Proteomics : Principles And Techniques

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Lecture No. # 25
Quantitative Proteomics: iTRAQ and TMT

Welcome to the proteomics course. Today we will talk about quantitative proteomics, and discuss about iTRAQ and TMT techniques. The quantitative proteomics aims to answer various questions including the identification of bio markers 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 dirgewhich we have discussed in the last module, and mass spectrometry based measurements. In last class we talked about iTRAQ which was first generation MS based quantitative approach for quantization of relative levels of proteins in various control and treatment samples, the iTRAQ reagent as we discussed was consist of three components, the thiol functional groups, a linker group and a biotin moiety.

Two samples treated with IE CAT reagents light or heavy isotope d 0 or d 8,where selectively alkylated for cysteine residues. These samples were combined peptide digested, affinity purified based on evident, and then analyzed by the l c MS based approach. So, the ion abundance ratio of light and heavy isotope labeled peptides could provide therelative abundance of these proteins. So, ICAT was the first quantitative MS based approach which was initiated, but because of the only two samples could be analyzed from thisapproach, new approaches came considering the sample multiplexing, because one limitation of ICAT was that only two 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 development of 2 or 4 plex isotope coded protein label i c p l 4 or 8plex isobaric tagging for relative and absolute quantitation iTRAQ and 2 or 6 plex tandem mass tag TMT labeled based techniques. So, today we will continue our discussion on iTRAQ and TMT techniques which can compare upto 8 or 6 samples in a single analysis. So these techniques can increase the throughput, iTRAQ or isobaric tag for relative and absolute quantification, the identification and quantification of complex protein mixtures have been facilitated by the mass spec based

quantitative proteomic techniques. The iTRAQ reagent consist of amine specific stable isotope reagents that can label peptides of upto 4 or 8 different biological samples. Although both ICAT and iTRAQ are similar in the overall concept, the ICAT method relies on the tagging cysteine residues whereas, the iTRAQ method based on the tagging on the primary amines.

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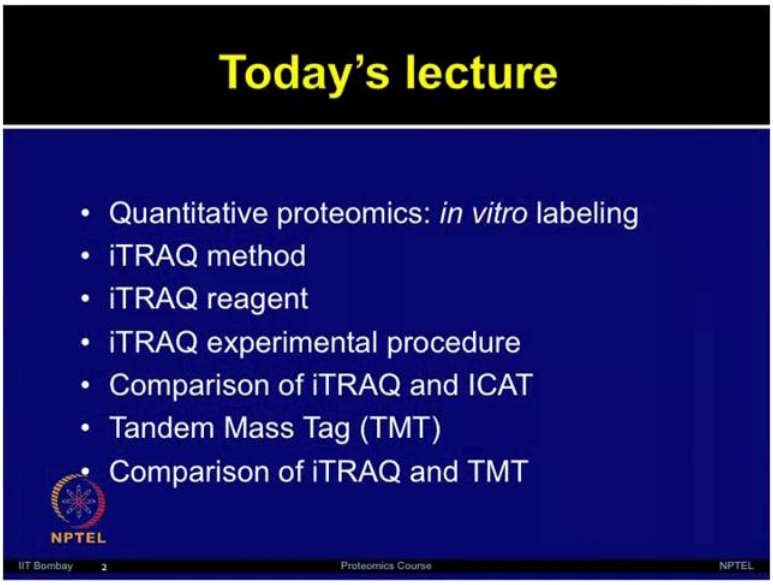

So, we will continue today's lecture onquantitative methods the in vitro labeling based approaches, we will discuss in detail about iTRAQ method, I will talk about the composition of iTRAQ reagent, then I will try to give you an experimental overview for the iTRAQ procedure we will compare the iTRAQ and ICAT techniques, then I will talk about tandem mass tag also known as TMT's, and then we will compare Iraq and TMT.

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 type 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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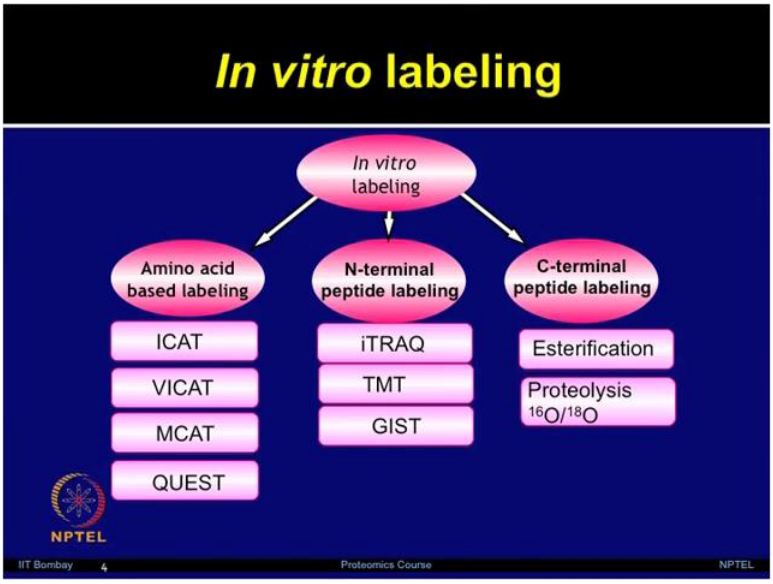

So, in vitro labeling can be three different types amino acid based labeling; and terminal peptide labeling; c terminal peptide labeling, in the amino acid based labeling such as ICAT or isotope coded affinity tag, there are other amino acid based labeling methods as well such as v i cat or visible isotope coded affinity tag, mcat or mass coded abundance tagging, quest or quantization using enhanced signal tags, then there are different type of n terminal peptide labeling methods such asiTRAQ and TMT which we are discussing today in detail, iTRAQ also known as isobaric tagging for relative and absolute quantization, TMT's are tandem mass tags, and gist global internal standard technology. 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 varieties of in vitro labeling basedapproaches available, anddepending upon your quantitative application there is possibility of using different type of tagging strategies, in our today's discussion we will mainly focus on iTRAQ and TMT's. So, as we discussed that mass spectrometry has played a very major role in proteomics 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 absolutequantization of proteins present in upto4 samples or upto 8 samples depending upon the type of iTRAQ tags, and these labels can be provided in the proteins whether n terminal.

The iTRAQ labels from the applied based system and tandem mass tags labels are available from the thermo fisher, they are currently the only commercially available tagging technologies, where quantation can be carried out in the MS/MS mode, the iTRAQ labels available from the applied bio systems, and tandem mass tags available from the thermo fisher are currently the only tagging technologies which are commercially available, where quantation is carried out in MS/MS mode, the iTRAQ technique was first time described by rose toll in 2004, I have given you reference in thebottom in my slide, and it was subsequently commercialized by the applied bio systems.

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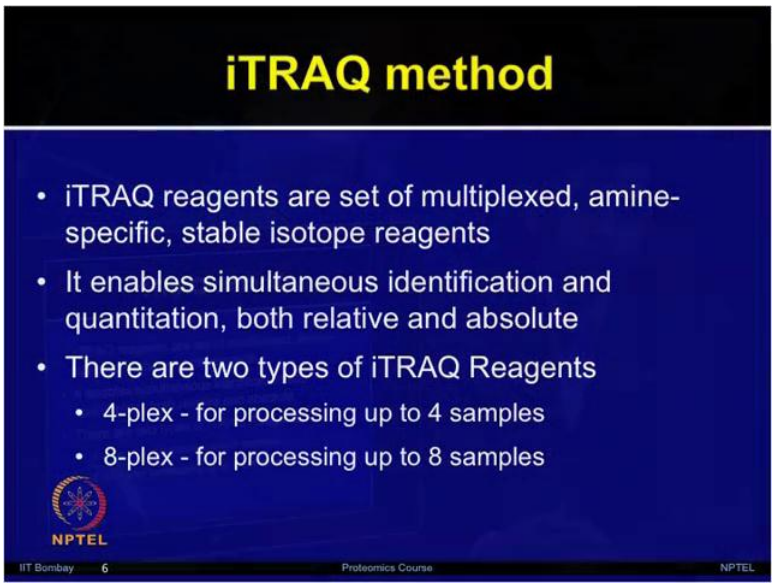

So, these iTRAQ reagents are set of multiplexed amine specific stable isotope reagents, it enables simultaneous identification and quantization both relative and absolute, there are two different type of iTRAQ reagents currently available,4-plex for sampling upto 4 samples and 8-plex for analysis of upto 8 samples, in iTRAQ method the derivative 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 a reporter ion signature ion for which differs in mass and charge ratio. So, the reporter ions are used to track thequantization and can be used to monitor the relative quantitation for proteins.

Now, discuss about the iTRAQ reagent, there are set of four isobaric amine specific labeling reagents,114,115,116 or 117, the iTRAQ reagents consist of reporter group; a balancer

group; and a peptide reactive group p r g, the protein reactive group labels the n terminals of all peptides as well as the free amine group of lysine side chains, the neutral balance portion and reporter group they provide total mass of 145, so this method canallow the multiplexing of upto 4 or 8 different samples in a single l c 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 release of and the release in mass spec.

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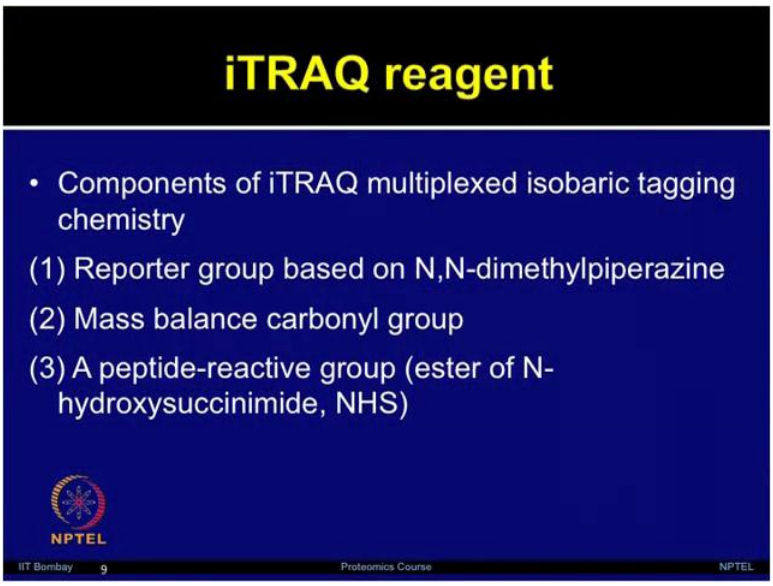

So, as I briefly talked there arethree major components of the iTRAQ reagent, one is a reporter group, which is based on the N,N-dimethylpiperazine or d m p, second is a mass balance carbonyl group, and third a peptide-reactive group, which is an ester of N-hydroxysuccinimide, NHS.

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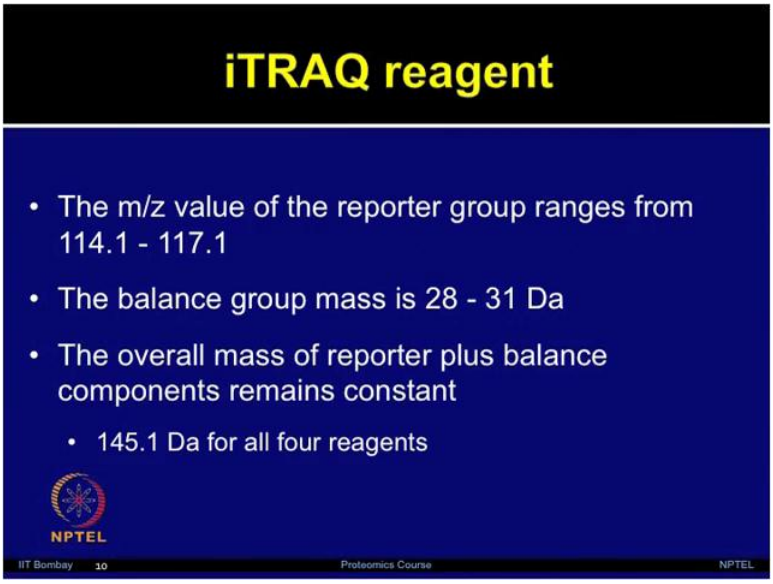

So, the iTRAQ reagent the mass to charge value of these reporter groups, they range from 114.1 to 117.1, if you are using a 4-plex reagent, the balance group of masses 28 to 31 Dalton, the overall mass of reporter plus balance components remains constant. So, 145.1 Dalton will remain for all the 4 reagents.

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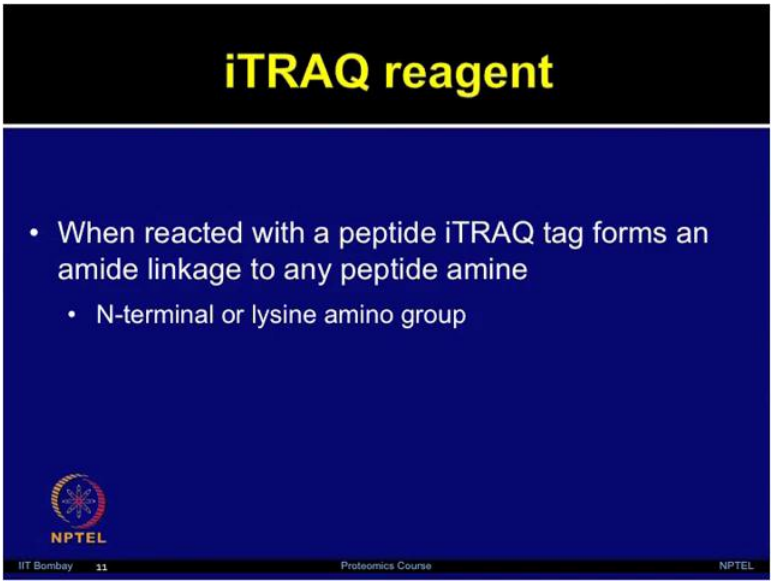

So, when reacted with a peptide iTRAQ tag forms an amine linkage to any peptide amine,N-terminal or lysine amino group.

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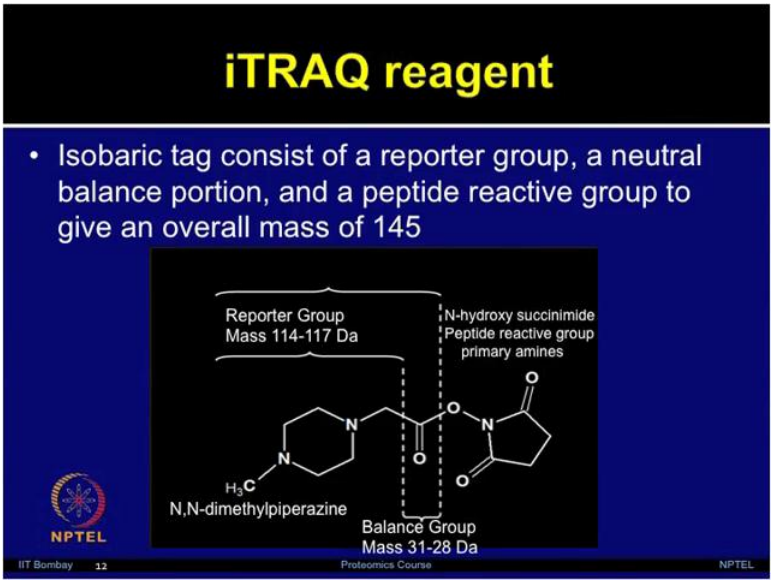

Now, this is the structure of the iTRAQ reagent, where you can see that it consist of a reporter group, a neutral balance portion, and a peptide reactive group, and overall mass will remain 145.

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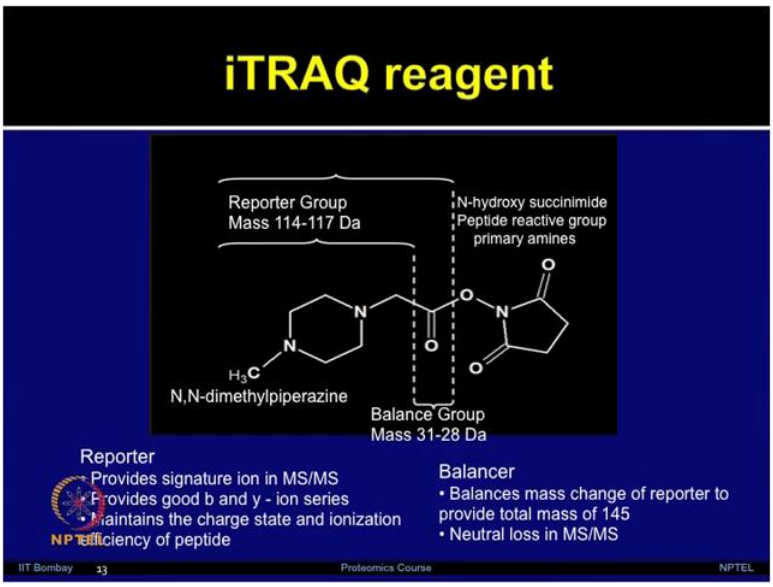

The reporter group provides signature ions in MS/MS mode, it providesgood b and y ion series, and it maintains the charge state and ionization efficiency of peptides, whereas, the balancer group it balances the mass change of reporter to provide total mass of 145 neutral loss in MS/MS mode.

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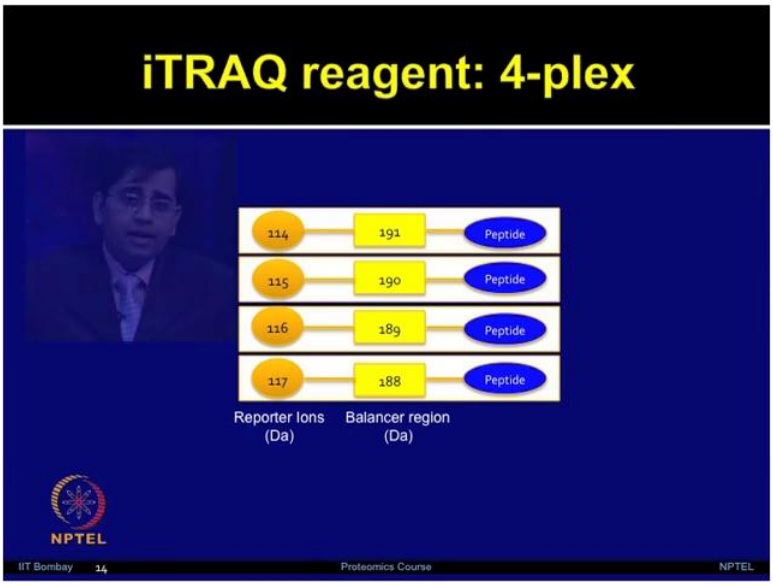

The iTRAQ label is an isobaric tagging compound consisting of a reporter ion series, here I have shown you 4-plexiTRAQ reagent with 114 to 117 Dalton, a balancer region 191 to 188 Dalton, so that the total mass of isobaric tag is 305.

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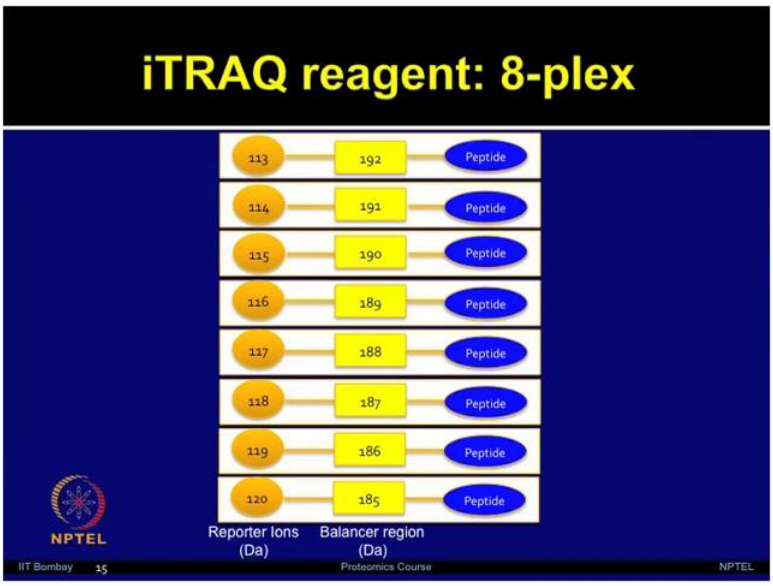

Now, let us look at the structure of iTRAQ reagent 8-plex, in 8-plexing reporter ions the variable masses from 113 to 120 Dalton, and the balancer region is from 185 to 192 Dalton, so that the total mass of isobaric tag remains 305. We will now talk about how to perform aniTRAQ experiment. So, in iTRAQ experiment the control and treated protein samples

they are first reduced alkylated and digested with trypsin, the digested samples are reacted with different altered reagents 4-plex or 8-plex,2 samples are then combined, in fact, control and 3 treatments or it can be four different type of time coursesamples. So, four samples can be combined and analyzed by the l c MS/MS. So, as per the guidelines provided by the applied bio systems, the protein samples can be prepared, and now we will discuss thatstep by step in the next few slides.

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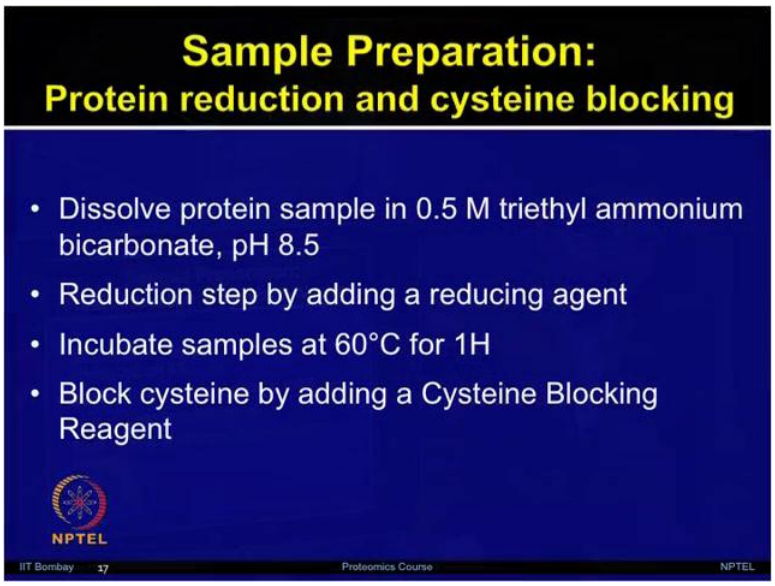

So, in the sample preparation first part is protein reduction and blocking of cysteines. So, first dissolve the protein sample in 0.5 molar tritely ammonium bicarbonate at PH 8.5, then perform reduction step by adding a reducing agent, incubate the samples at 60 degrees for an hour after that add a cysteine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

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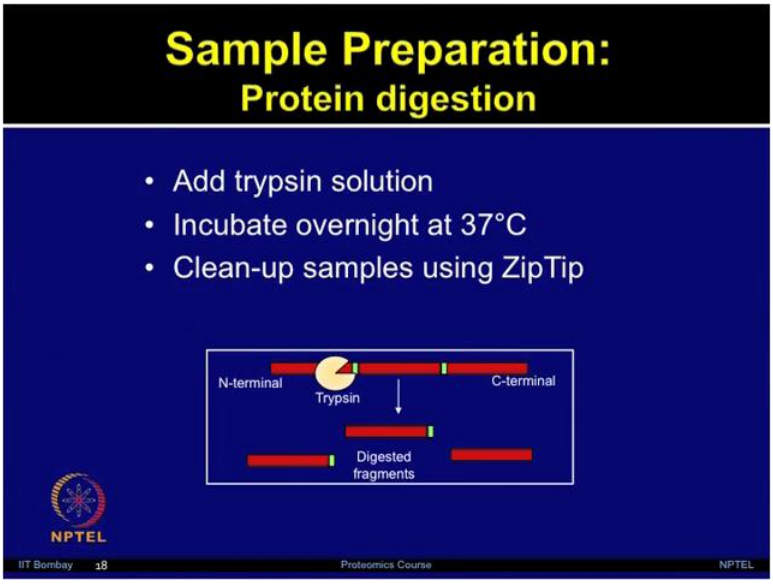

Once reduction and cytosine blocking is done thenprotein digestion can be performed, so add try sin solution incubate overnight at 37 degrees, so that these proteins can be digested into the peptides, you may have discussed these things briefly in the previous module when we talked aboutin gel digestion for doing the gel based proteomics, similar type of concepts are applied here, but this one is in solution digestion. Once you have done the overnight digestion then a cleanup step can be performed by using zip tip, so that some salts and contaminants can be removed.

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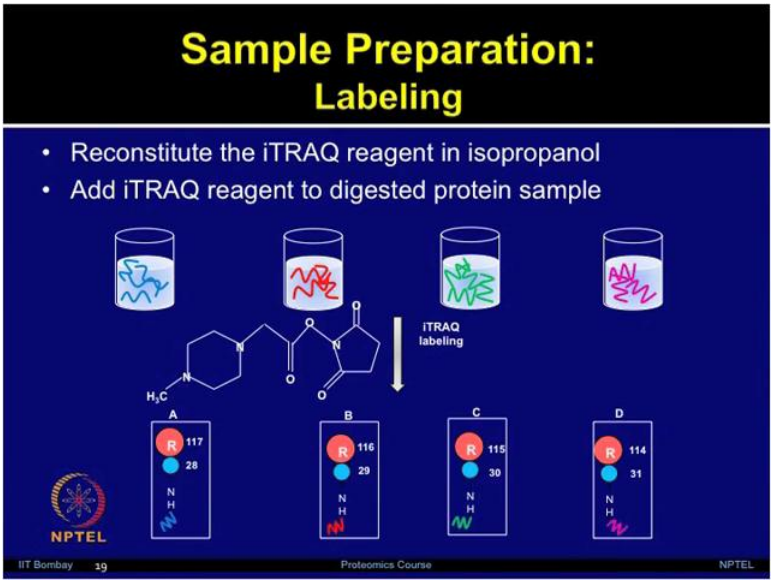

Now, next step is labeling adding these iTRAQ reagents to the four different samples, so first reconstitute the iTRAQ reagent in isopropanol, add iTRAQ reagent to the digested protein samples, if you have four samples you can use with the 4-plex iTRAQ reagents,114,115,116 and 117 as I have shown in this p p t.

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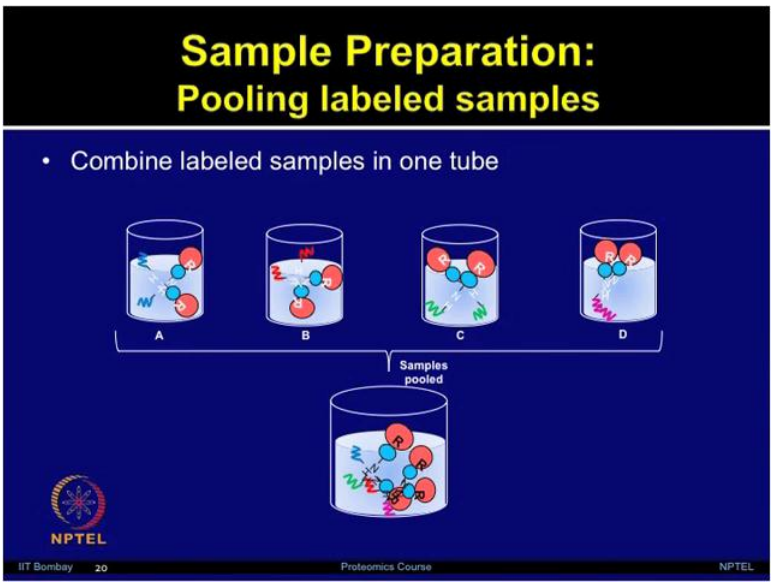

Now, combine these labeled samples in 1 tube, so that later on sample processing will not have any mineral artifact, so the labeling is performed separately, but after that all the labeled samples are pooled in one common tube.

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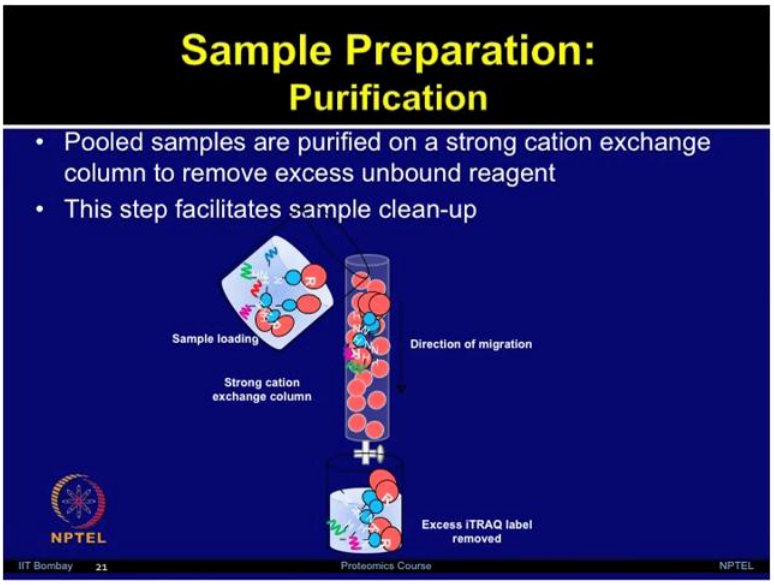

Now, these pooled samples can be further purified on a strong cation exchange column, so that their excess unbound reagents can be removed, this is an optional step, but thisdoes help inproper cleaning and getting the better signal, so this step facilitates sample clean up and this is also not essential, but recommended to perform this step. So, now, let us have a look on the overview of this protocol.

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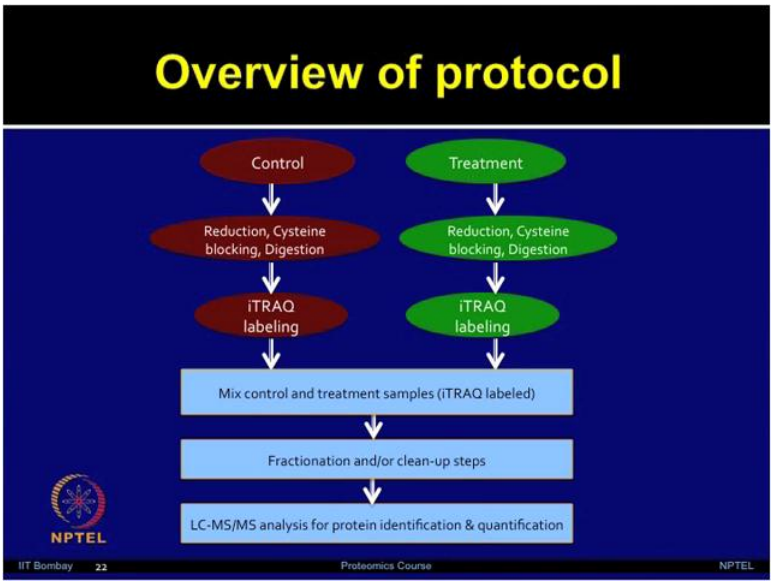

So, we had control and treatment populations, each of those samples were first reduced cysteine blocked and digested, after that iTRAQ labeling was performed for each sample, then control and treatment populations were all mixed together with the iTRAQ labels, fractionation and or clean up steps were performed, and then l c MS/MS analysis can be performed for the protein identification and protein quantification.

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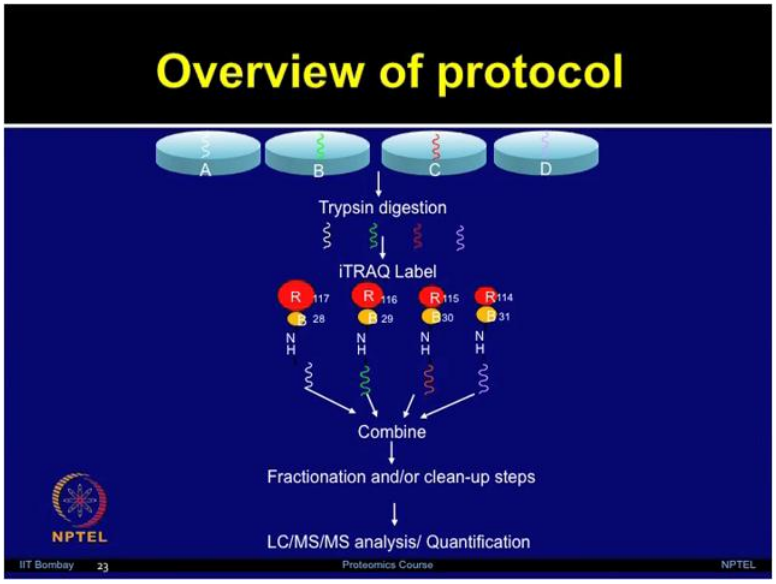

Now, I think in this image it will be more clear to you the overallsteps, you have four different populations a, b, c and d, each population is reduced cysteine blocked trypsin digested and then further labeled with the 4 different type ofiTRAQ label, combine fractioned and further l c MS analysis can be performed and quantation can be obtained. Once the sample preparation is done then further mass spectrometry analysis can be start, the peptides which are differentially labeled they kind of mix together and measured by the mass spectrometer.

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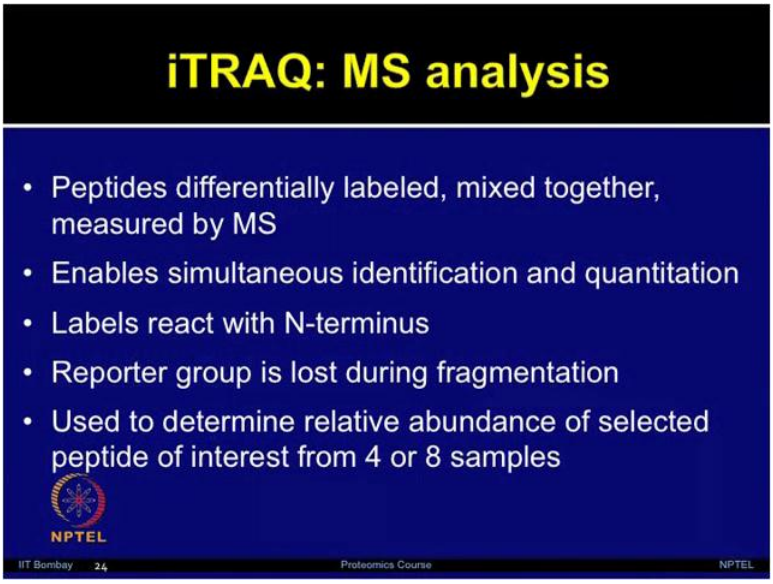

This method enables simultaneous identification and protein quantification, the labels react with the N-terminals and reporter group is lost during the fragmentation, so this method can be used to determine the relative abundance of selected peptides of interest from 4 or 8 samples,therefore, high multiplexing can be obtained usingi TRAQ method.

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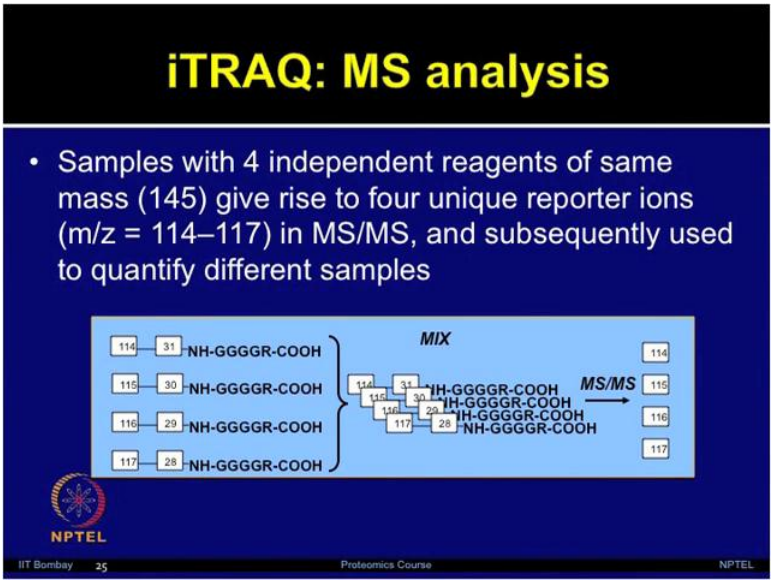

Four independent reagents of same mass 145 can give rise to 4 unique reporter ions, m by z 114 to 117 in MS/MS analysis, and subsequently these reporter ions can be used for quantification of different samples.

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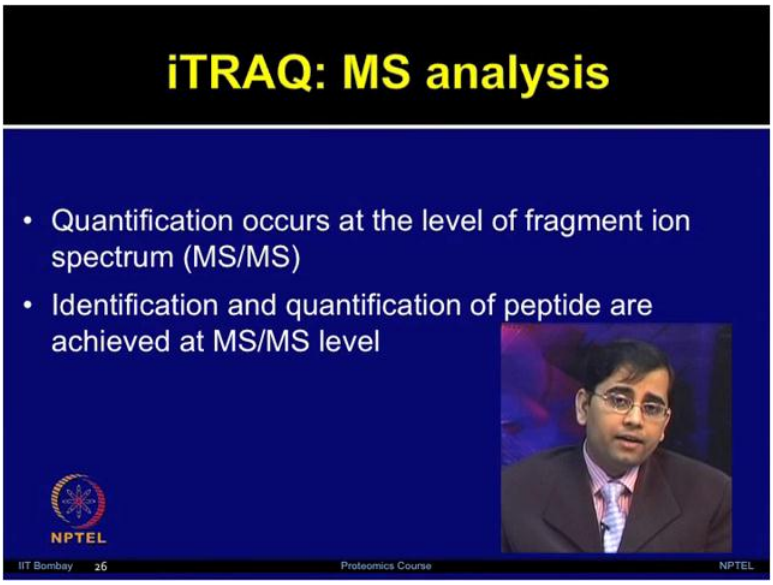

So, in iTRAQ the quantation occurs at the level of fragment ion spectrum at the MS/MS level, the peptides with the identical sequence, but there were ties with different isobaric reagents are indistinguishable during the MS spectrum alone, that is where only during the MS/MS phase the quantation can 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 quantization. So, identification and quantification of peptidesare achieved during the MS/MS analysis, the iTRAQ enables the quantitative protein profiling of multiplex samples without making MS spectra complicated, it reduces the redundancy to selecting MS precursor ions for collision induced disassociation and enhances the efficiency for MS/MS analysis and peptide identification.

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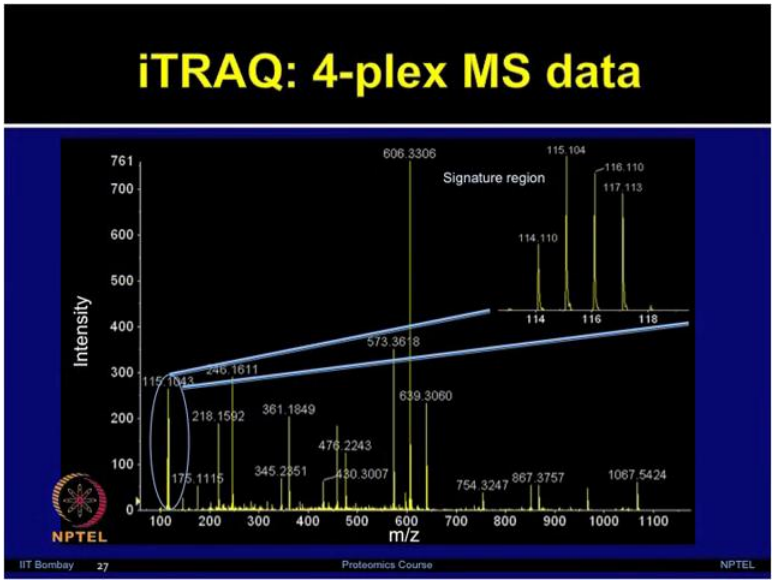

So, this is a representative spectra for the 4-plexiTRAQ experiment, the MS data is shown and MS/MS spectrum is showing the reporter region, the signature of these 4-plexiTRAQ labeled peptides,114,15, 16 and 17. Now, we will have a look on the 8-plex MS data. So, in MS/MS spectrum, now here we are showing the reporter region signature of a 8-plexiTRAQ region showing 113.14, 114.15, 115.15, 116.15,117.16, 118.16, 119.16 and121.16 (( )) ions, I will now describe you the over all iTRAQ technique by showing you an animation, in this animation we will talk about iTRAQ technique

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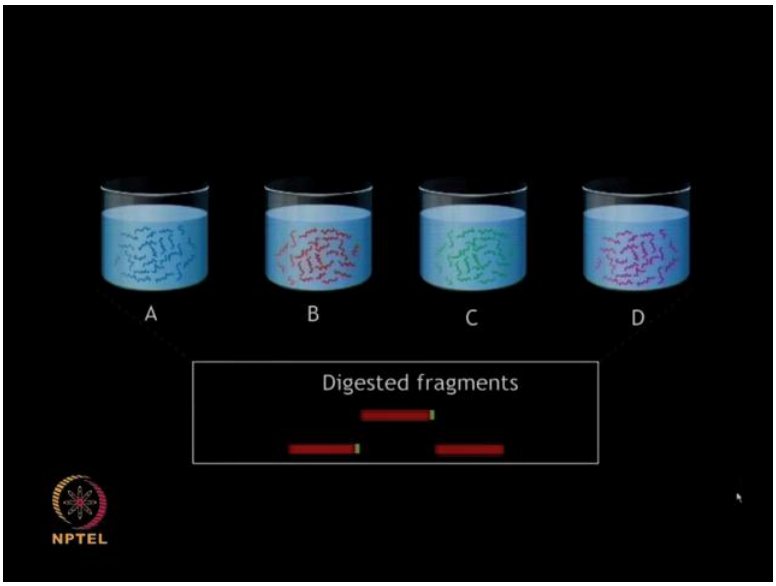

The protein samples to be analyzed are first digested with trypsin into smaller peptide fragments, the trypsin cleavesthe proteins at the c terminal of lysine and argentine residues unless they are followed by a praline residue.

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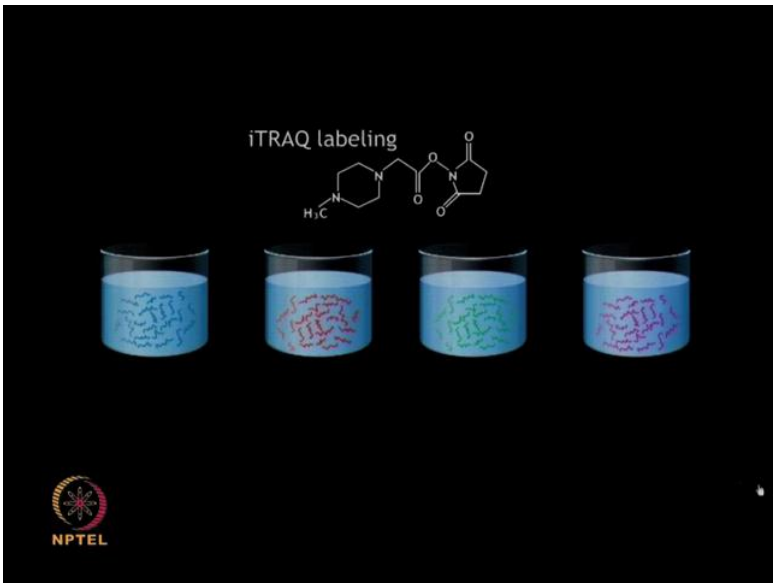

The iTRAQ reagent consist of a reporter group, a balance portion, and peptide reactive group, that interacts with the end terminals of the peptide or free amino group of lysine residues, giving an overall mass of 145, the reporter group used to label each peptide sample is

unique,and the mass are varying between the 114 to 117, thereby enabling thelabeling and quantification of up to four samples simultaneously.

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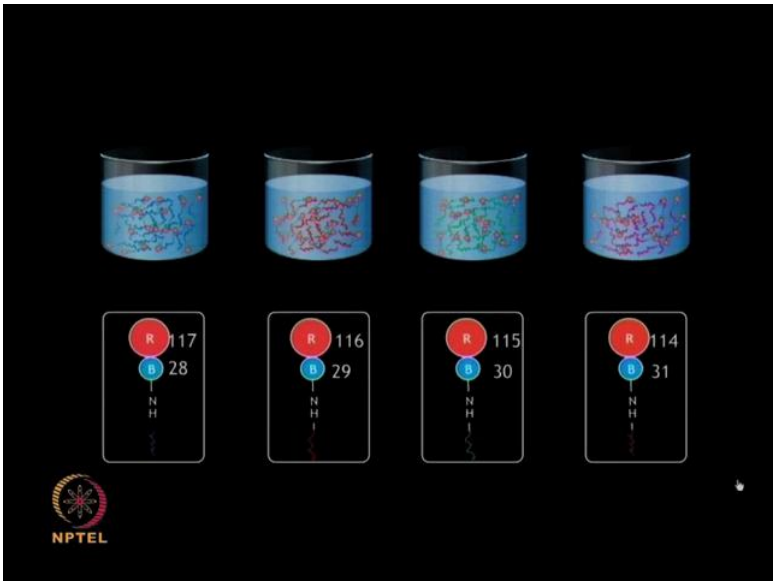

As you can see in these animations, and first one the reporter group 117, and balancer group 28, in second sample reporter group 116, balancer group 29,in third sample reporter group 115 and balancer group 30, in fourth the reporter group 114 and balancer group 31, overall 145 Dalton, this method has now further been improved to allow labeling of 8 samples simultaneously, although we are showing here the 4-plex experiment.

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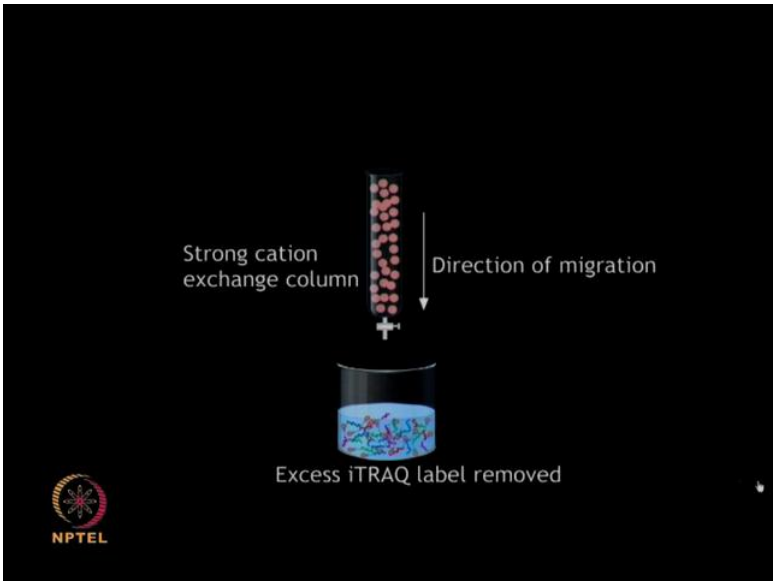

The labeled samples can be pooled together, the pooled samples are purified on a strong caution exchange column to remove any excess unbound iTRAQ reagent, this facilitates the sample clean up prior to further final separation.

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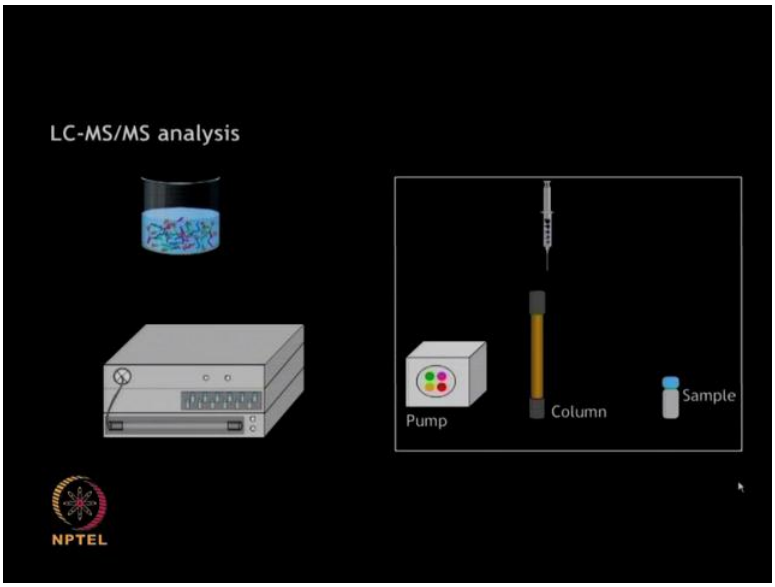

And purification using reverse phased chromatography, further purification of strong cation exchange purified samples is carried out by reverse phase liquid chromatography, wherein the sample is passed through a column containing a packed station rephrase matrix that selectively adsorbs only certain analyze samples, the eluted fractions are further characterized by MS.

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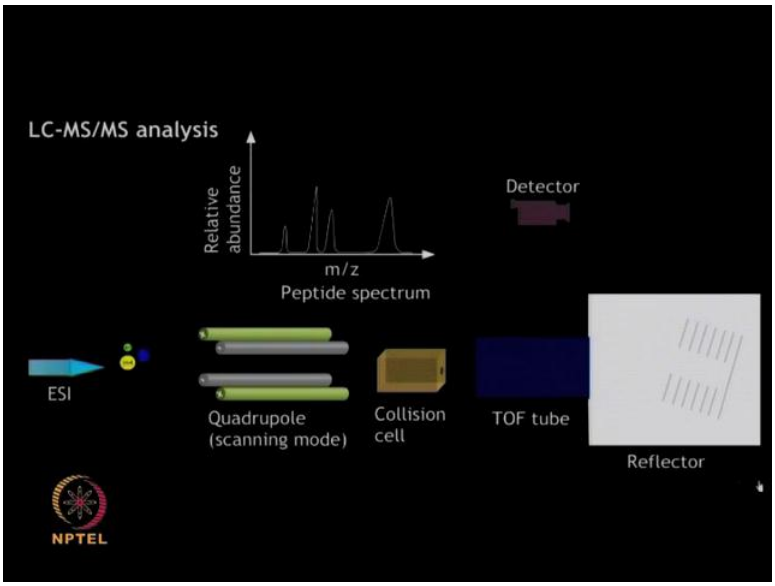

The purified label peptide fragments can be analyzed by MS/MS, the different masses of the reporter group allows the peptide fragments to be identified, the reporter group is lost during fragmentation step, the relative quantification of four samples or 8 samples can now be performed in this way.

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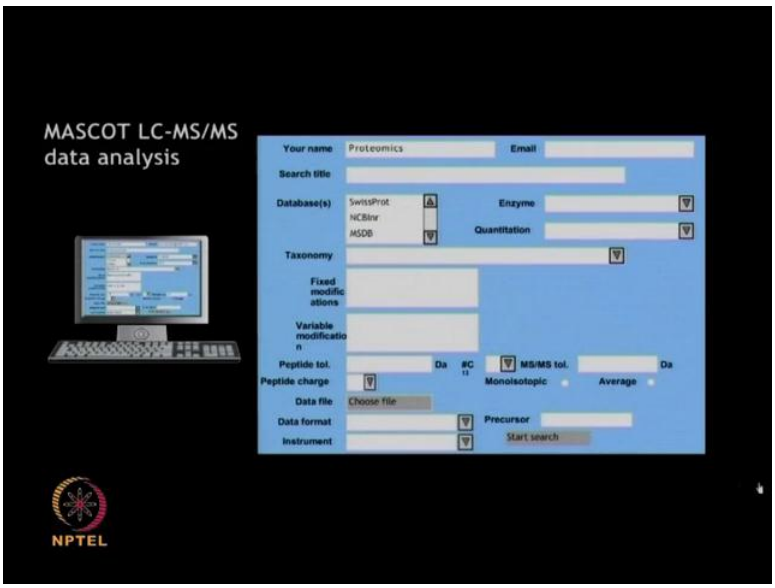

Once iTRAQ experiment is completed, the data obtained from tandem mass spectrometry can be analyzed by using mascot and other software's, the MS/MS data analysis requires inputs from the user regarding the experimental parameters such as enzyme cleavage, protein

name, modifications, instrument used, peptide charge etcetera, the commonly used protein databasesagainst which the MS information is processed to retrieve the sequel data include n c b I, MS d b and sysprot, the data file generated from the MS uploaded and the search can be carried out.

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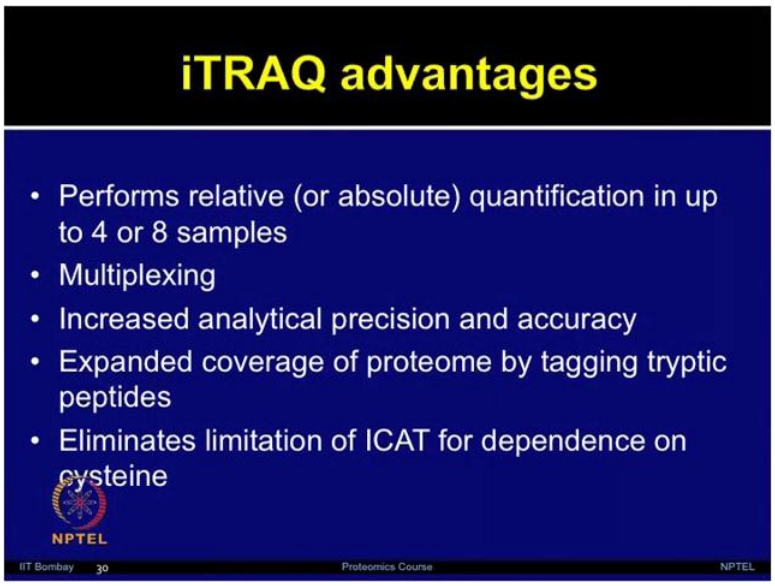

So, I hope the animation was informative, and now you are able to understand all the steps involved in the iTRAQ experiment. So, then let us talk about the advantages of using iTRAQ method, the this method performs relative and absolute quantization in upto 4 or 8 samples, it gives good multiplexing 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 limitation of the previously discussed method the ICAT for dependence on 16 residues.

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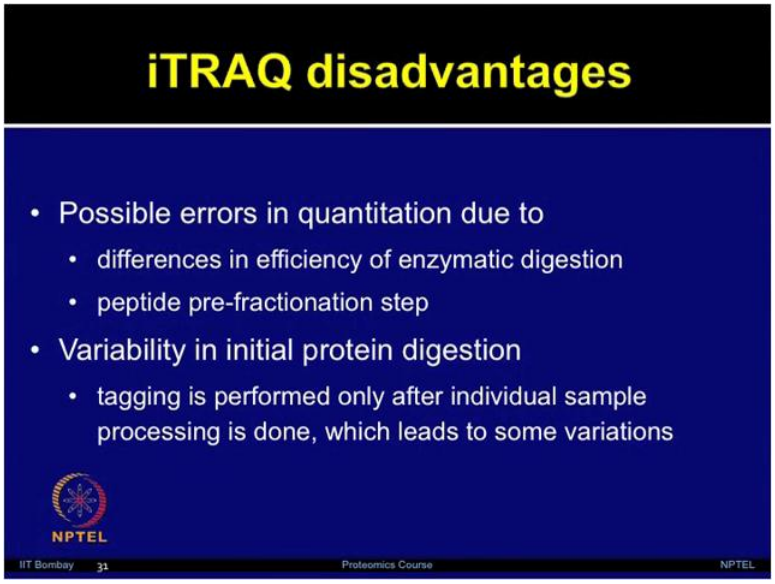

But this method has several disadvantages as well first of all there is possibility of errors in the quantification due to the difference in the efficiency of enzymatic digestion, because we are performing that step separately for 4 samples, if you remember we talked about sample a, b, c and d, each one of those we 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 thesame enzyme lot and if you have done the quantification for the proteins properly ideally that should not matter, but there is some possibility that some errors could becoming from that efficiency of enzymatic digestion.

The peptide prefractionation step could be another possible way of introducing some variation. So, variability in the initial protein digestion and then tagging is performed only after individual sample processing is done, so if we cando the multiplexingin some way that tryptic digestion cannot be performed separately that can reduce the overall chances of error, but that will actually not be possible, because we need to label each samples separately with the iTRAQ reagents. So, these are still some of the possible errors and disadvantages of iTRAQ method.

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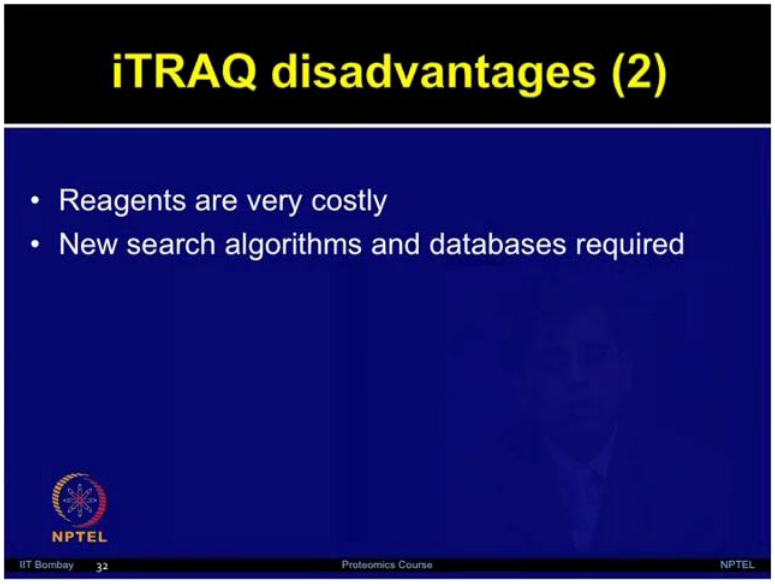

Now, these reagents are quiet costly, so that is also a limiting factor for many laps to notable to use that, and there are various search algorithm and databases arerequired; obviously, with more and more new studies arehappening, so now we have better software's and better information available for performing these experiments, but still that is one of the limitation. So, we will now talk about comparison of iTRAQ with iso with ICAT techniques, isotope coded affinity tagging with the iTRAQ method.

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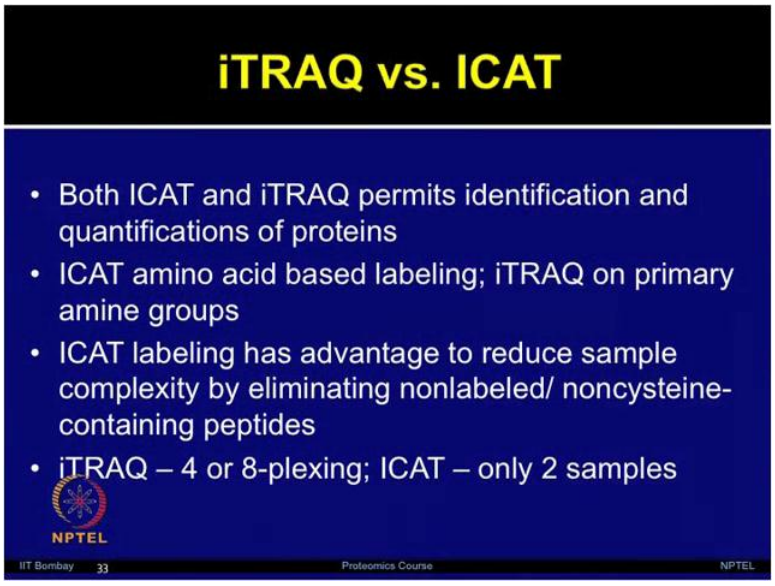

So, both ICAT and iTRAQ they allow the identification and quantification of the proteins, the ICAT is an amino acid based labeling method which we discussed earlier, and iTRAQ we talked today based on the primary amine groups, so iTRAQ potentially allows tagging of all tryptic peptides, the ICAT labeling have advantage to reduce the sample complexity by eliminating the non labeled or noncysteine peptides, iTRAQ provides multiplexing 4 or 8 plexing capability, where ICAT provides only two sample comparison, for example if you want to compare tumor progression various stages of tumor progression simultaneously from the normal to the cancerous precancerous stage, now iTRAQ is the good system which can be used, if you want look for different developmental stages of an organism again iTRAQ is a good way to do that part, because multiplexing is not possible by using ICAT.

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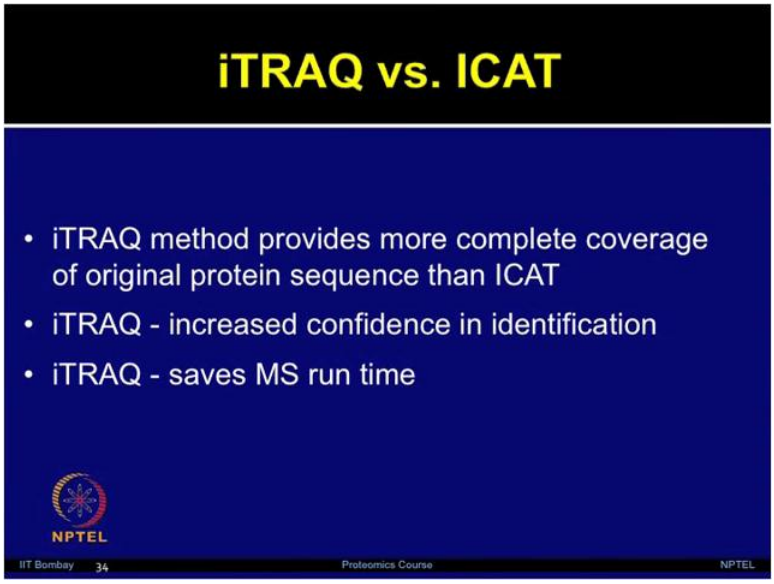

So, iTRAQ has definitely other advantages, now iTRAQ method provides more complete coverage of original protein sequence than the ICAT method, because every proteolytic peptides is tagged at the end terminals, so tagging is not limited the cysteine residues as was the case with the ICAT. IniTRAQ there is increased confidence in the identification of the proteins, in trap all the precursor ions they are isobaric, so it saves some MS runtime, there is no need to spend l c MS/MS run to sequentially select the differentially labeled precursor overtime course of hp l c peak, in order to obtain the MS/MS spectra to identify the proteins, the same MS/MS spectrum can be provided information on peptide sequence in spite of what label it is present.

So, the data required for relative quantification can be obtained via relative signal intensities of report of fragmentize, let us briefly look at the iTRAQ applications, and I will show you this in this animation. In this animation we will look at one application of iTRAQ method, a study performed by (( )) in 2010 used the iTRAQ method for identification of candidate biomarkers in ovarian calcium serum.

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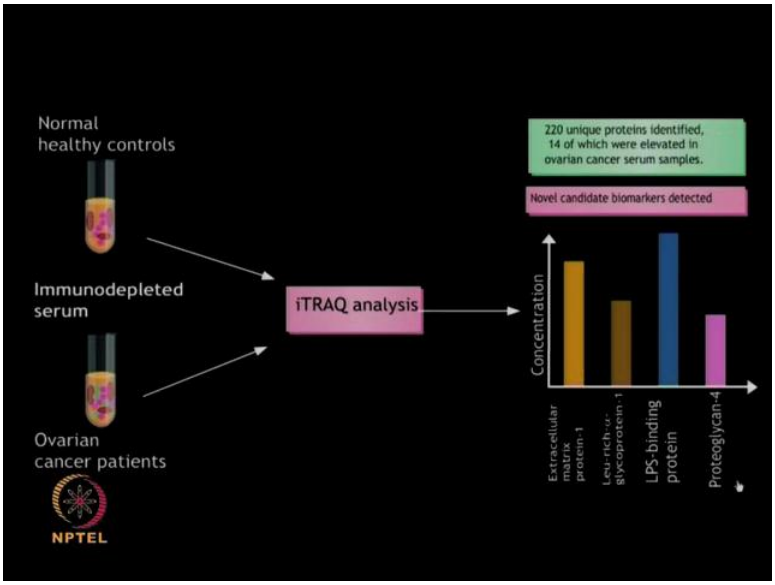

So, serum sample of control and cancer patients where first of all depleted by using a multiple affinity removal system to carry out immunodepletion of the serum samples, from normal controls and ovarian cancer samples, this step helped in removing the high abundance protein leaving behind only the medium and low abundance protein for iTRAQ analysis, the immune depleted serum samples where then labeled with iTRAQ reagents, and further analyzed in MS/MS, the authors detected a total of 220 unique proteins of which 14 were found to be elevated in over in cancer serum samples as compared to the healthy controls, and 4 novel candidate biomarkers were first time reported, these results were further validated by the western immune blotting, this gel gives you an overview of how iTRAQ regions can be used for various type of applications including in biomarker.

So, now, let us talk about tandem mass tag or TMT, this method is similar to the iTRAQ which we just discussed, TMT is also MS/MS based quantitative technique 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 the slide.

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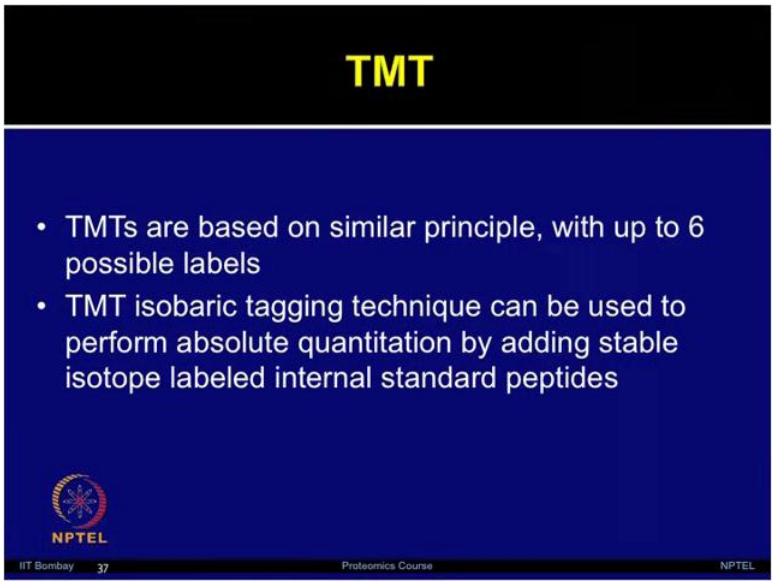

So, these tandem mass tags they are based on the similar principle of iTRAQ, here the possibility for multiplexing is up to six possible labels, the TMT isobaric tagging technique can be used to perform absolute quantification by adding stable isotope labeled internal standard peptides, it can be done by comparing the peptides from a target protein to a known amount of labeled standard peptide psyched into a sample, in that way the absolute quantification can be obtained.

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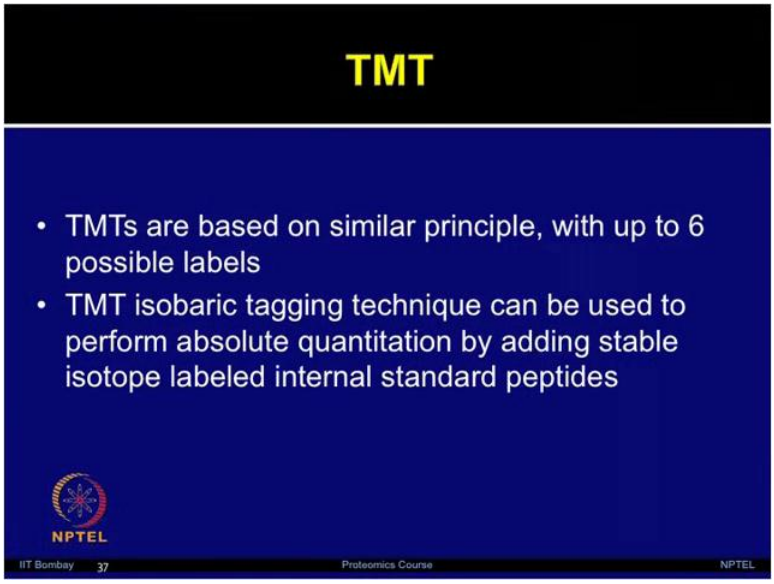

The N-terminal amine and lysine residuesare labeled through the NHS group, there are family of chemical tags which are based on the common structures, the series of TMT tags available, TMT0, TMT2-plex, TMT6-plex, so these TMT's are an innovative set of isobaric mass tagsfor labeling the proteins and peptides at amine functions and mixing of up to 6 different protein samples are possible, why duplex and 6-plex labels TMT differ by the number of isotopic substitutions.

The TMT0 is a 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 quantization, the TMT6-plex each tag adds a mass of 229 Daltons per labeled adding to the protein, the TMT duplex and TMT0 share TMT complex structures, let us look at the TMT0 label structure in more detail.

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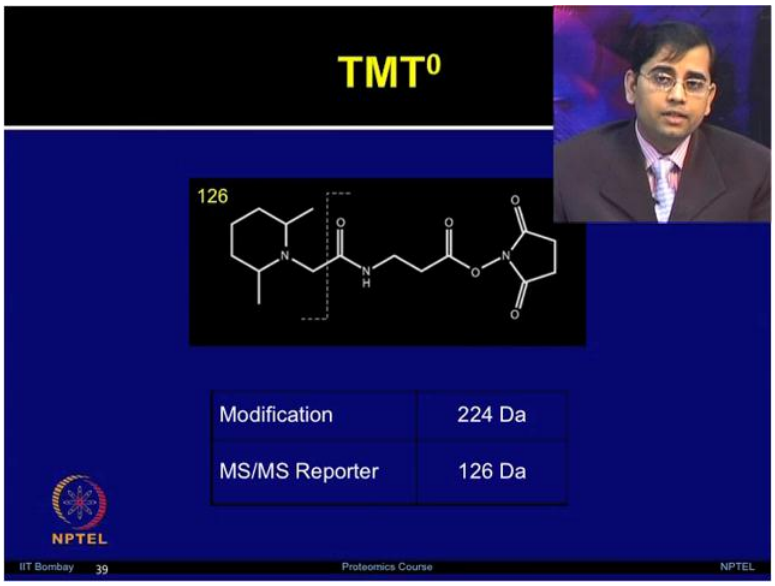

The TMT0 tag is used for testing and optimization of sample preparation, labeling, fractionation, and MS fragmentation for peptide identification, and reporter detection, the modification is 224 Daltons, and MS/MS reporter ions is 126 Daltons.

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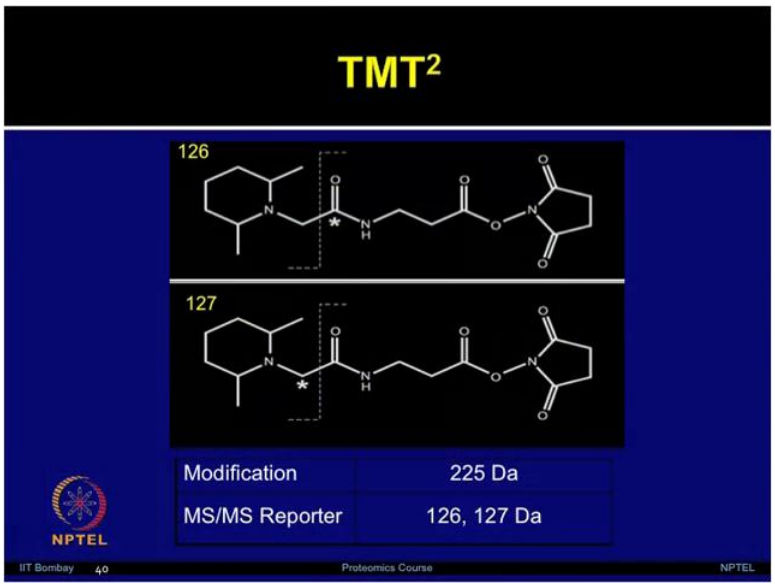

Now,let us look at TMT duplex, the TMT duplex reagent allows for the comparison of two samples, 126 and 127 these are two different MS/MS reporter ions available and modification is 225 Daltons.

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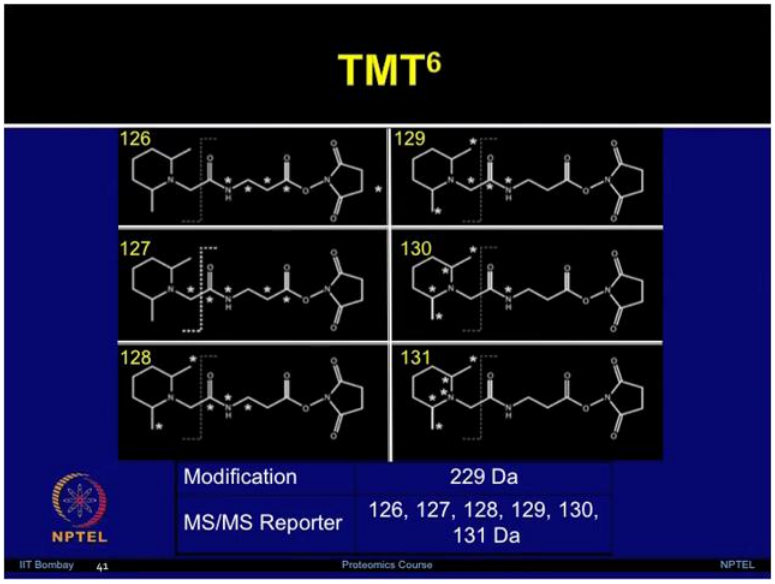

Let us now look at the TMT6-plex reagent, it allowsthe comparison of up to six conditions, the MS/MS reporter ions as you can in the structure are from 126,127,128, 129, 130 and 131 Daltons. So, the TMT6-plex reagent allows comparison of up to six conditions, it could be useful for studying about time course, drug dose responses, replicates or looking for multiple sample disease comparison, the modification is two 229 Daltons.

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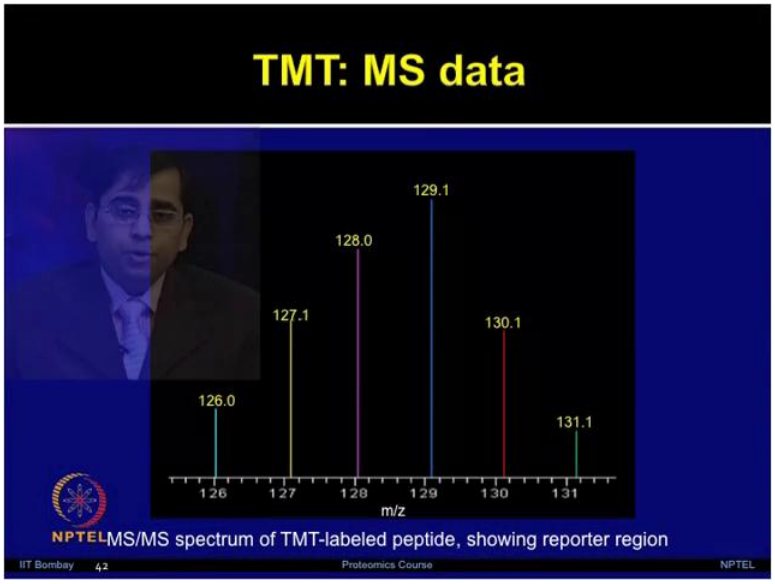

I am showing onerepresentative MS/MS spectrum of TMT labeled peptide, which is showing a reporter region, the relative abundance of target protein or peptide fragment in

such different samples can be easily measured by comparing these signature mass peaks which are generated by the different mass tags.

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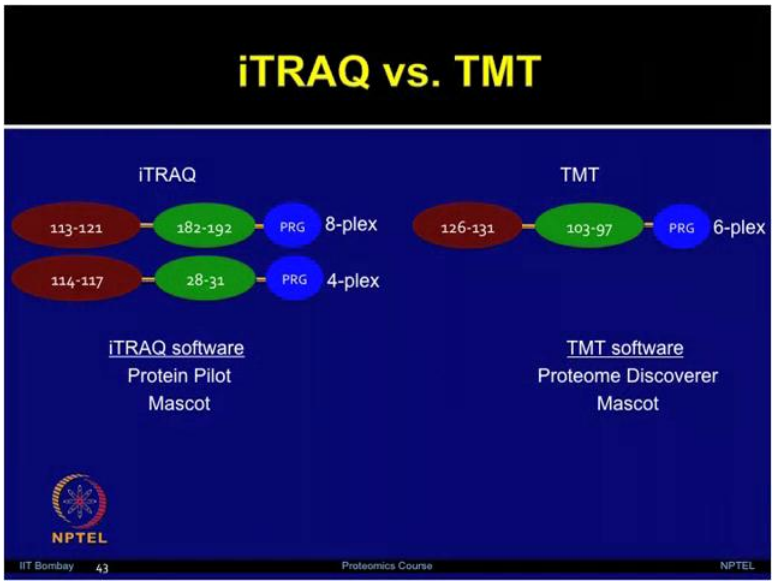

Let us now look at the comparison of iTRAQ and TMT tags. So, in iTRAQ as we talked there are two different type of regions available, 4-plex and 8-plex, in both there is a reporter group, a balancer group, and then there is a protein reactive group, same concept is also in the TMT tags where we have a reporter group, a balancer group, and p r g, I have shown you comparison with a 6-plex TMT tag, but as you seen earlier there is TMT duplex and TMT0 tags are also available. Now, in iTRAQ the reporter in the 4-plex consist 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 report groups having 126 to 132 Daltons, and the balancer consist of 97 to 103, so tags are quite similar in the overall structure, the iTRAQ analysis can be performed by using the software such as protein pellet and also the mascot, the TMT based analysis can be performed from software's such as protein discover and mascot.

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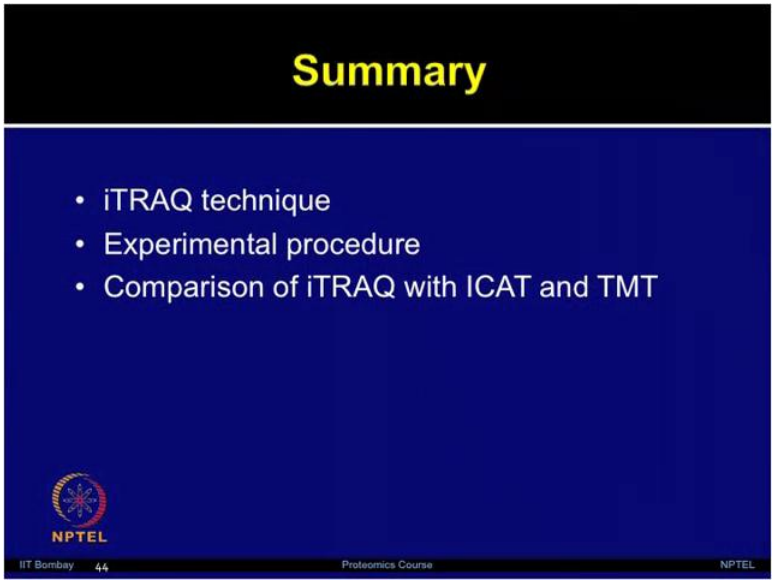

So, overall in today's lecture we talk about iTRAQ technique, we compared iTRAQ with ICAT and TMT, and during the discussion we also looked in much more detail about how to perform the iTRAQ experiment, so as you know with this t d advances in the mass spectrometry the quantitative proteomics has progressed dramatically in the past few years, the efforts to analyze proteome of many species both qualitative and quantitative have generated an abundance of data in a variety of biological samples, from bacteria to human.

The chemical labeling using isobaric tags for relative and absolute quantification iTRAQ or tandem mass tag, TMT reagents, the quantization is based on the extraction of reporter ions from tandem mass spectrometry MS/MS spectra, these methods can be used on all kinds of biological samples, and provide high level of reliability for the quantitative data. So, we will continue our discussion about quantitative proteomic techniques in the next lecture on in vivo based stable isotope labeling methods, and then we will try to compare the in vitro and in vivo based labeling method .Thank you.