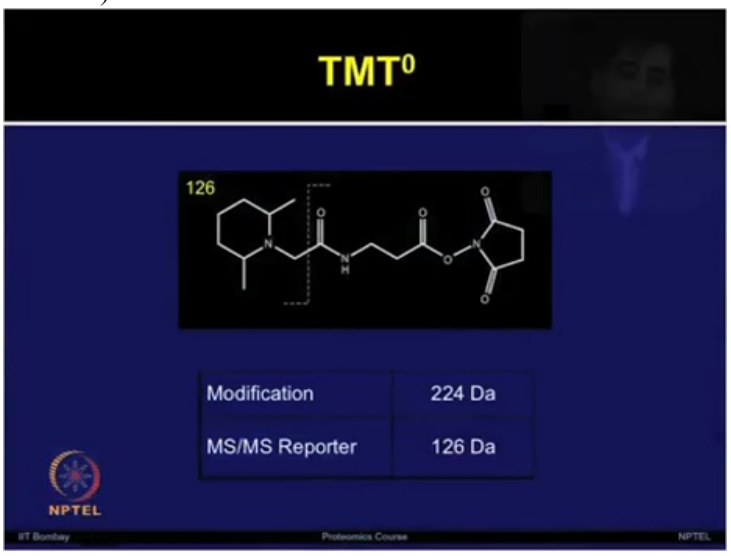

The TMT0 tag is used for testing and optimization of sample preparation, labeling, fractionation and MS fragmentation for peptide identification and reporter detection. The modification is 224 Daltons and MS/MS reporter ion is 126 Daltons.

Now let's look at TMT duplex.

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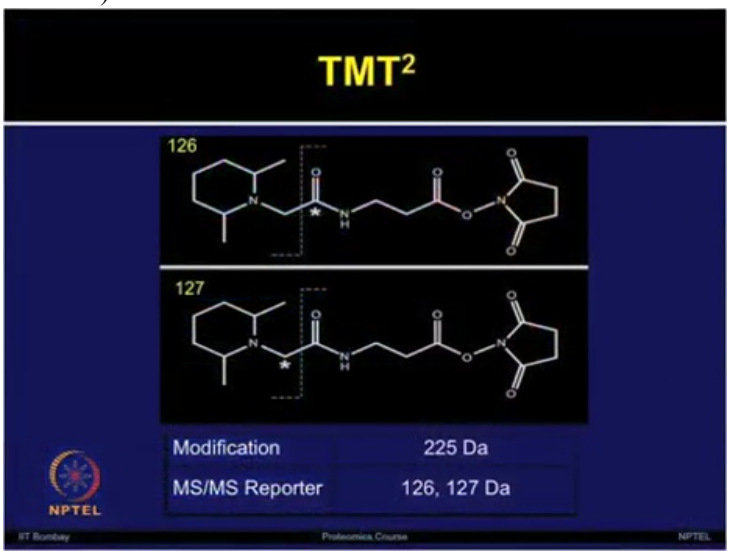

The TMT duplex reagent allows for the comparison of two samples. 126 and 127. These are two different MS/MS reporter ions available and modification is 225 Daltons.

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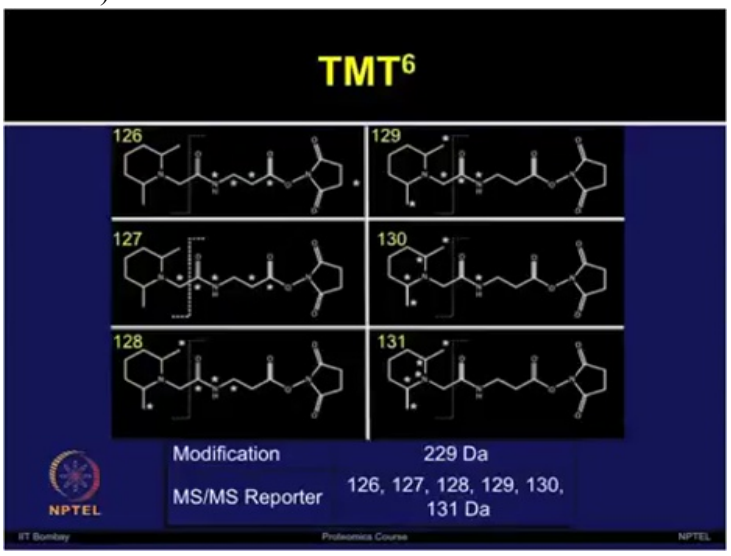

Let's now look at TMT 6-plex reagent. It allows the comparison of up to 6 conditions. The MS/MS reporter ions as you can see in the structure are from 126, 127, 128, 129, 130 and 131 Daltons.

So the TMT 6-plex reagent allows the comparison of up to 6 conditions. It could be useful for studying about time course, drug-dose responses, replicates or looking for multiple sample disease comparison. The modification is 229 Daltons

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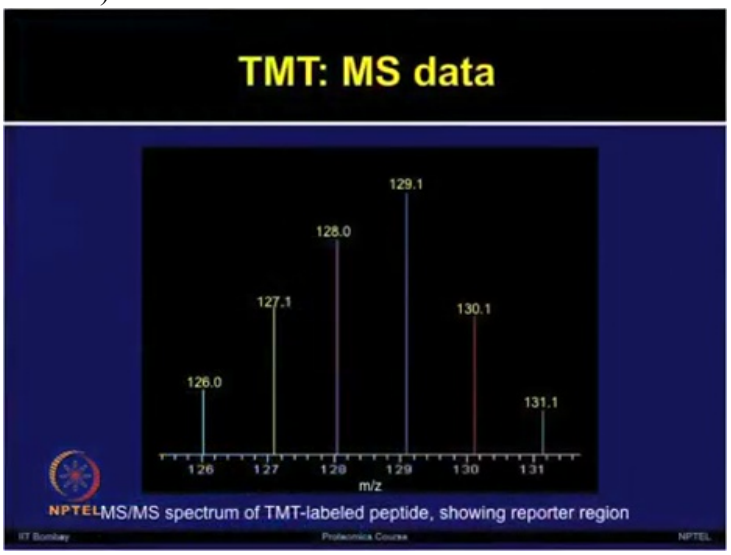

I am showing you one representative MS/MS spectrum of TMT labeled peptide which is showing a reporter region.

The relative abundance of target protein or peptide fragment in 6 different samples can be easily measured by comparing these signature mass peaks which are generated by the different mass tags.

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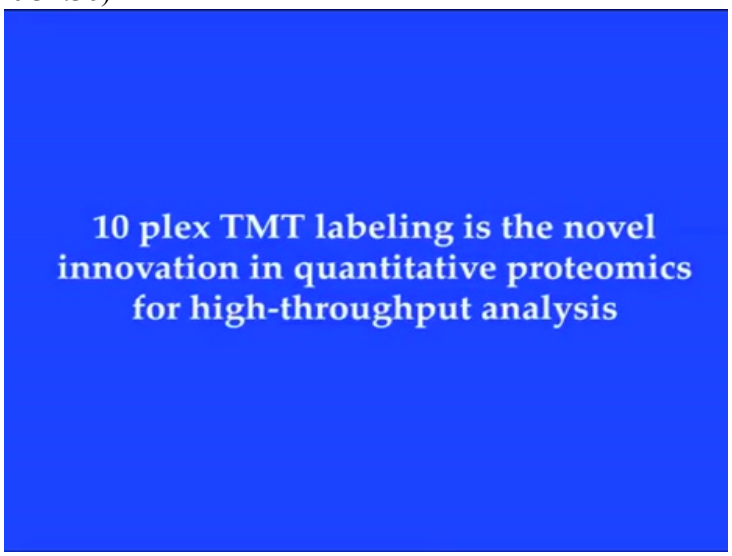

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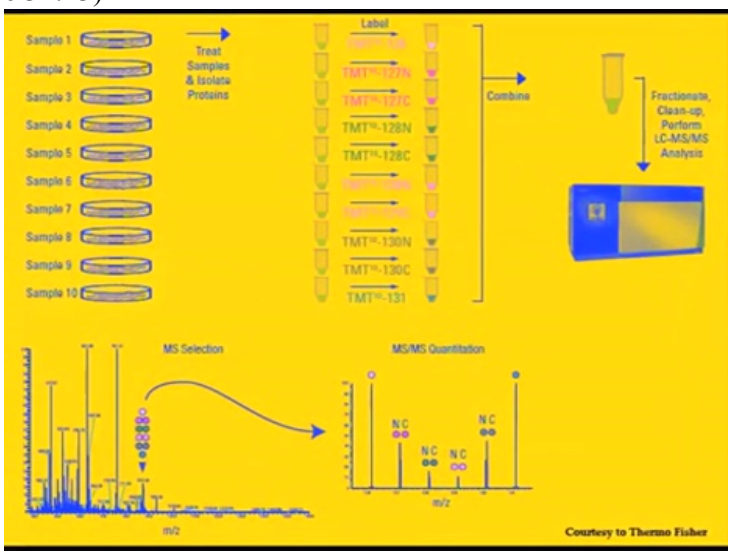

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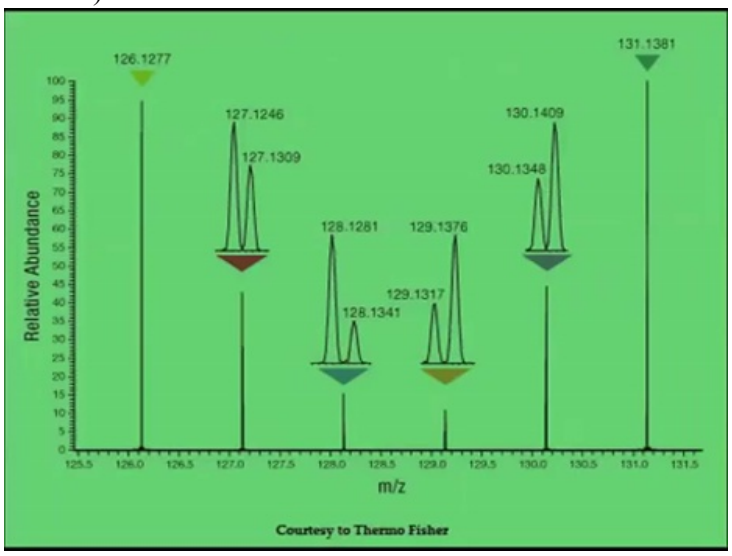

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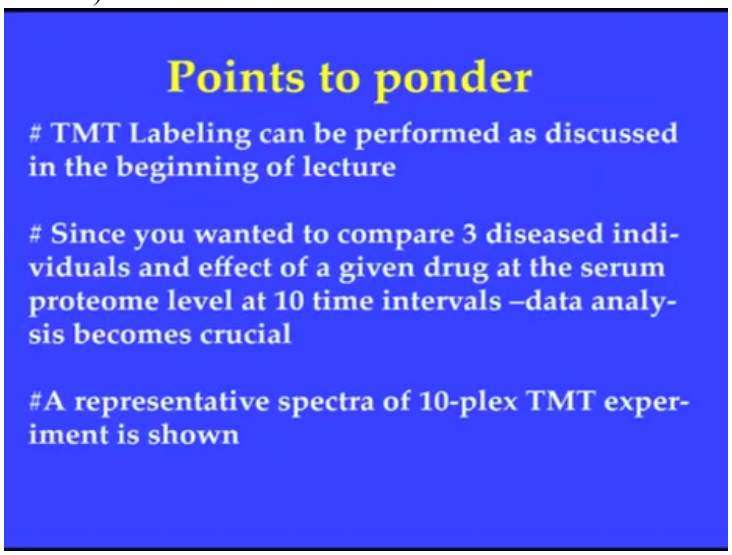

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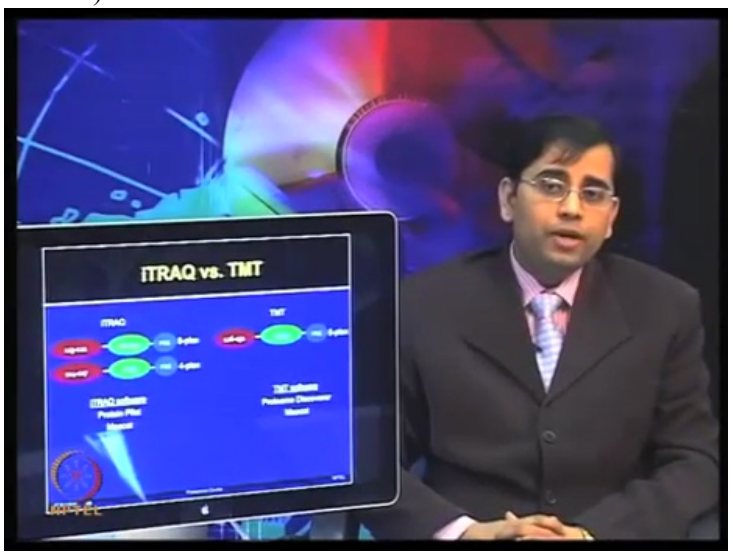

Let’s now look at the comparison of iTRAQ and TMT tags.

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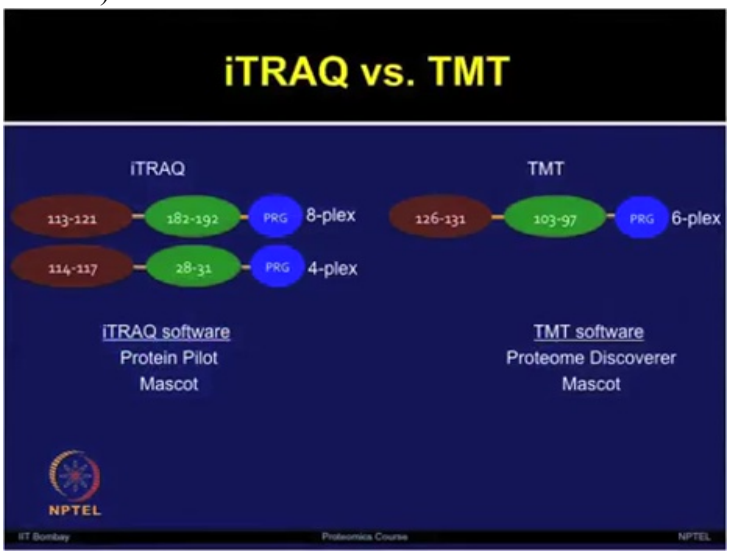

So in iTRAQ, as we talked, there are two different type of reagents available, 4-plex and 8-plex.

In both there is a reporter group, a balancer group and then there is a protein-reactive group. Same concept is also in the TMT tags where we have a reporter group, a balancer group and PRG.

I have shown a comparison with 6-plex TMT tag but as you have seen earlier there is TMT duplex and TMT0 tags are also available.

Now in iTRAQ the reporter in the 4-plex consists of either from 114 to 117 Dalton, the balancer is between 28 to 31 Dalton whereas in 8-plex it is from 113 to 121 and the balancer is from 182 to 192.

In case of TMT, the reporter group has 126 to 132 Daltons and the balancer consists of 97 to 103. So tags are quite similar in overall structure.

The iTRAQ analysis can be performed by using the software such as Protein Pilot and also the Mascot. The TMT based analysis can be performed from software such as Proteome Discoverer and Mascot.

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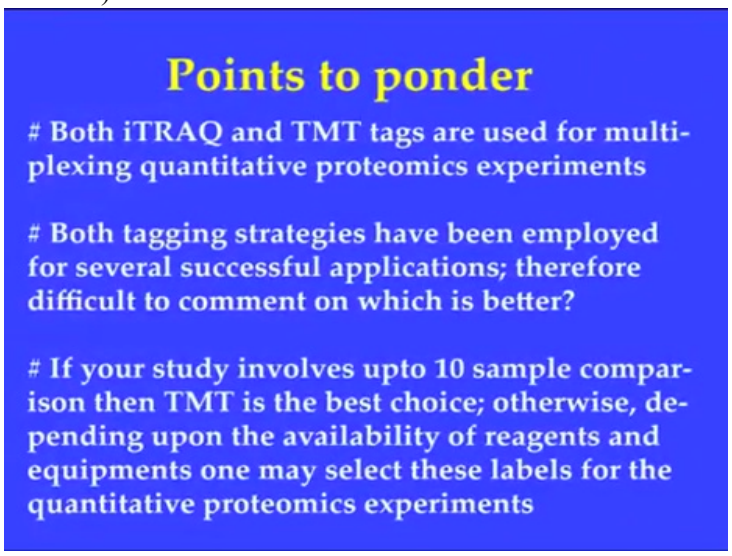

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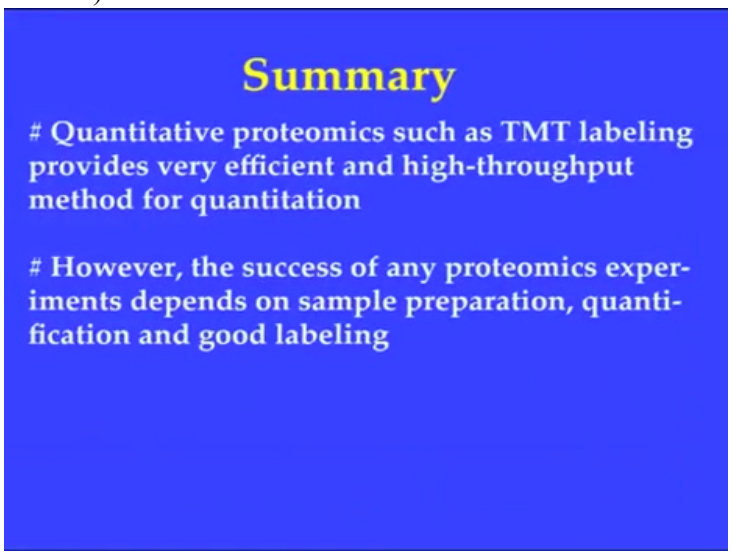

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