Mass Spectrometry Based Proteomics
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Mod 03 Lecture Number 14

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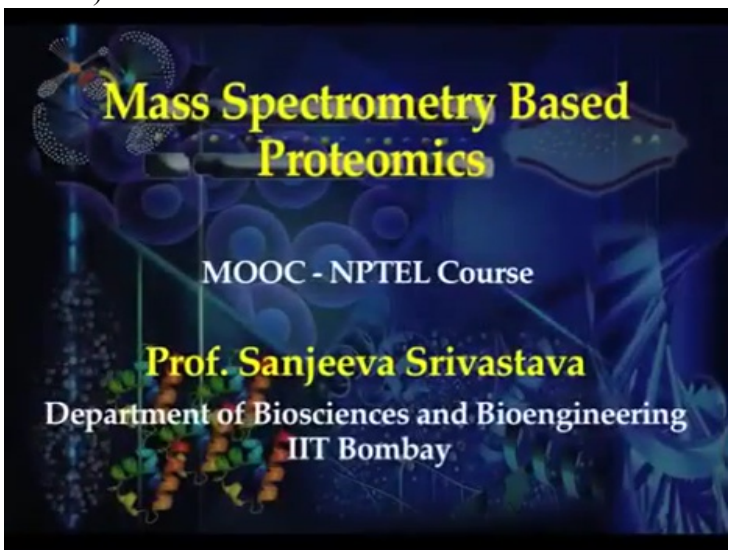

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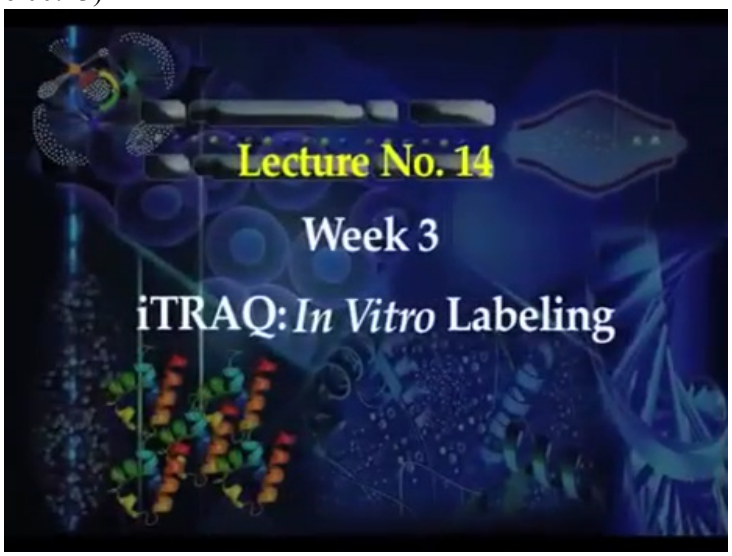

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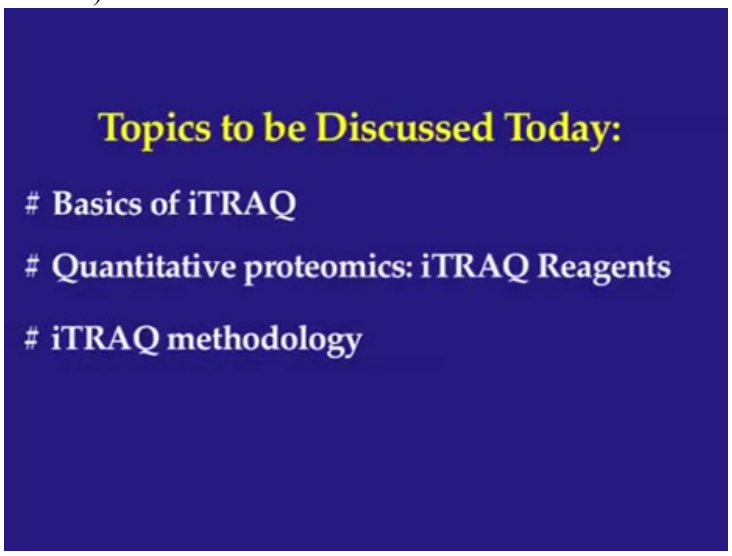

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Today we will talk about

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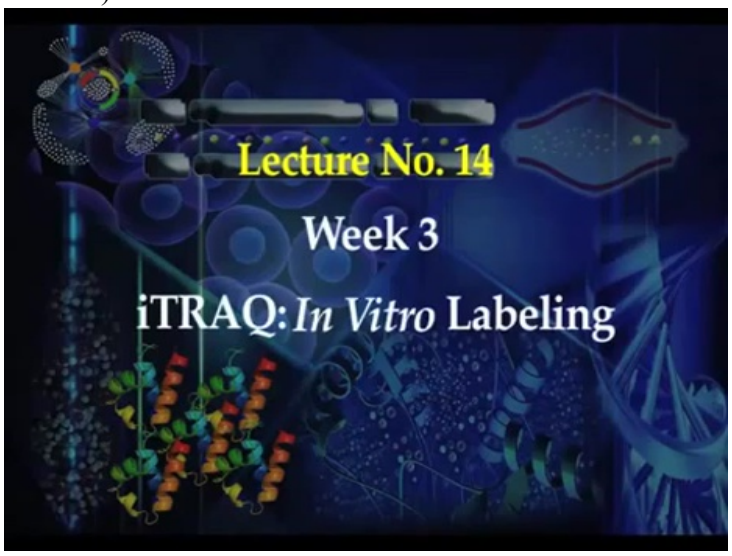

…quantitative proteomics and discuss about iTRAQ.

The quantitative proteomics aims to answer various questions including the identification of biomarkers for various diseases and target identification for early diagnostic intervention.

Various techniques or strategies have been developed for quantitative proteomics including the gel-based approaches such as DIGE and mass spectrometry-based measurements, ICAT which was first generation MS-based quantitative approach for quantitation of relative levels of proteins in various control and treatment samples.

The ICAT reagent, as we discuss, was consist of three components, the thiol functional groups, a linker group and a biotin moiety. Two samples treated with ICAT reagent, light or heavy isotope, D0 or D8, were selectively alkylated for cysteine residues.

These samples were combined, tryptic digested, affinity purified based on avidin and then analyzed by LC-MS based approach. So the ion abundance ratio of light and heavy isotope-labeled peptides could provide the relative abundance of these proteins.

So ICAT was the first quantitative MS-based approach which was initiated but because of the, only 2 samples could be analyzed from this approach, new approaches came considering the sample multiplexing.

Because one limitation of ICAT was only 2 labels are available, therefore more samples cannot be compared in a given experiment.

So there is a need to compare large number of treatments that led to the development of 2 or 4-plex Isotope Coded Protein Label ICPL, 4 or 8-plex isobaric Tagging for Relative and Absolute Quantitation iTRAQ and 2 or 6-plex Tandem Mass Tag TMT based techniques.

So, today we will continue our discussion on iTRAQ and TMT techniques which can compare up to 8 or 6 samples in a single analysis. So these techniques can increase the throughput.

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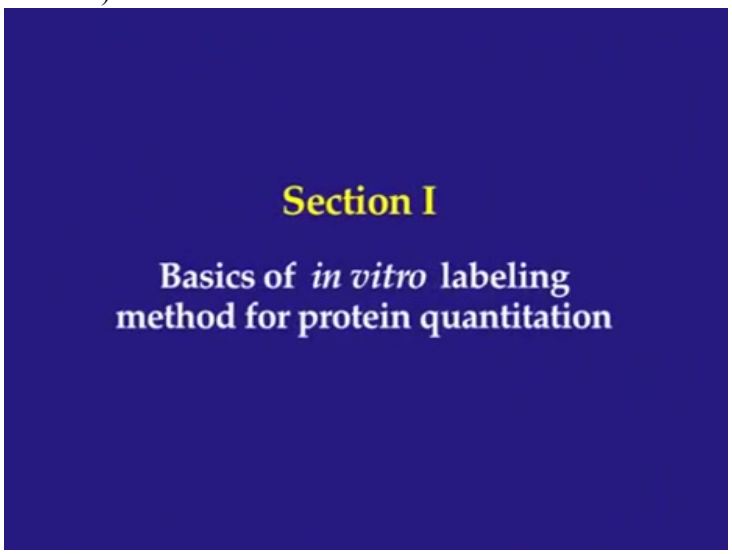

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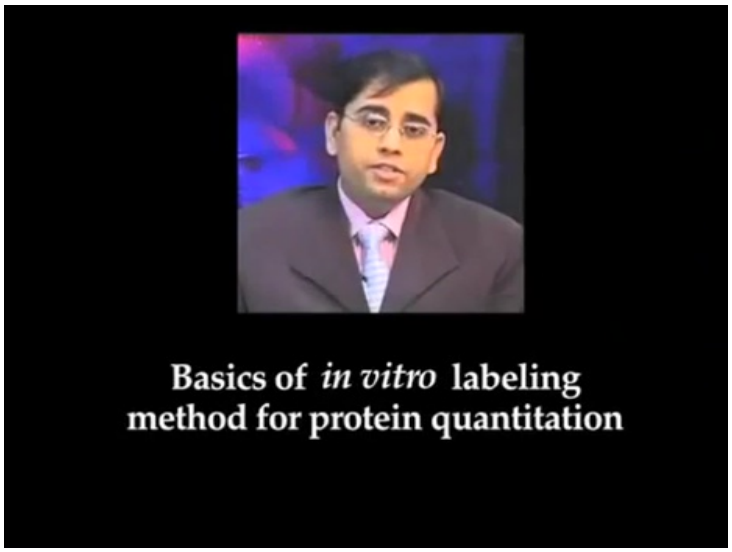

So let us talk about quantitative proteomics and different in vitro labeling methods. The in vitro labeling methods rely on use of labeling reactions at a specific site in proteins or peptides.

Based on various labeling chemistry, different types of strategies have been developed to introduce isotopes at either protein or peptide level. And these strategies, we will discuss in the next slide.

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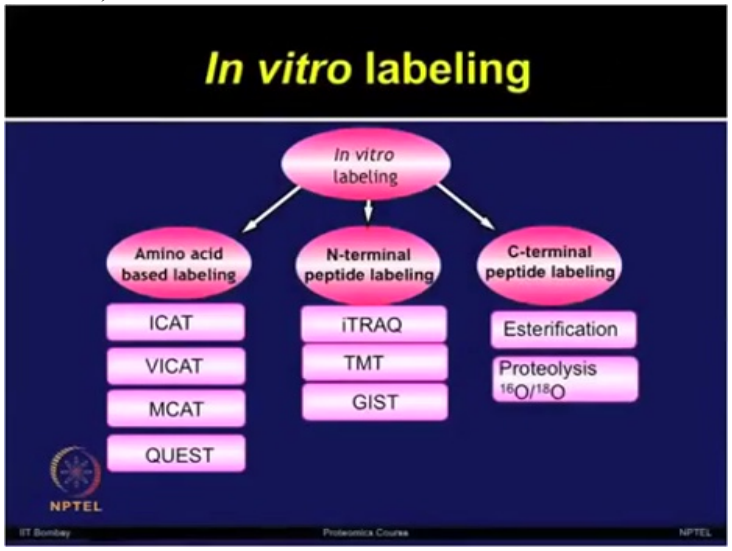

So, in vitro labeling can be 3 different types; amino acid based labeling, N-terminal peptide labeling, C-terminal peptide labeling. In the amino acid based labeling such as ICAT or Isotope Coded Affinity Tag, there are other amino acid based-labeling methods as well such

as VICAT or Visual Isotope Coded Affinity Tag, MCAT or Mass Coded Abundance Tagging, QUEST or Quantitation Using Enhanced Signal Tags.

Then there are different type of N-terminal peptide labeling methods such as iTRAQ and TMT which we are discussing today in detail. iTRAQ also known as isobaric Tagging for Relative and Absolute Quantitation, TMTs are Tandem Mass Tags and GIST, Global Internal Standard Technology (GIST).

Then there are C-terminal based peptide labeling methods such as esterification and proteolysis using 16 or 18 Oxygen, using different light and heavy form of oxygen.
So there are variety of in vitro labeling based approaches available and depending upon your quantitative application there is a possibility of using different type of tagging strategies.

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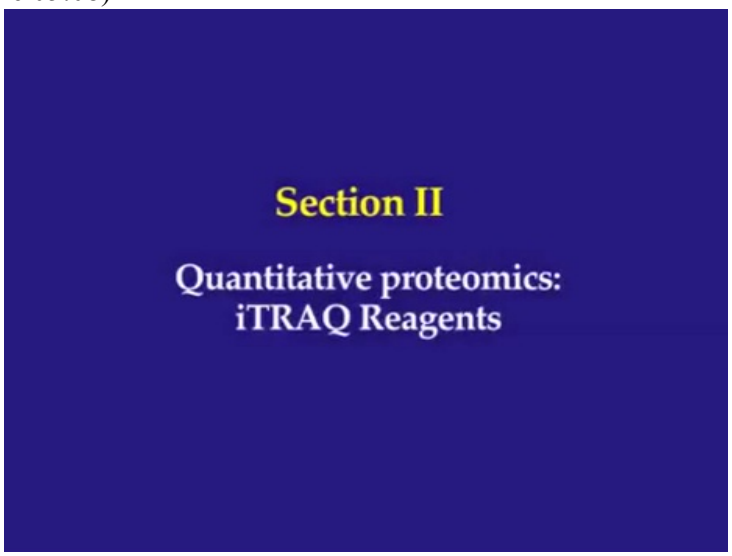

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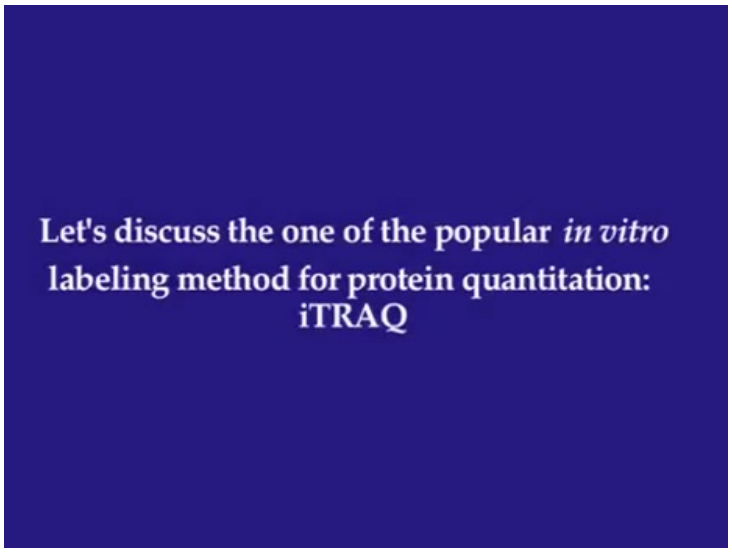

So as I discussed that Mass Spectrometry has played a very major role in proteomics

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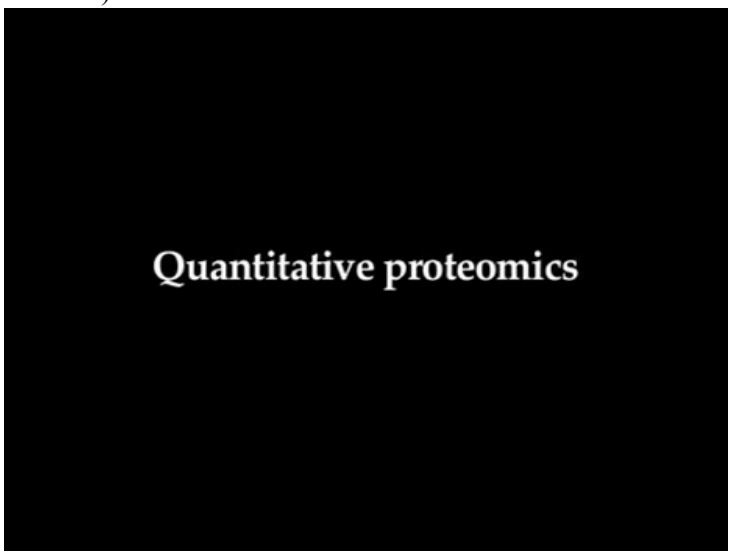

and now it is becoming a very essential tool to study the complex biological system in various diseases. iTRAQ is a Mass Spec based technique for relative and absolute quantitation of proteins present in up to 4 samples or up to 8 samples depending upon the type of iTRAQ tags.

And these labels can be provided in the proteins where there are N-terminal. The iTRAQ labels from Applied Biosystems and Tandem Mass Tags labels are available from Thermo Fisher; there are currently the only commercially available tagging technologies where quantitation can be carried out in the MS/MS mode.

The iTRAQ labels available from Applied Biosystems and Tandem Mass Tags available from Thermo Fisher are currently the only tagging technologies which are commercially available where quantitation is be carried out in the MS/MS mode.

The iTRAQ technique was first time described by Ross et al. in 2004, I have given you reference in the bottom in my slide and it was subsequently commercialized by Applied Biosystems.

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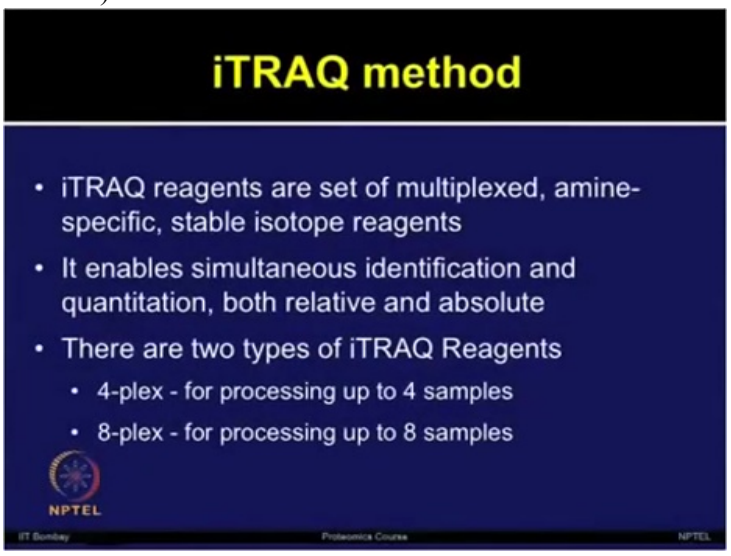

So these iTRAQ reagents are set of multiplexed, amine-specific stable isotope reagents. It enables simultaneous identification and quantitation, both relative and absolute.

There are two different types of iTRAQ reagent currently available, 4-plex for processing up to 4 samples and 8-plex for analysis of up to 8 samples.

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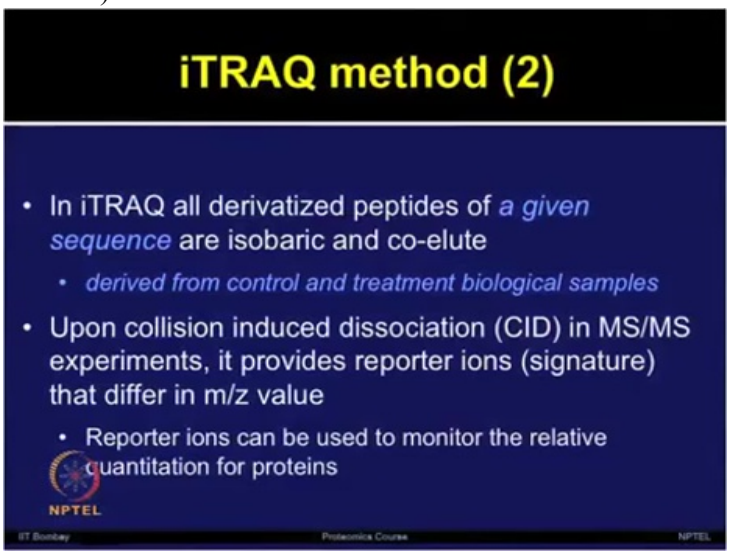

In iTRAQ method the derivatized peptides of a given sequence, they are isobaric and they are co-eluted because they are derived from the control and treatment biological samples. So in the Mass Spec, upon collision induced dissociation during the MS/MS experiments, it provides reporter ion, signature ion for… which differs in mass and charge ratio.

So the reporter ions are used to track the quantitation and can be used to monitor the relative quantitation for proteins. Now we will discuss about the iTRAQ reagent

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There are set of 4 isobaric amine specific labeling reagents, 114, 115, 116 or 117.

The iTRAQ reagent consists of a reporter group, a balancer group and a peptide reactive group PRG. The protein reactive group labels the N-terminals of all the peptides as well as the free amine groups of lysine side chains. The neutral balance portion and reporter group, they provide total mass of 145.

So this method can allow the multiplexing of up to 4 or 8 different samples in a single LC-MS/MS experiment. The different distribution of isotopes between the reporter and balance group makes the label isobaric and it enables the detection upon fragmentation and their release in Mass Spec.

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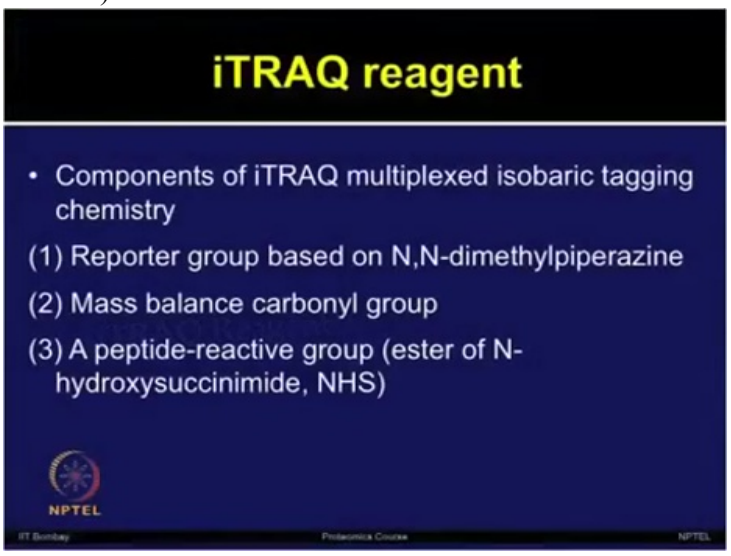

So as I briefly talked, there are three major components of the iTRAQ reagent. One is the reporter group which is based on N, N-DiMethylPiperazine or DMP. Second is a mass balance carbonyl group and third a Peptide Reactive Group which an ester of N-HydroxySuccinamide NHS.

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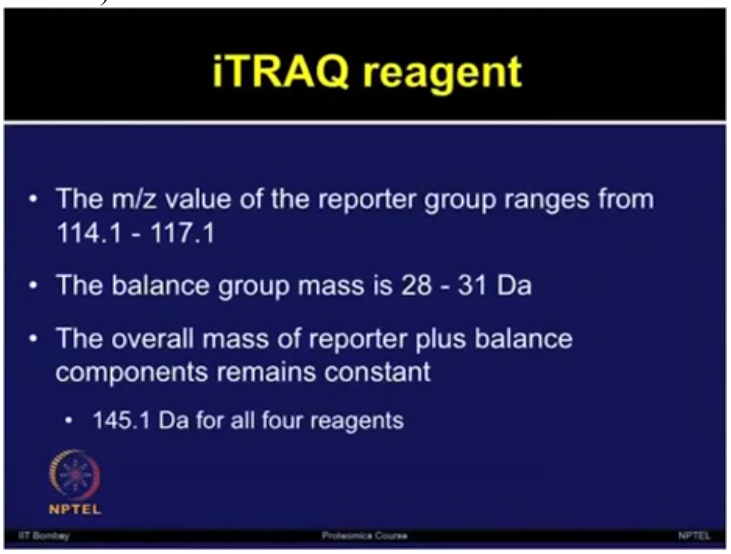

So the iTRAQ reagent, the mass to charge value of these reporter groups, they range from 114.1 to 117.1 if you are using a 4- plex reagent.

The balance group of mass is 28 to 31 Dalton. The overall mass of reporter plus balance components remains constant. So 145.1 Dalton will remain for all the 4 reagents.

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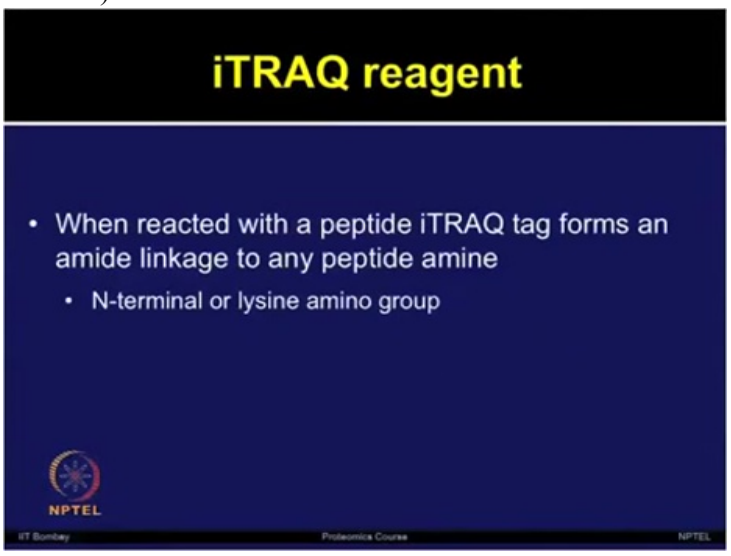

So, when reacted with a peptide iTRAQ tag forms an amide linkage to any peptide amine; N-terminal or lysine amino group.

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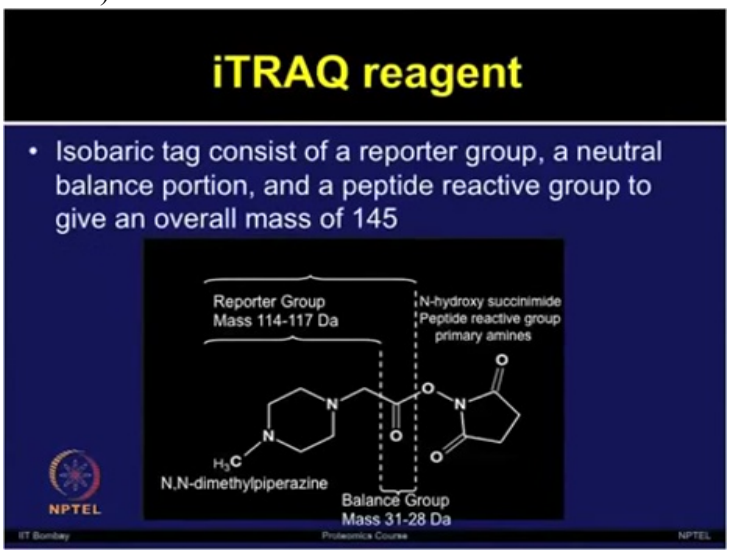

Now this is the structure of iTRAQ reagent where you can see that it consists of a reporter group, a neutral balance portion and a peptide reactive group. Overall mass will remain 145.

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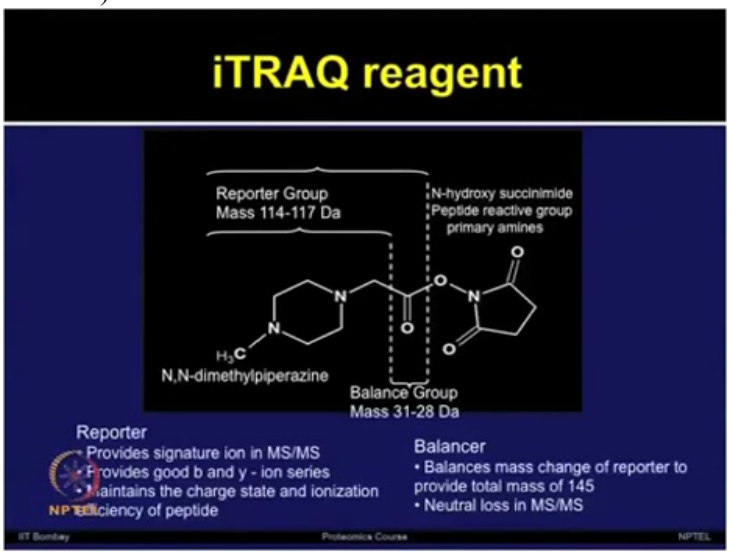

The reporter group provides signature ions in MS/MS mode. It provides good b and y ion series and it maintains the charge state and ionization efficiency of peptides.

Whereas the balancer group, it balances the mass change of reporter to provide total mass of 145 neutral mass in MS/MS mode.

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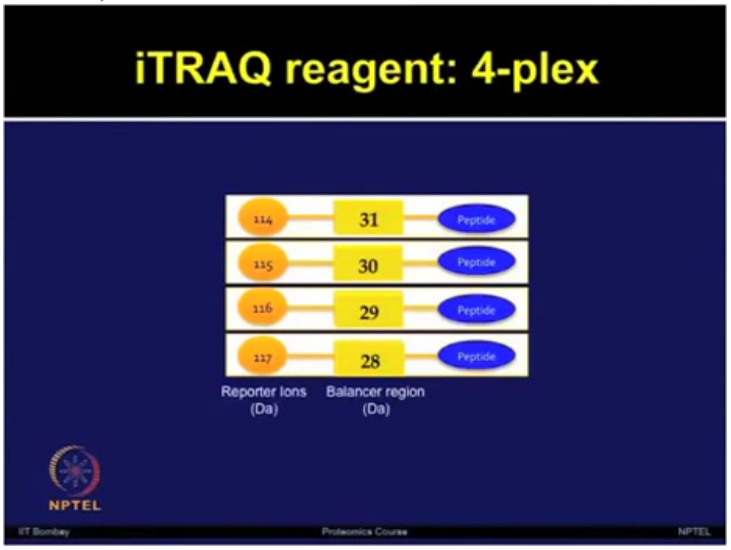

The iTRAQ label is an isobaric tagging compound consisting of reporter ion series. Here I have shown you a 4-plex iTRAQ reagent with 114 to 117 Dalton, a balancer region 191 to 188 Dalton, so that the total mass of isobaric tag is 305.

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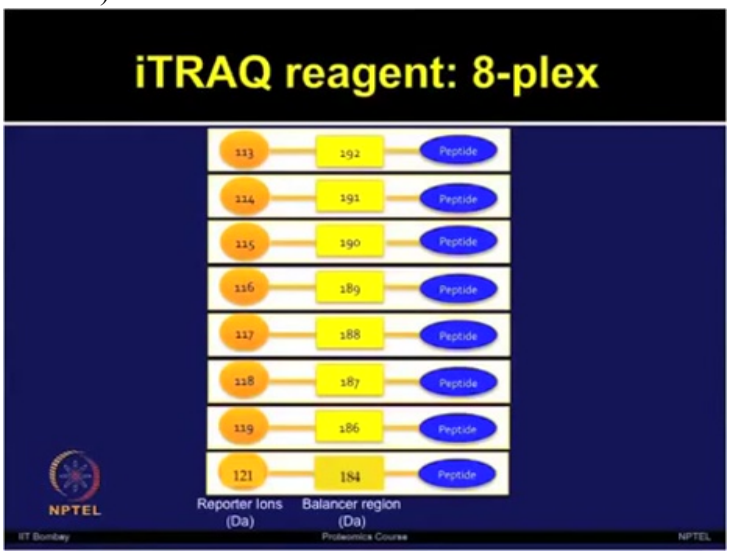

Now let us look at the structure of iTRAQ reagent 8-plex. In 8-plexing reporter ions, the variable masses from 113 to 120 Dalton and the balancer region is from 185 to 192 Dalton so that the total mass of isobaric tag remains 305.

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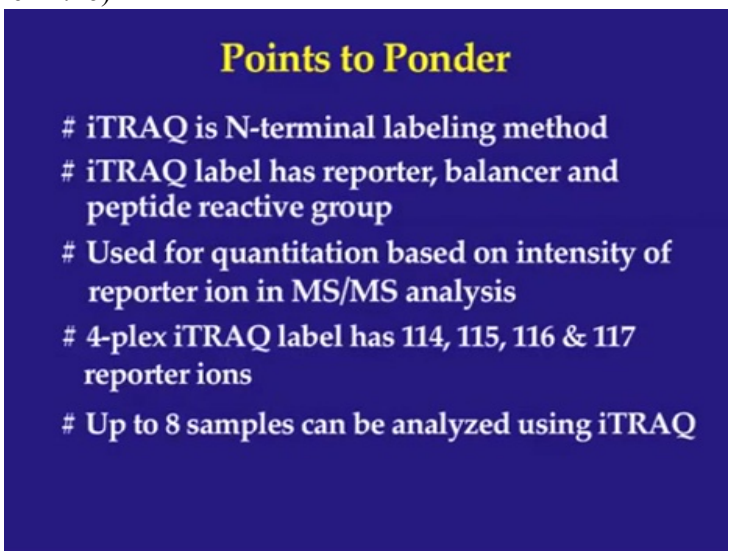

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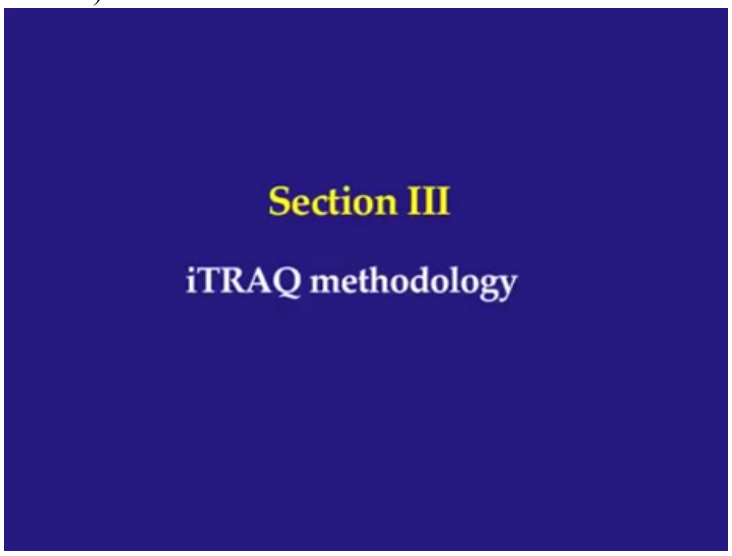

We will now talk about how to perform an iTRAQ experiment. So, in an iTRAQ experiment, the control and treated proteins samples, they are first reduced, alkylated and digested with trypsin.

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The digested samples are reacted with different iTRAQ reagents, 4-plex or 8-plex. Two samples are then combined; in fact control and 3 treatments or it can be 4 different type of time-course samples.

So 4 samples can be combined and analyzed by LC MS/MS. So as per the guidelines provided by Applied Biosystems, the protein samples can be prepared and now we will discuss that step-by-step in the next few slides.

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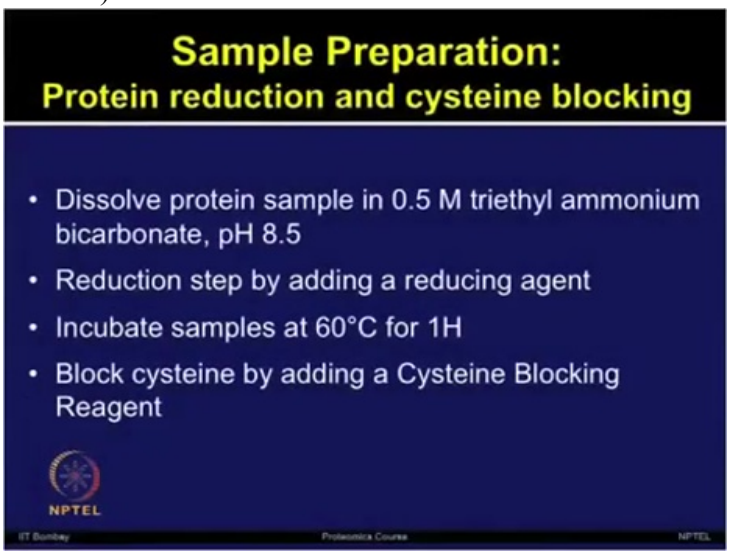

So in the sample preparation first part is protein reduction and blocking of cysteines. So first, dissolve the protein samples in 0.5 molar triethyl ammonium bicarbonate at pH 8.5. Then perform a reduction step by adding a reducing agent. Incubate the samples at 60 degrees for an hour. After that, add a cysteine blocking reagent so that the cysteine residues can be blocked.

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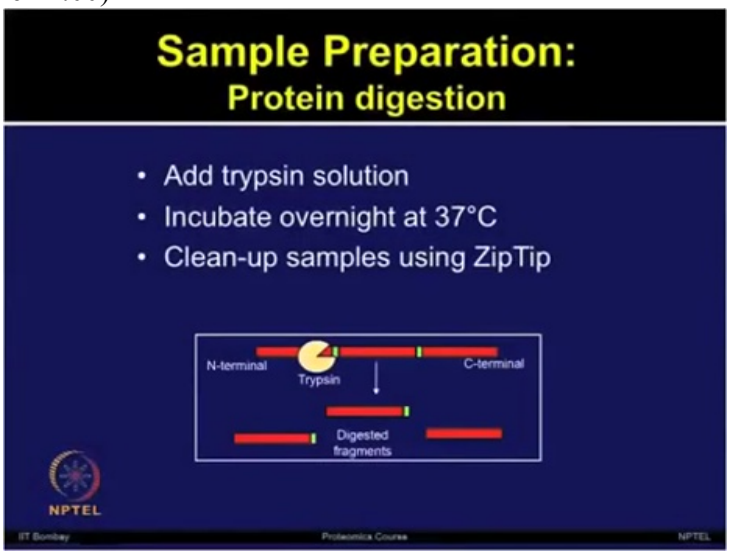

Once reduction and cysteine blocking is done then the protein digestion can be performed. Add trypsin solution; incubate overnight at 37 degrees so that these proteins can be digested into peptides. You may have discussed this thing briefly in the previous module when we talked about in-gel digestion for doing the gel-based proteomics.

Similar types of concepts are applied here but this one is in-solution digestion. Once you have done the over-night digestion then a cleanup step can be performed by using ZipTip so that some salts and contaminants can be removed.

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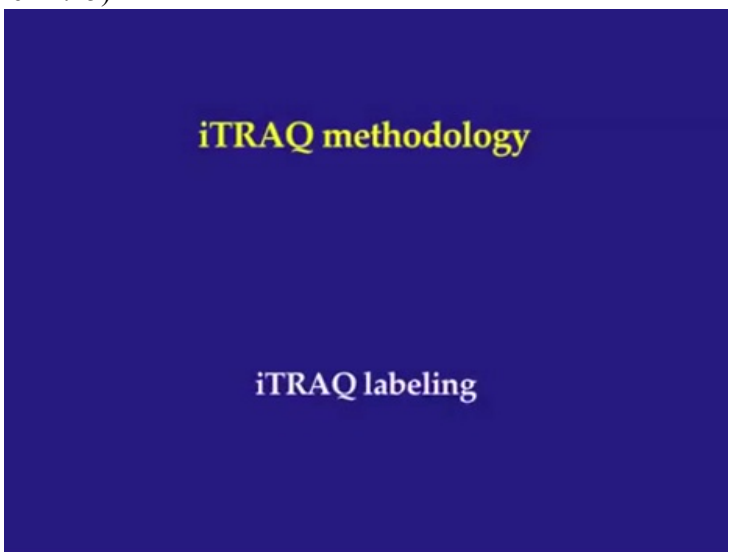

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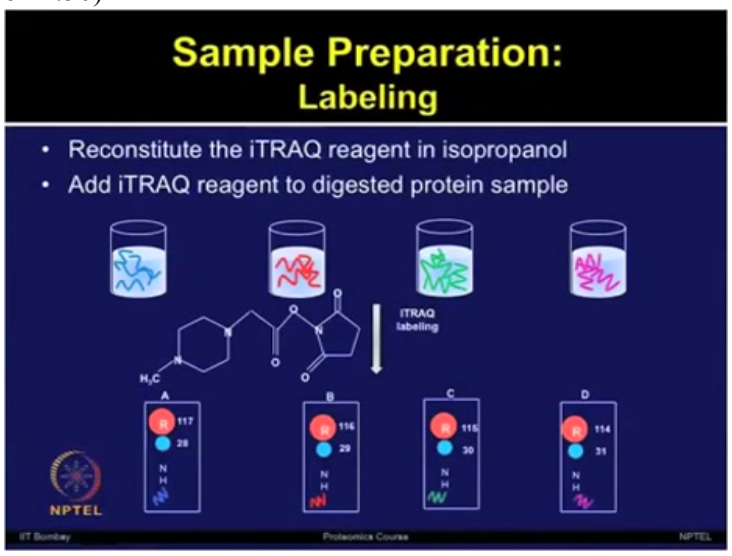

Now next step is labeling, adding these iTRAQ reagents to 4 different samples

So first reconstitute the iTRAQ reagent in isopropanol. Add iTRAQ reagent to the digested protein samples. If you have 4 samples you can use with the 4-plex iTRAQ reagents, 114, 115, 116 and 117, as I have shown in this PPT.

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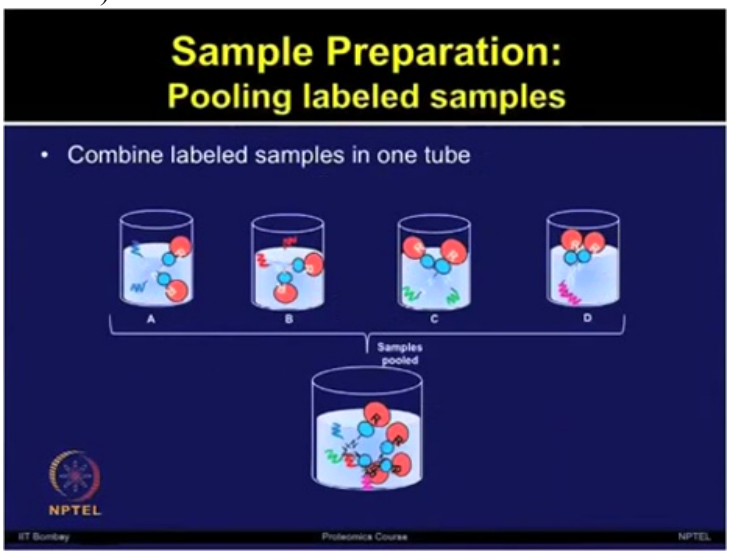

Now combine these labeled samples in one tube so that later on sample processing will not have any manual artifact. So the labeling is performed separately, but after that all the labeled samples are pooled in one common tube.

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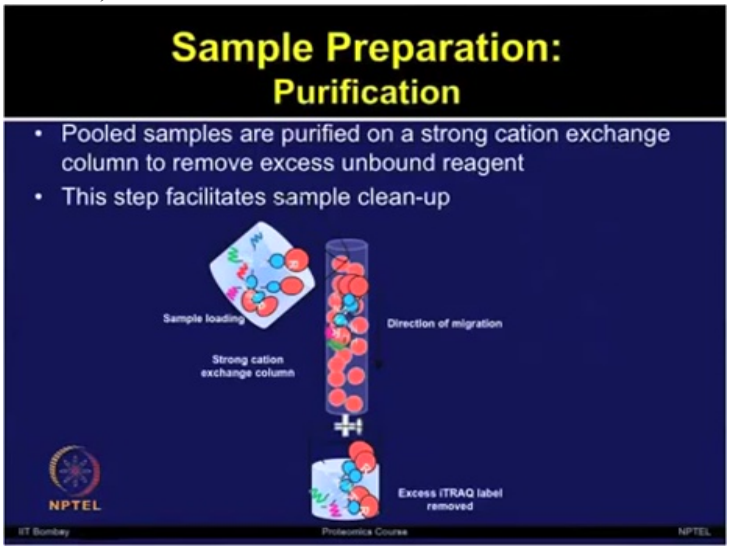

Now these pooled samples can be further purified on a strong cation exchange column so that the excess unbound reagents can be removed. This is an optional step but this does help in proper cleaning and getting a better signal. So this step facilitates sample clean-up.

And this is, although not essential, but recommended to perform this step.

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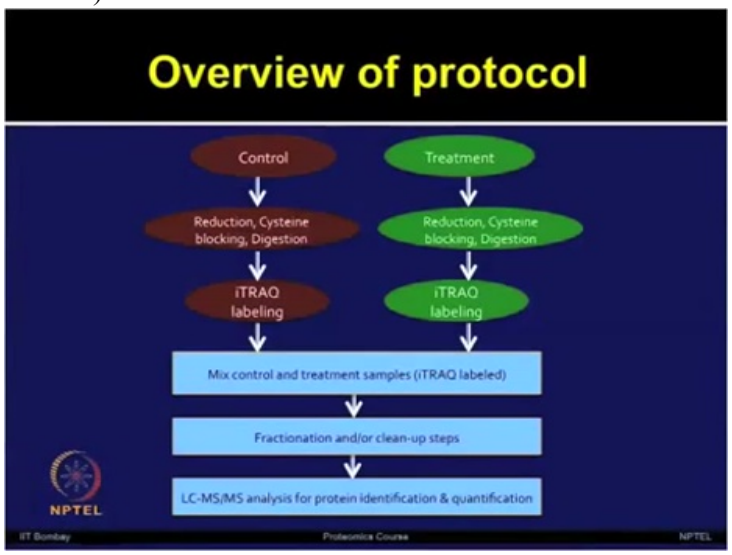

So now Let us have an overview of this protocol. So we have control and treatment populations. Each of those samples were first reduced, cysteine blocked and digested. After that, iTRAQ labeling was performed for each sample.

Then control and treatment populations were all mixed together with the iTRAQ labels. Fractionation and/ or clean-up steps were performed and then LC-MS/MS analysis can be performed for protein identification and protein quantification.

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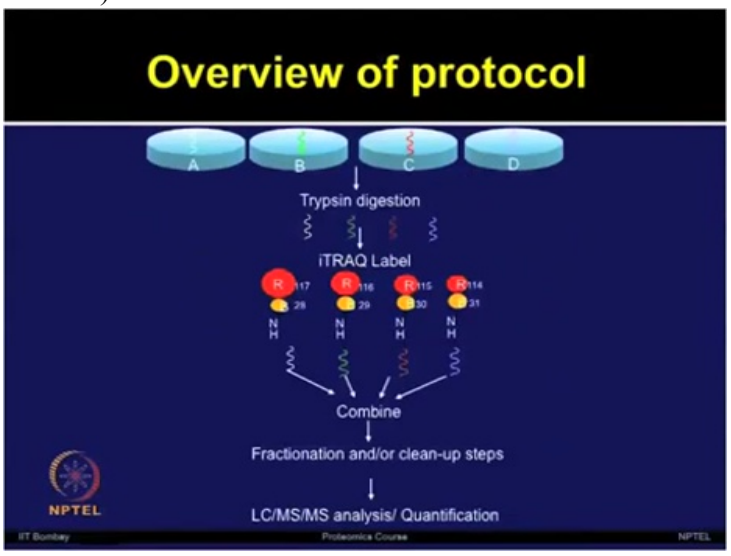

Now I think in this image it will be more clear to you the overall steps. You have four different populations A, B, C and D.

Each population is reduced, cysteine blocked, trypsin digested and then further labeled with 4 different types of iTRAQ labels, combined, fractionated and further LC-MS analysis can be performed and quantitation can be obtained.

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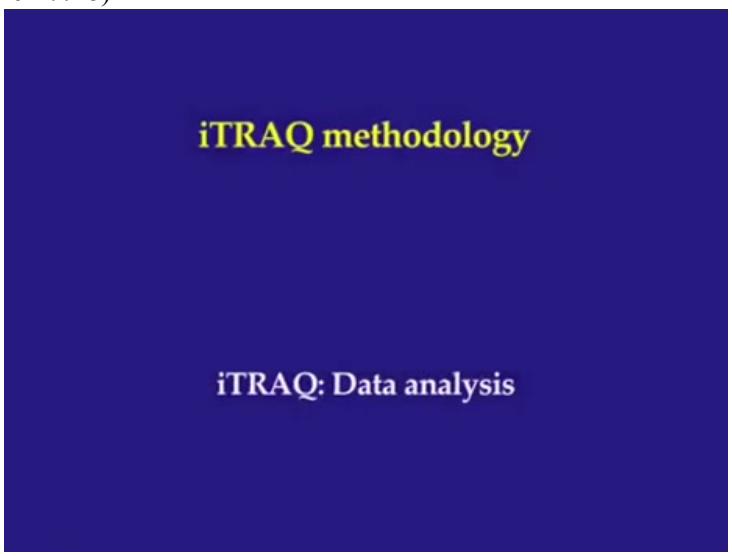

Once the sample preparation is done then further Mass Spectrometry analysis can be started. The peptides which are differentially labeled, they can be mixed together and measured by mass spectrometer.

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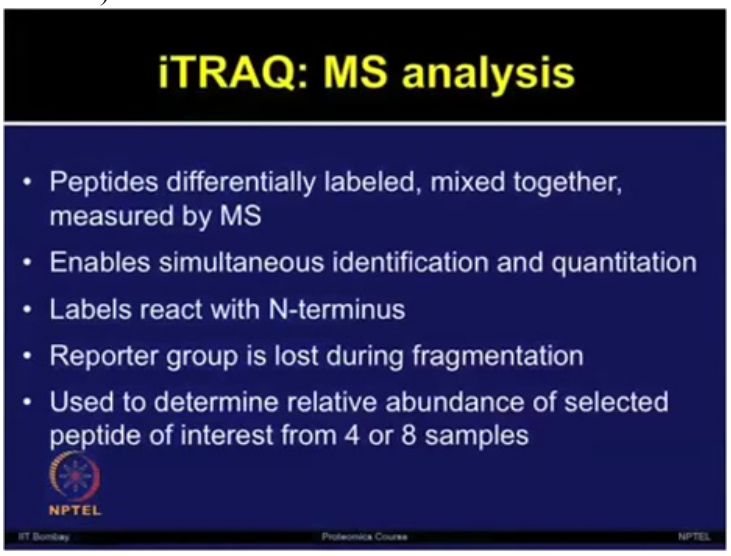

This method enables simultaneous identification and protein quantification. The labels react with the N-terminals and the reporter group is lost during the fragmentation.

So this method can be used to determine the relative abundance of selected peptides of interest from 4 or 8 samples. Therefore, high-multiplexing can be obtained by using iTRAQ method.

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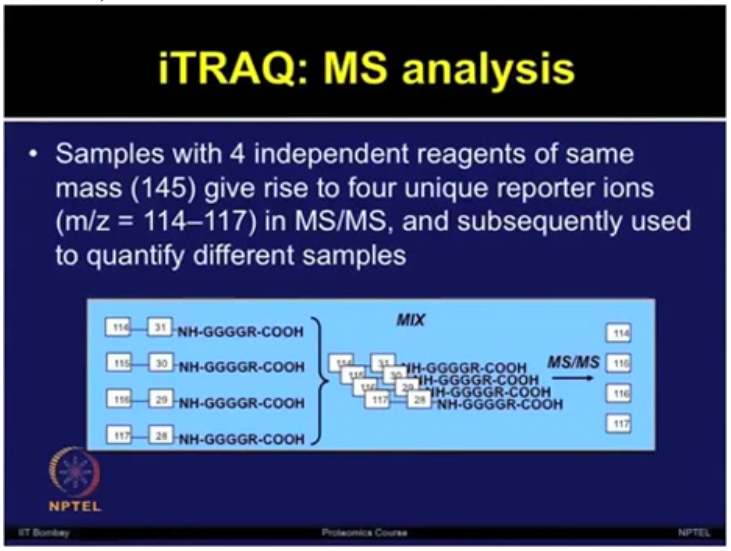

4 independent reagents of same mass, 145 can give rise to 4 unique reporter ions, m by z 114 to 117, in MS/MS analysis and subsequently these reporter ions can be used for quantification of different samples.

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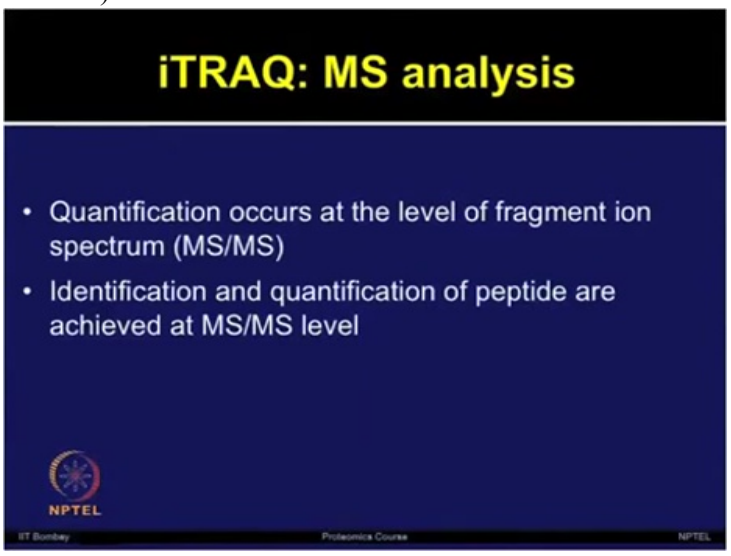

So, in iTRAQ the quantitation occurs at the level of fragment ion spectrum at the MS/MS level. The peptides with the identical sequence but derivatized with different isobaric reagents are indistinguishable during the MS spectra alone.

That is where only during the MS/MS phase the qunatitation can happen. So it shows that identical fragment ion series for the peptide-derived fragments but it shows low mass reagent derived fragment ions, that indicate that sample of origin and permit the quantitation.

So the identification and quantification of peptides are achieved during the MS/MS analysis. The iTRAQ enables the quantitative protein profiling of multiplex samples without making the MS spectra complicated. It reduces the redundancy to selecting the MS precursor ions for collision induced dissociation and enhances the efficiency for MS/MS analysis and peptide identification

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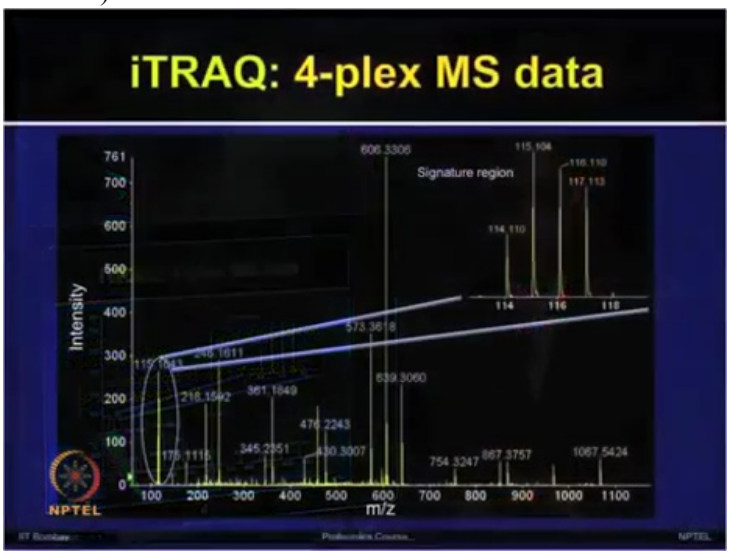

This is a representative spectra for the 4-plex iTRAQ experiment, the MS data is shown and MS/MS spectrum is showing the reporter region, signature of these 4-plex iTRAQ labeled peptides 114, 15, 16 and 17.

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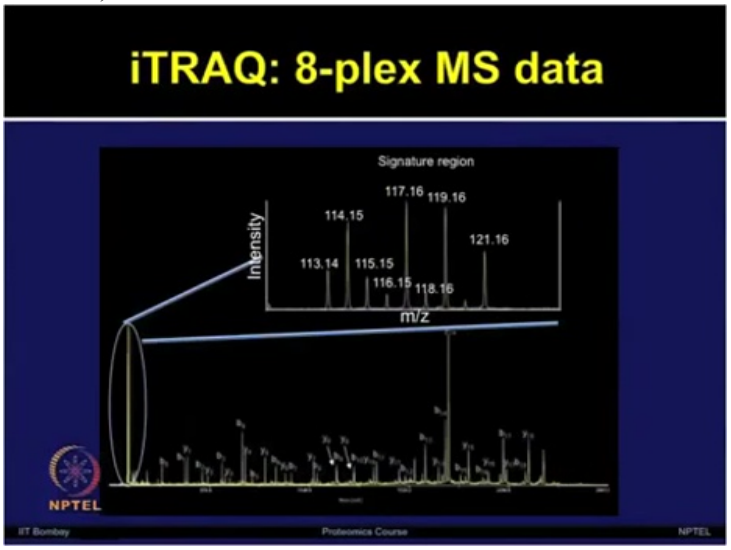

Now we will have a look on the 8-plex data. In the MS/MS spectrum, here we are showing the reporter region signature of a 8-plex iTRAQ reagent showing 113.14, 114.15, 115.15, 116.15, 117.16, 118.16, 119.16 and 121.16 reporter ions.

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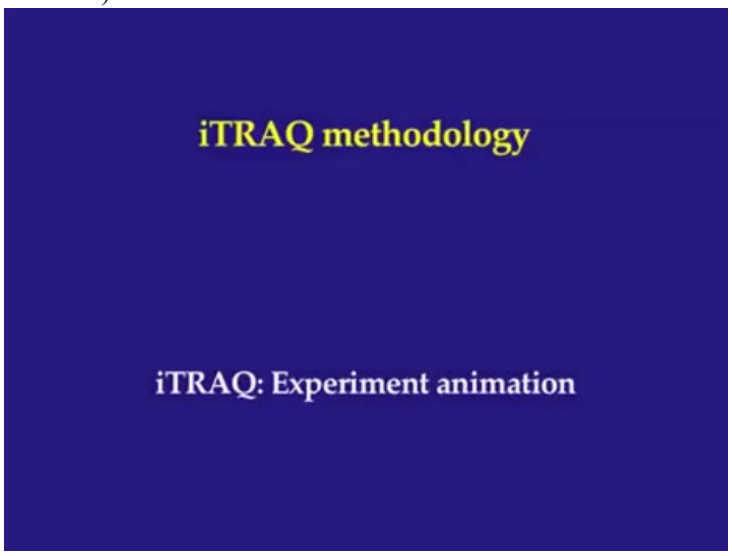

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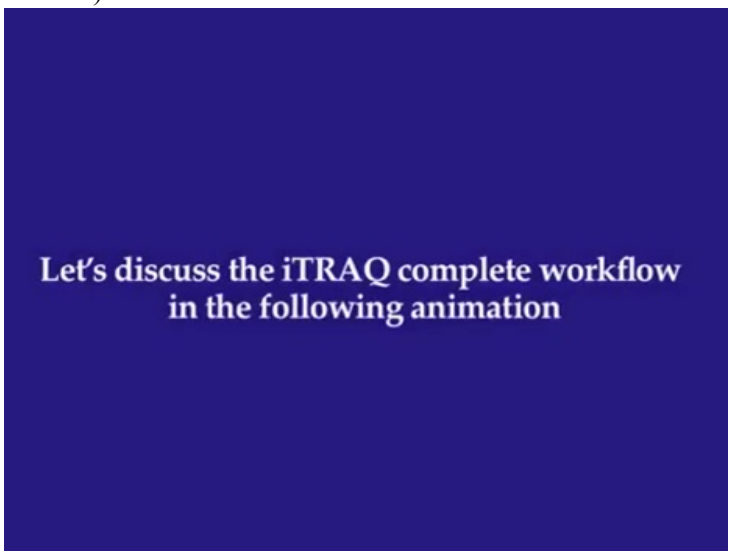

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I will now describe you the overall iTRAQ technique by showing you an animation.

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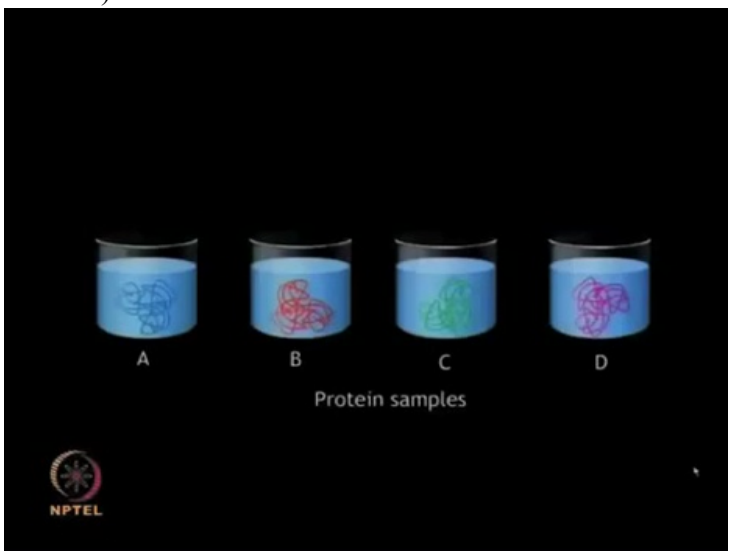

In this animation we will talk about iTRAQ technique. The protein samples to be analyzed are first

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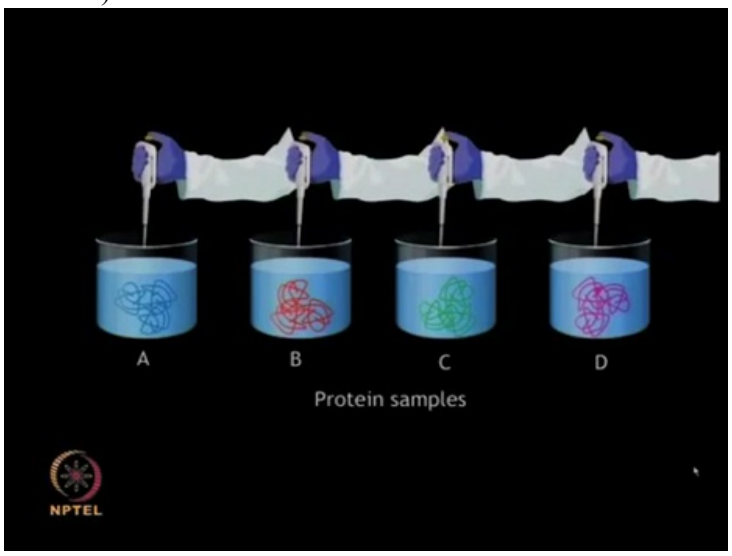

digested with trypsin into smaller peptide fragments

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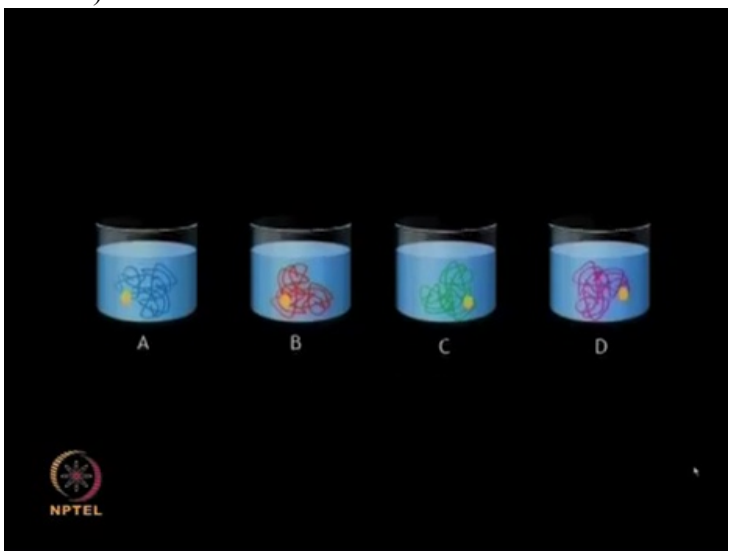

The trypsin cleaves the proteins at the C-terminal of lysine and arginine residues

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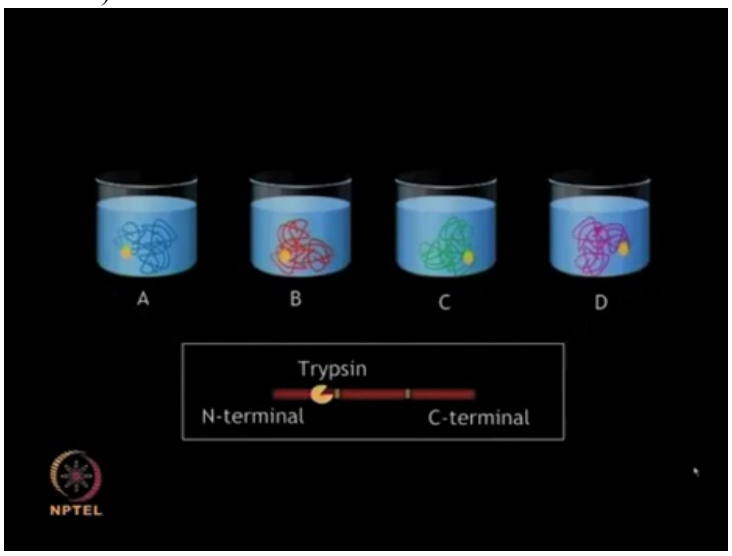

unless they are followed by a proline residue

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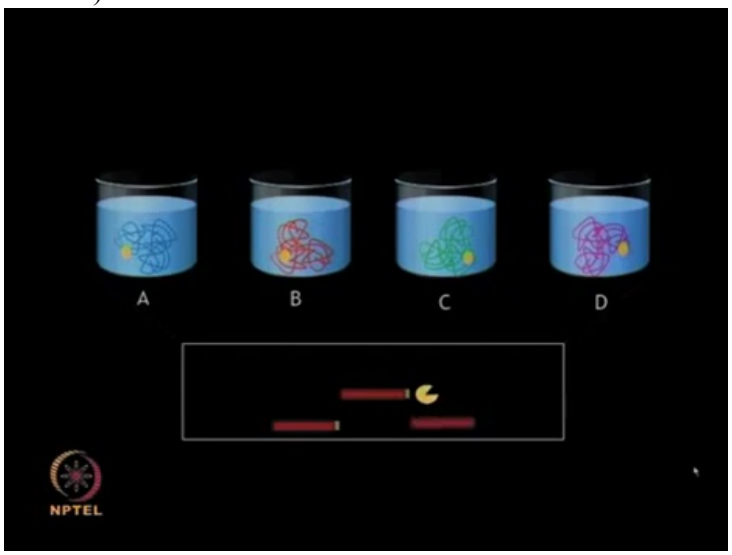

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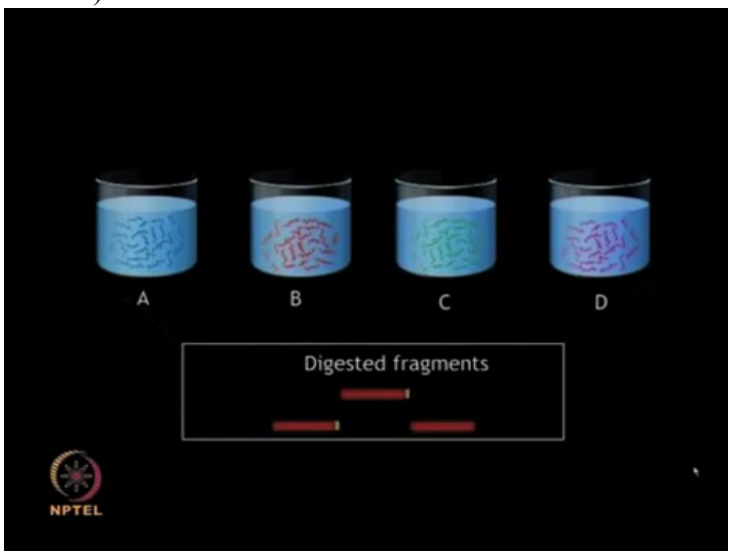

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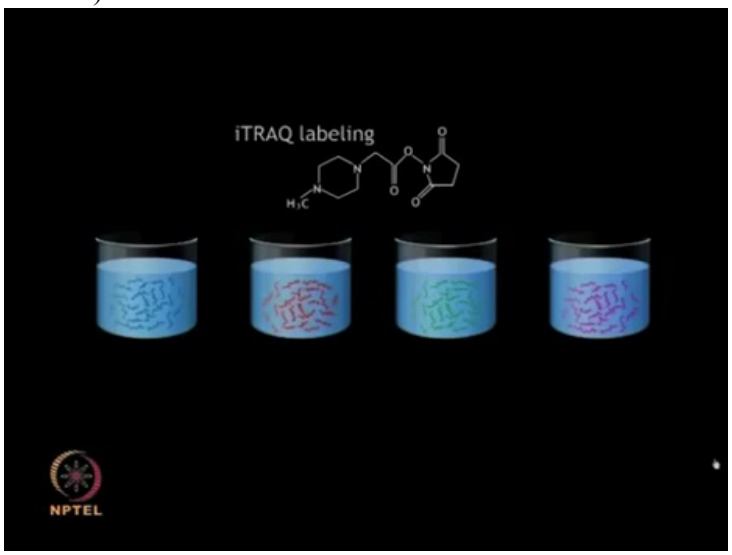

The iTRAQ reagent consists of a reporter group, a balance portion and a peptide reactive group that interacts with the N-terminals of the peptide or free amino group of Lysine residues,

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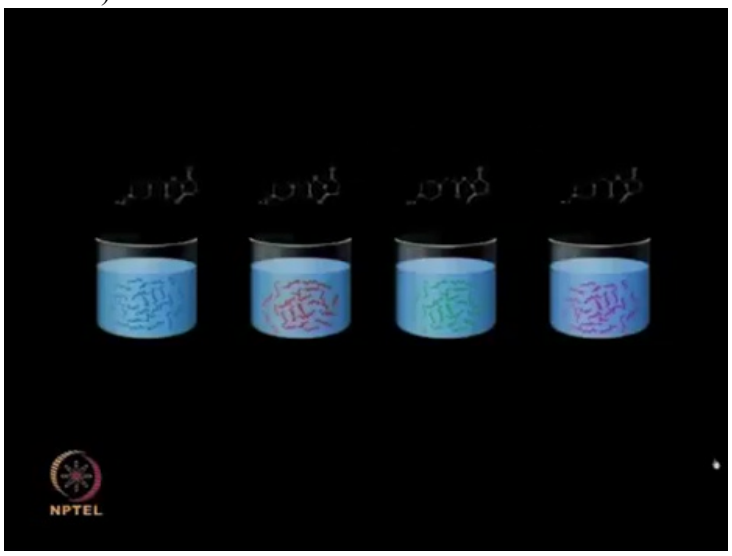

giving an overall mass of 145

The reporter group used to label each peptide sample is unique and the mass varying between the 114 to 117,

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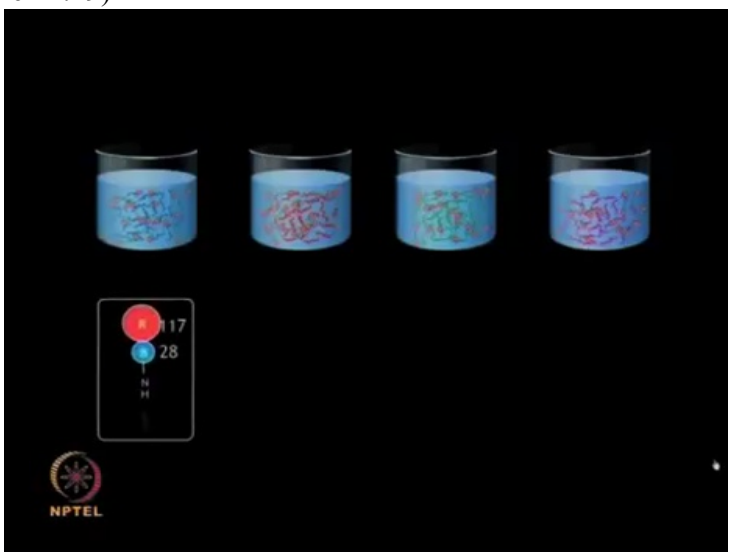

thereby enabling the labeling and quantification of up to 4 samples simultaneously

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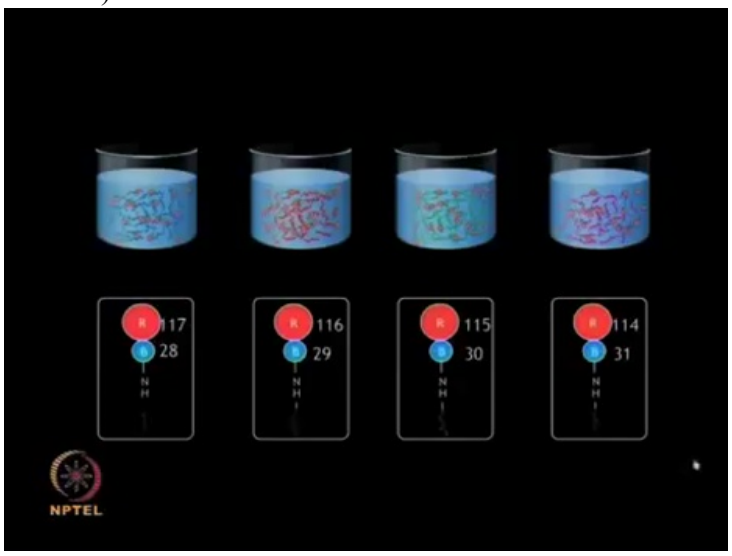

As you can see in this animation, and first one, reporter group 117, and balancer group 28, in second sample, reporter group 116, balancer group 29, in third sample, reporter group 115 and balancer group 30, and in fourth, the reporter group 114, and balancer group 31, overall 145 Dalton.

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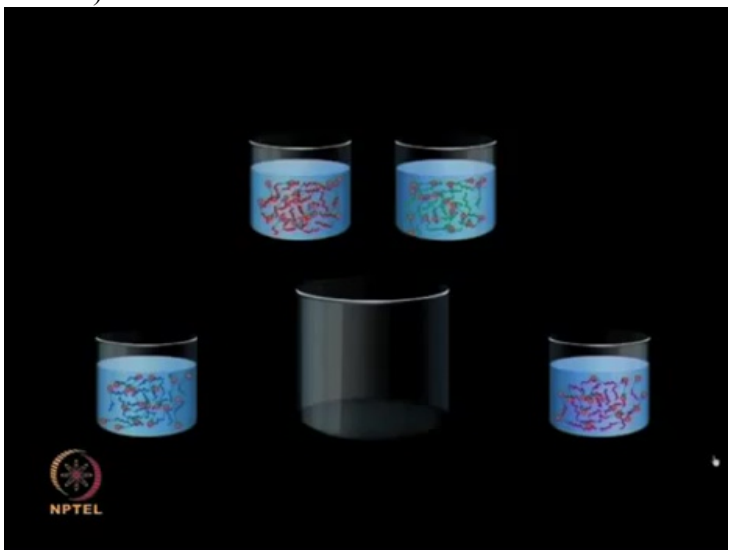

This has been further improved

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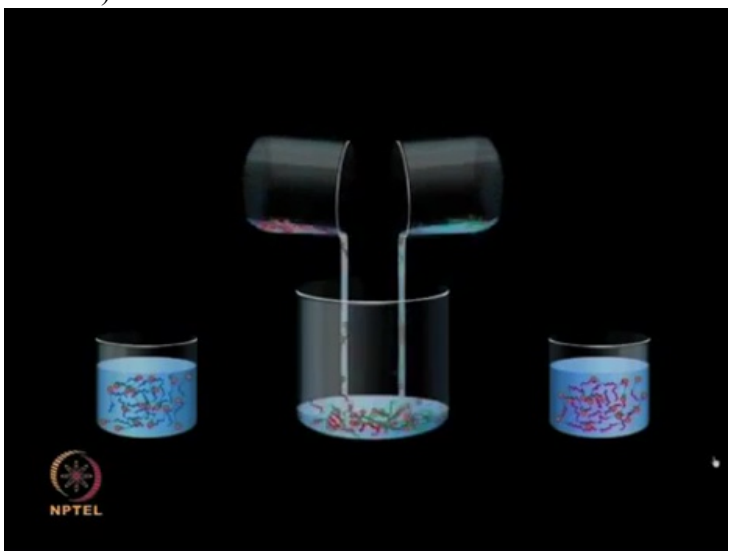

to allow labeling of eight samples simultaneously although we are just showing here the 4-plex experiment.

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The labeled samples can be pooled together.

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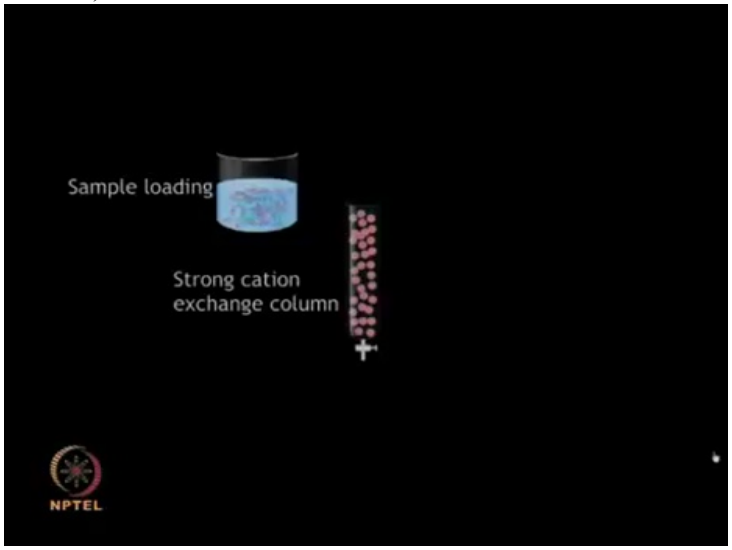

The pooled samples are

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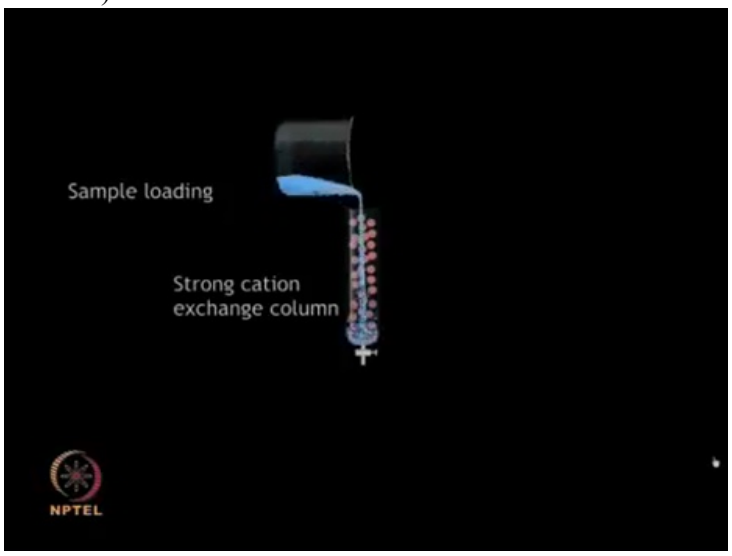

purified on a strong cation exchange column to remove any excess unbound iTRAQ reagent This facilitates sample cleanup

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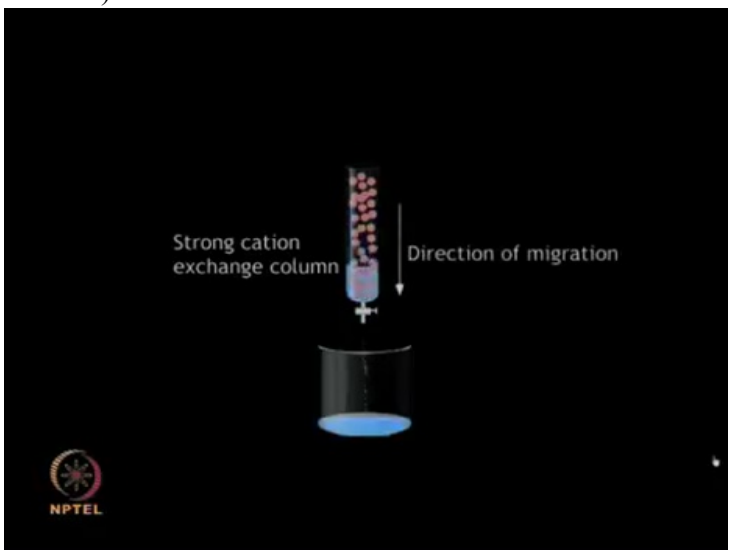

prior to further finer separation and purification using

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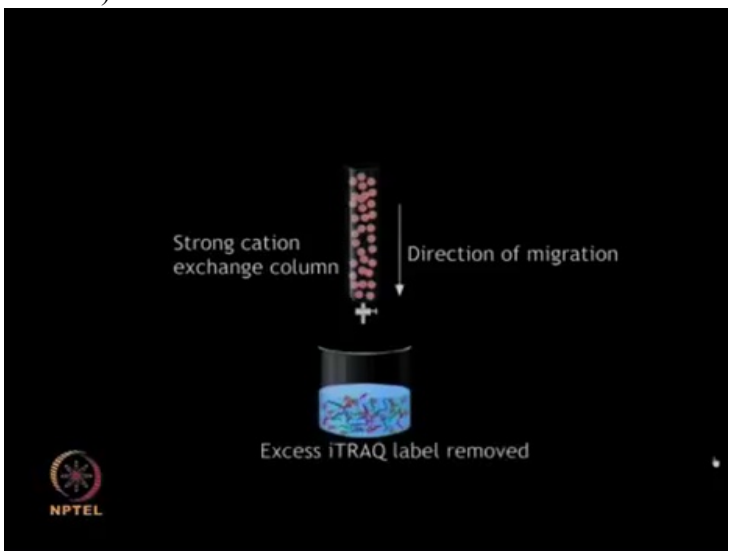

reversed phase chromatography.

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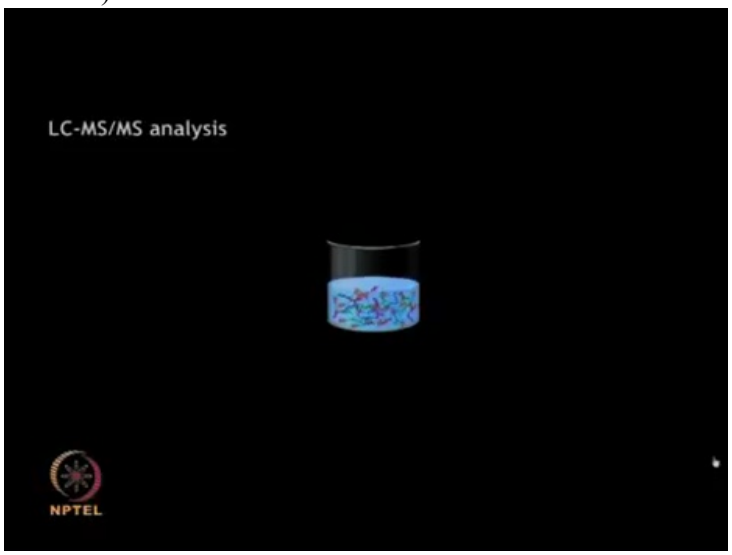

Further purification of the Strong Cation eXchange purified peptides is carried out by

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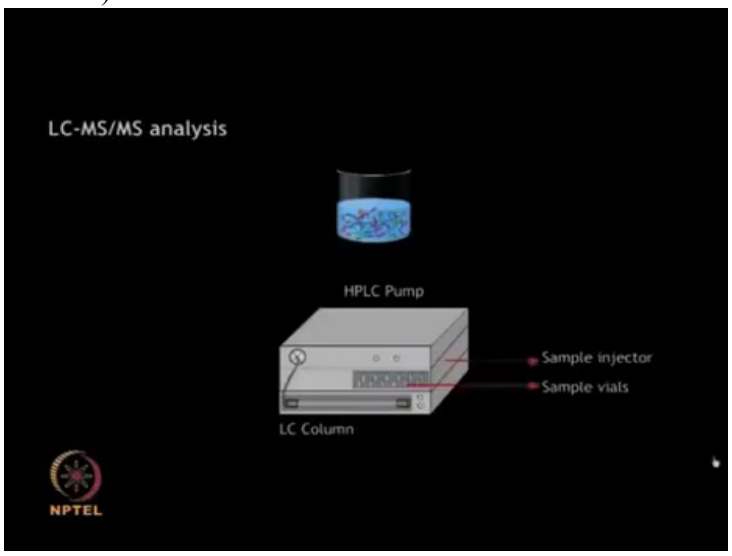

reversed phase liquid chromatography; wherein the sample is passed through

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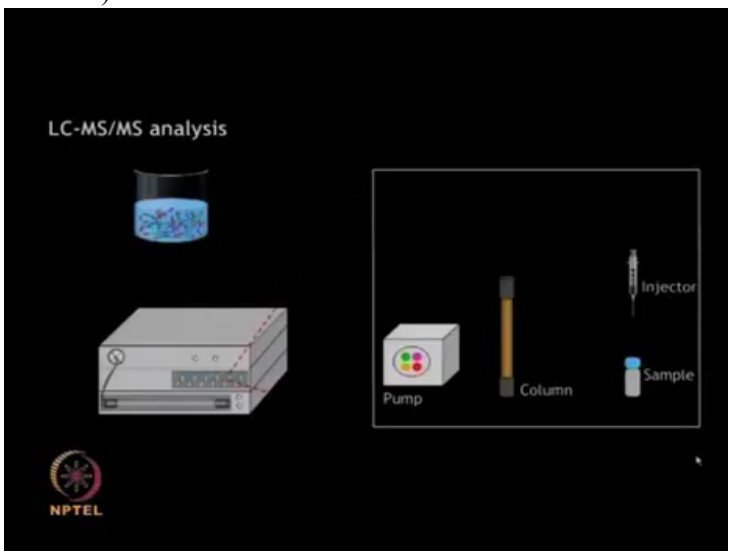

a column containing a packed stationary phase matrix that selectively adsorbs

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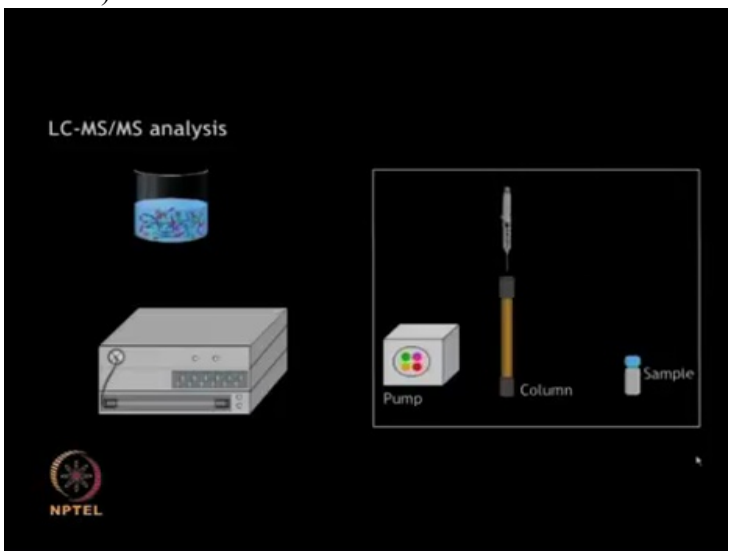

only certain analyte molecules

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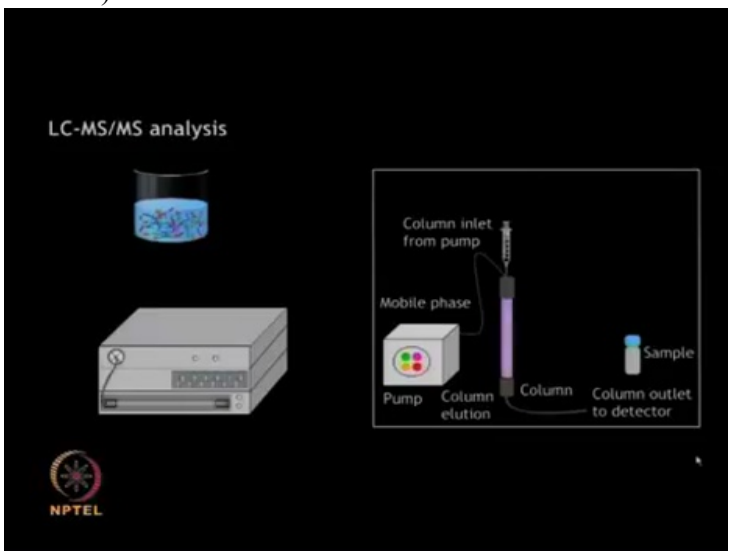

The eluted fractions are further characterized by MS.

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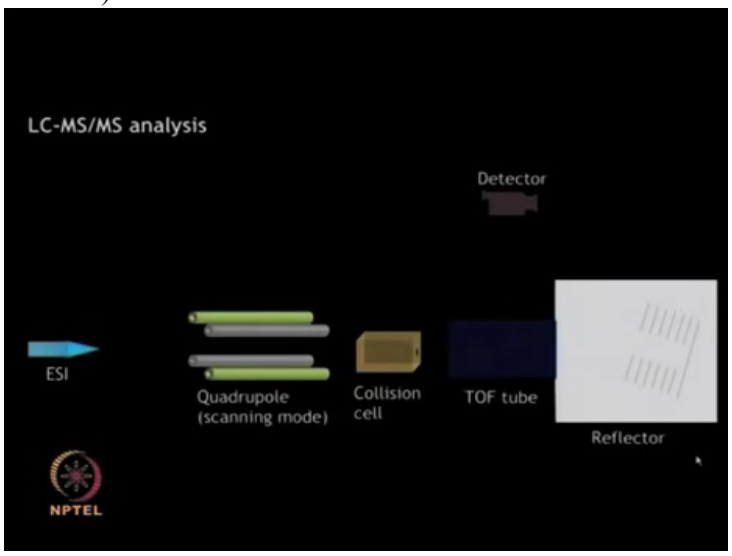

The purified labeled peptide fragments can be analyzed by MS/MS. The different masses of the reporter group allows the peptide fragments to be identified. The reporter group is lost

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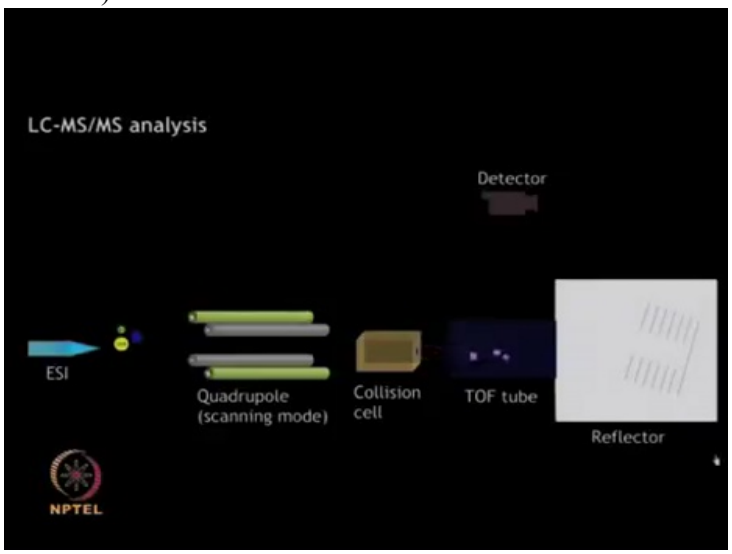

…during fragmentation step

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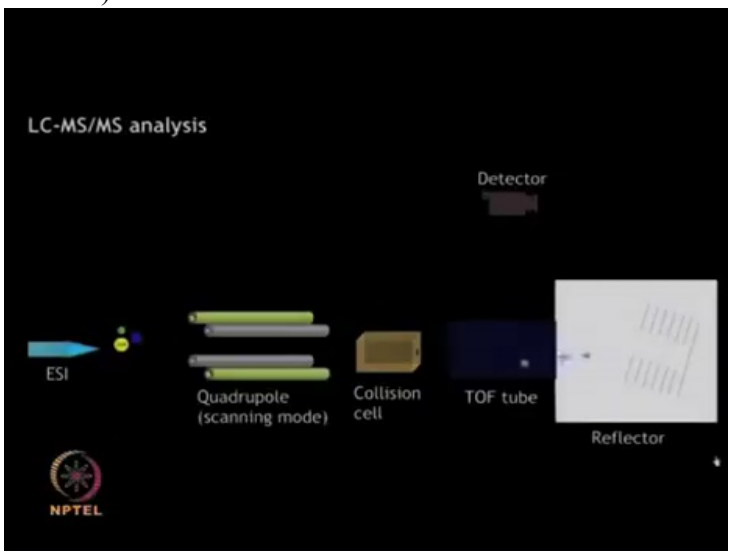

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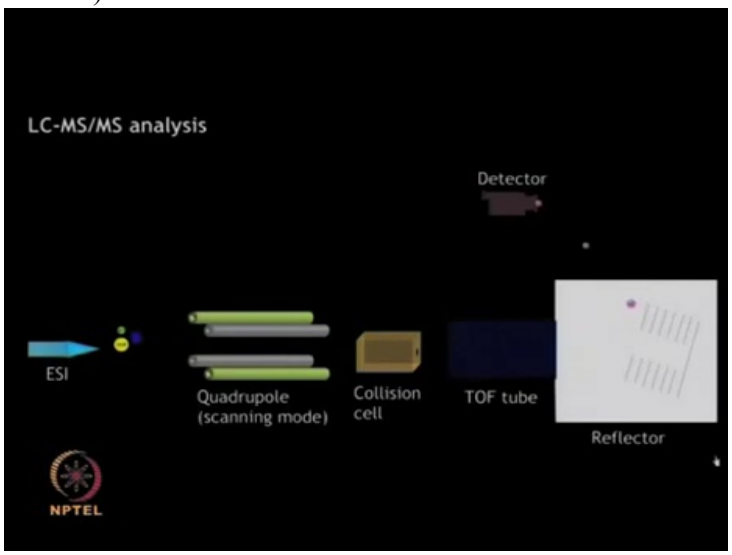

Relative quantification of

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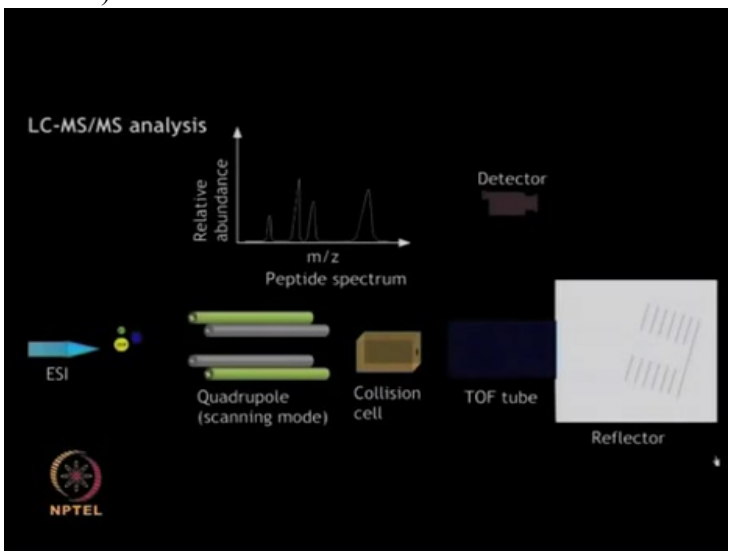

4 samples or 8 samples can now be performed in this way.

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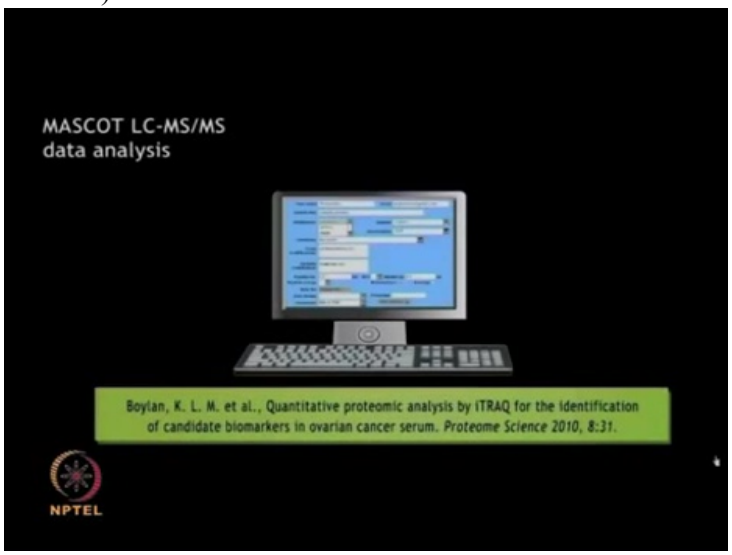

Once iTRAQ experiment is completed, the data obtained from tandem mass spectrometry can be analyzed by

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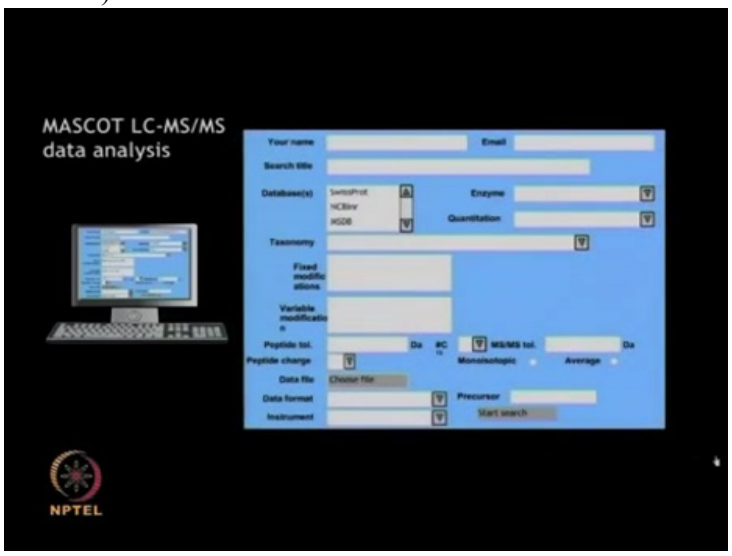

using Mascot and another softwares

The MS/MS data analysis requires inputs from the user regarding the experimental parameters such as enzyme cleavage, protein name, modifications, instrument used, peptide charge etc.

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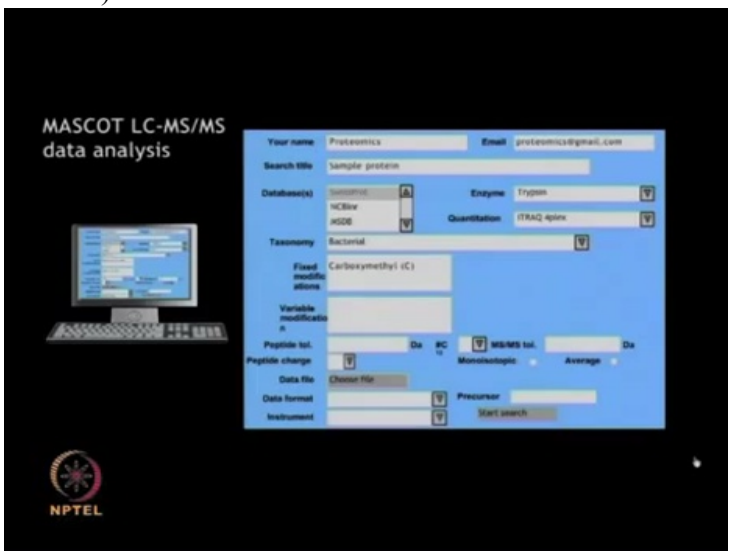

The commonly used protein databases against which the MS information is processed to retrieve sequence data include NCBI, MSDB and SwissProt. The data file generated from the MS is uploaded and the search can be carried out.

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Ok, so I hope that the animation was informative and now you are able to understand all the steps involved in the iTRAQ experiment.

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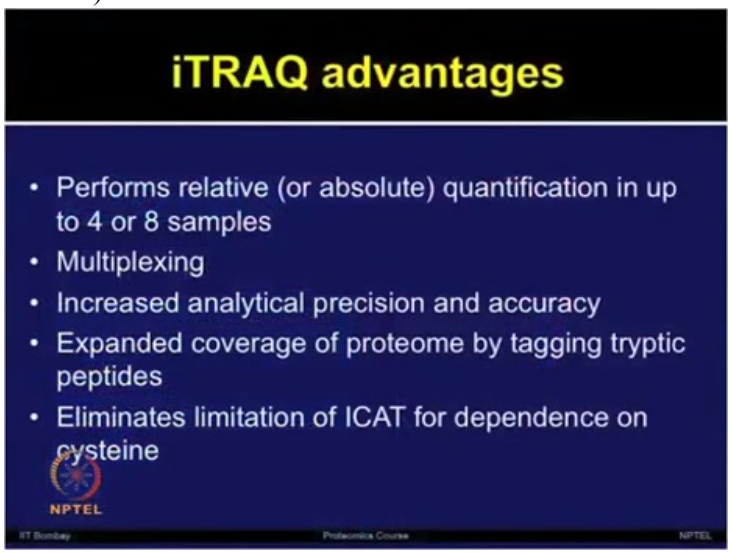

So now let us talk about the advantages of using iTRAQ method. This method performs relative and absolute quantitation up to 4 or 8 samples. It gives good multiplexing capability; it increases the analytical precision and accuracy.

The expanded coverage of proteome can be obtained by iTRAQ method by tagging the tryptic peptides. It eliminates the limitations of previously discussed method, the ICAT, for dependence on cysteine residues.

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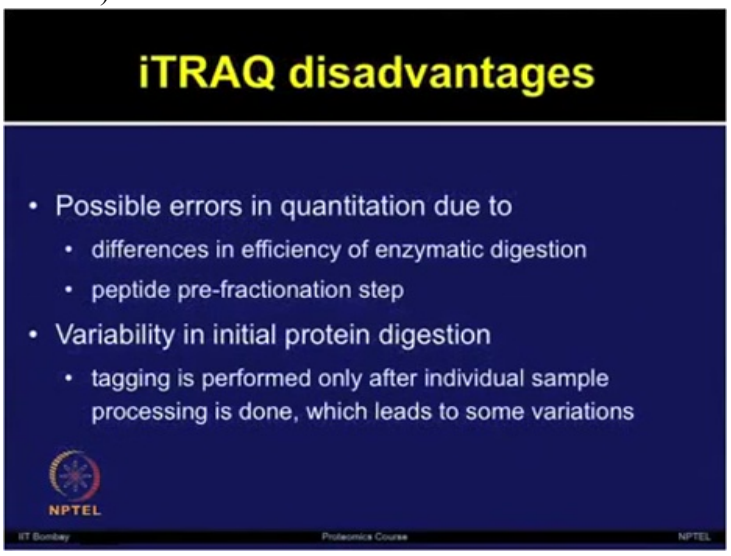

But this method has several disadvantages as well. First of all there is possibility of errors in the quantification due to the differences in the efficiency of enzymatic digestion, because we are performing that step separately for 4 samples.

If you remember, we talked about sample A, B, C and D; each of those was treated separately; and if during the tryptic digestion the efficiency was not exact for the digestion process, may be that could lead to some difference although if you start with the same enzyme lot and if you have done the quantification for the proteins properly. Ideally that should not matter.

But there's some possibility that some errors could be coming from that efficiency of enzymatic digestion. The peptides pre-fractionation step could be another possible way of introducing some variations.

The variability in the initial protein digestion and then tagging is performed only after the individual sample processing is done. So, if we can do the multiplexing in some way that the tryptic digestion cannot be performed separately, that can reduce the overall chances of error.

But that will actually be not possible because we need to label each sample separately with iTRAQ reagents. So these are still of the possible errors and possible disadvantages of iTRAQ method.

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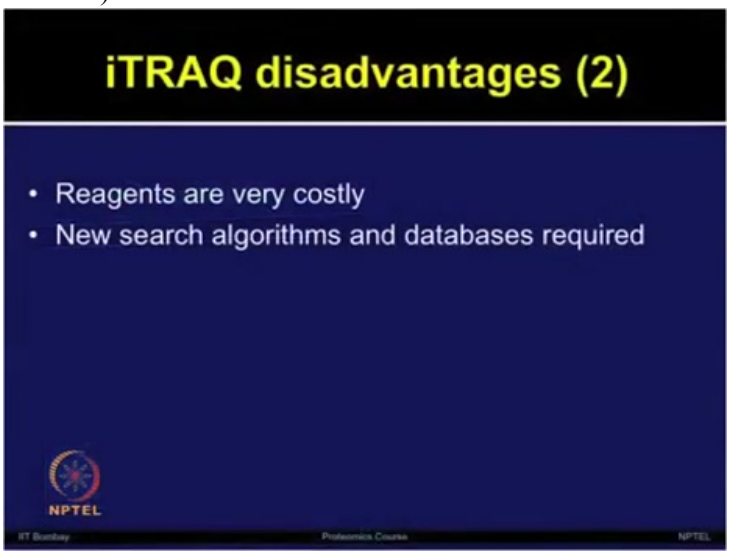

Now these reagents are quiet costly. That is also a limiting factor for many labs to not be able to use that. And various search algorithms and databases are required. Obviously more and more studies are happening. So we have now better software and information available for performing this experiment. But still that is one of the limitation.

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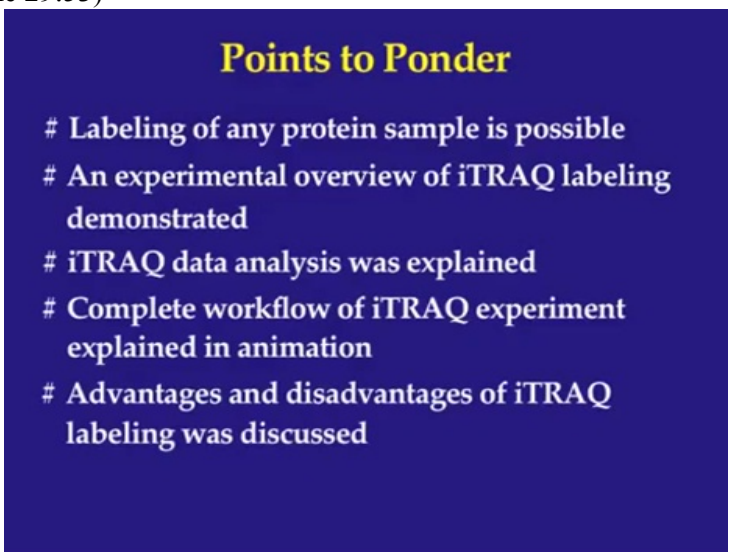

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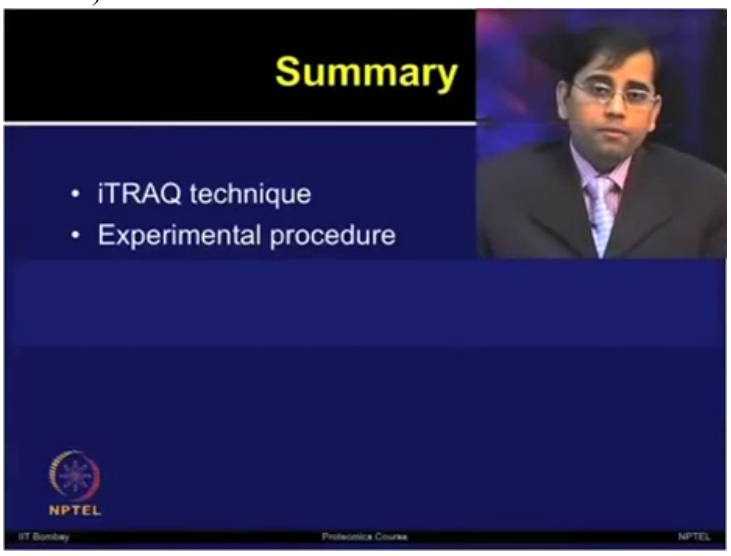

So overall in today's lecture we talked about iTRAQ technique. We compared iTRAQ with ICAT and TMT. And during the discussion we talked in much more detail about how to perform the iTRAQ experiment.

So as you know, that there is been steady advancement in the mass spectrometry, the quantitative proteomics has progressed dramatically in the past few years.

The efforts to analyze proteome of many species, both qualitative and quantitative have generated an abundance of data from a variety of biological samples, thank you.

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