Introduction to Proteomics
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Lecture - 31
Introduction to quantitative proteomics

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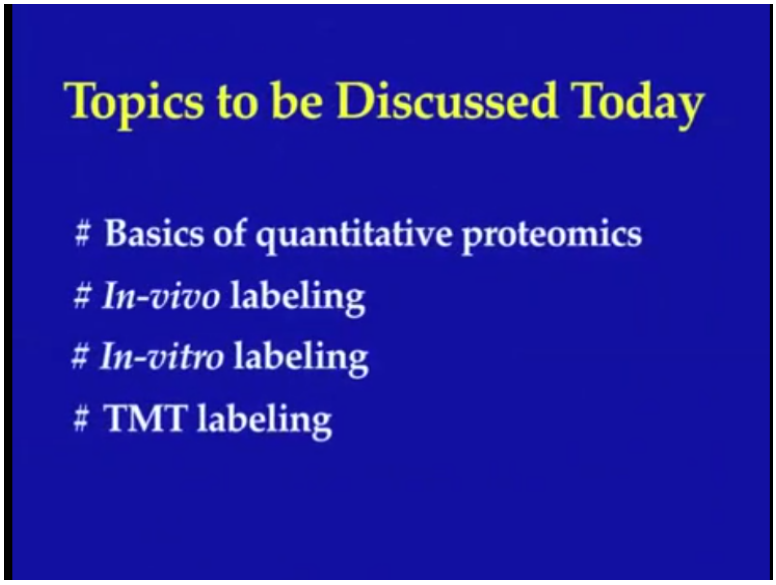

The complexity and dynamic nature of proteome present major technological challenges. Mass spectrometry advancements have improved the high-throughput identification and quantification of proteins. The mass spectrometers have advanced significantly and now offering opportunities to understand the human diseases and discover biomarkers. MS has proven to be an extremely powerful tool to characterize the protein complexes.

However, MS is not a quantitative technique to begin with and peptide ionization efficiency is unpredictable. So usefulness of this data for the quantitative purpose remains limited. Many advancements in MS during the last decade have provided new ways for protein analysis and facilitated the study of proteomic analysis of various biological system.

Advancement in MS include the development of highly sensitive mass spectrometers, fast scan rates, automation, nanoflow liquid chromatography as well as new techniques and methods to quantify proteins, its abundance for quantitative proteomic analysis.

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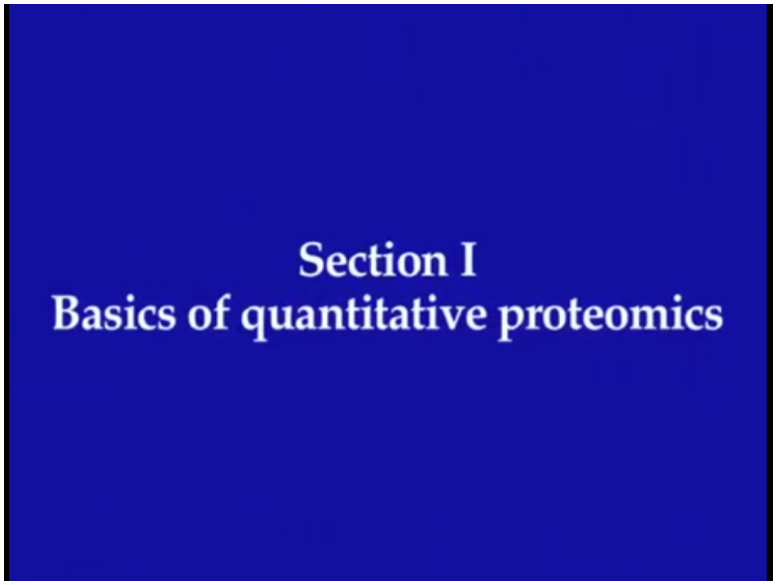

The protein labeling with stable isotopes are new and effective methods for quantitative proteome profiling using mass spec. These isotopic labels can be introduced in-vivo or in-vitro and provide relative abundance of proteins for proteomic analysis. The isotopically labeled peptides are chemically identical and they generate similar specific signal intensities in mass spectrometer.

The relative levels of isotopically labeled peptides are determined by comparing the signal intensities of paired peptides.

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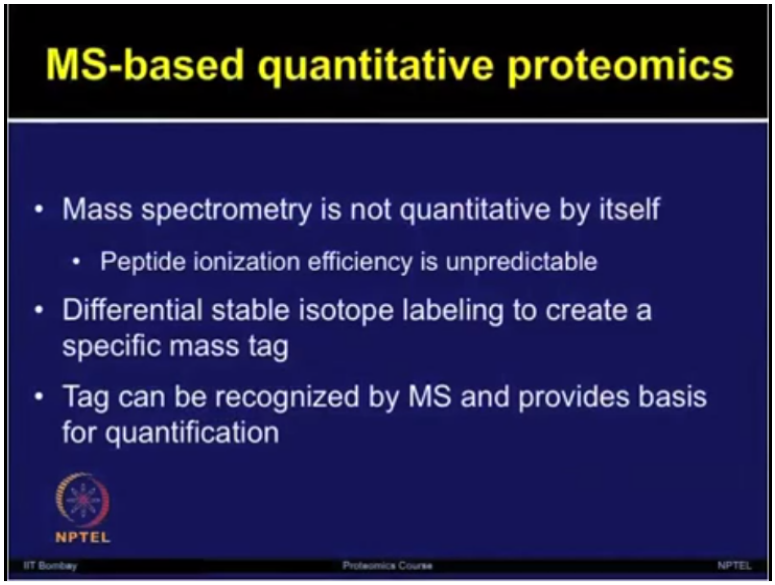

So as I discussed mass spectrometer is not quantitative technique by itself due to the peptide ionization efficiency, which is unpredictable. So the differential stable isotope labeling is used to create specific mass tags, a different type of mass tags, which are currently very promising

for various quantitative proteomic applications including ICAT, iTRAQ, SILAC as well as whereas other tags. So these tags can be recognized by MS and provide the basis for quantification.

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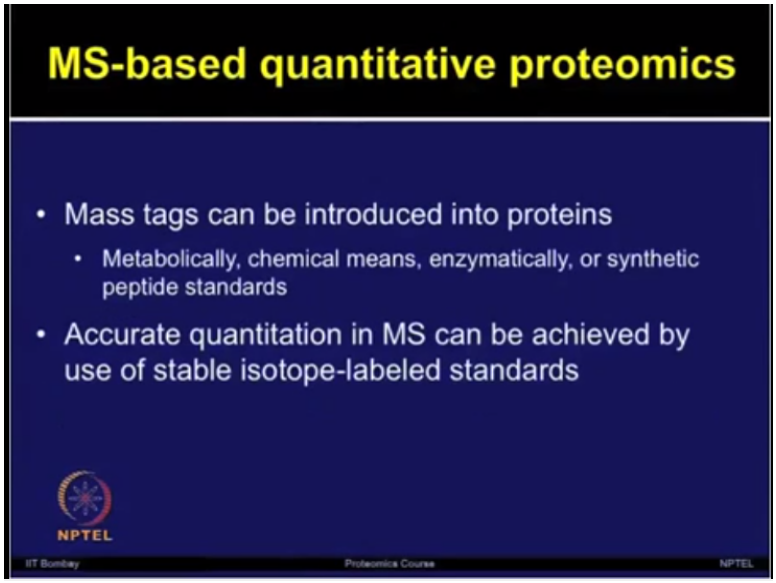

These mass tags can be introduced by various methods into the proteins such as metabolic labeling, chemical means of labeling, enzymatic methods or by using synthetic peptide standards. The accurate quantification in mass spec can be achieved by use of stable isotope labeled standards.

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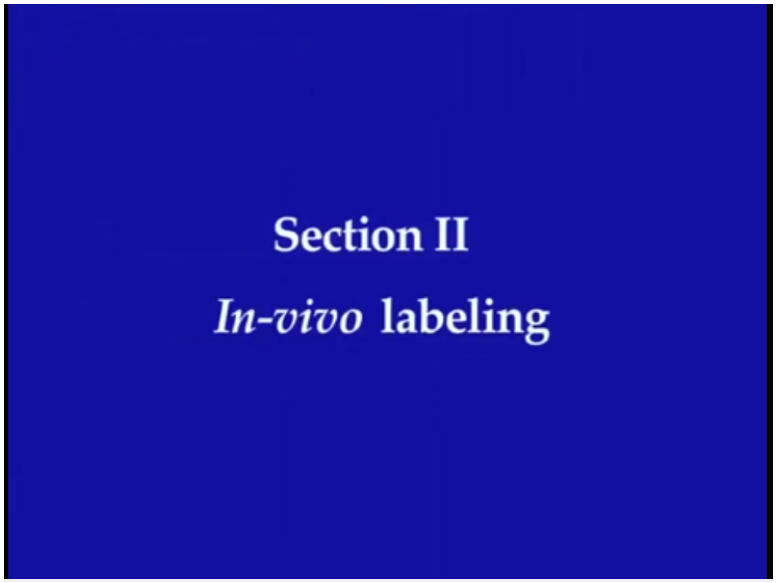

So there are different ways of quantitative proteomic analysis, in-vitro and in-vivo labeling methods. In today's lecture, we will focus on in-vivo labeling methods, so most of the quantitative proteomic approaches by MS utilize isotopic labels as a reference for either the

relative or absolute quantitation. These labels can be introduced in-vivo for example by growing an organism in a media enriched with specific isotopes.

The labels are also introduced by performing tryptic digestion in presence of heavy water. Many methods using isotopically labeled reagents that react with specific amino acids or the protein N-terminals are also developed. For comparative and quantitative proteomic analysis, the development of stable isotope tagging methods can allow the quantification of relative levels of proteins.

These differentially labeled peptides with stable isotopes can be distinguished by characteristic mass shift in MS.

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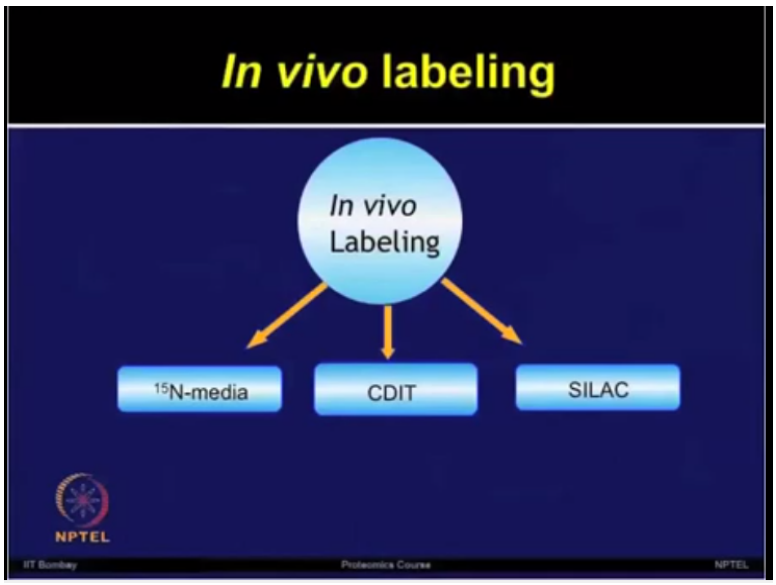

The different ways of in vivo labeling such as enrichment of 15N media, the culture derived isotope tags or CDIT, stable isotope labeling by amino acids in cell culture or SILAC. Although, we will discuss briefly about 15N media method and culture derived isotope tags and then will focus on mainly SILAC method for rest of the lecture.

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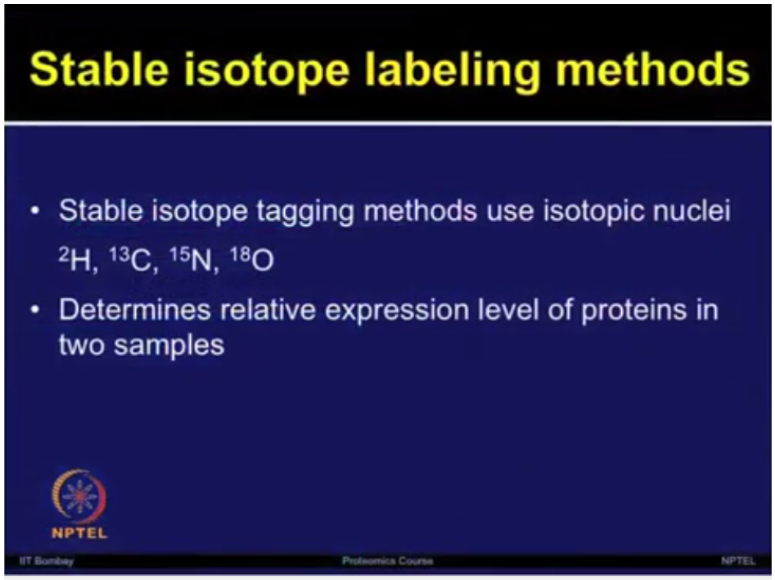

So the stable isotope tagging methods use isotopic nuclei such as 2 hydrogen, 13 carbon, 15 nitrogen and 18 oxygen. These stable isotopes are incorporated in place of natural abundance isotope in heavy standard. By using the stable isotope labeling methods, one can determine the relative expression level of proteins in 2 samples.

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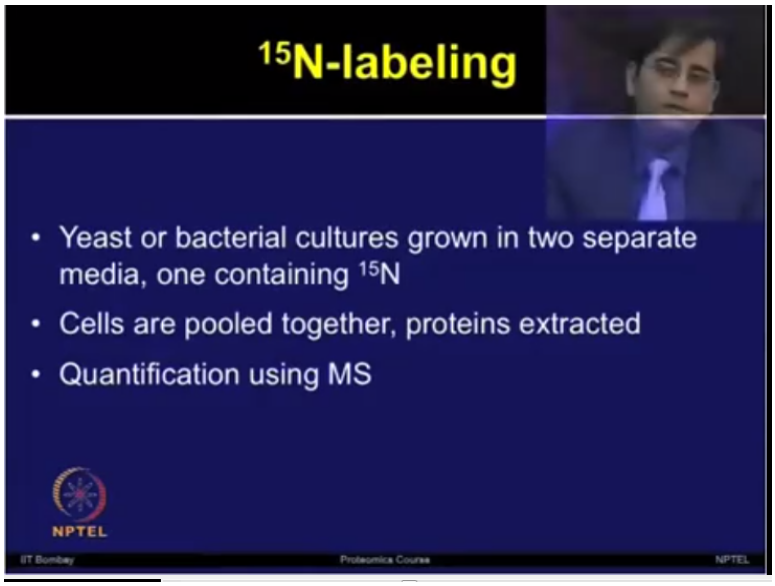

Let us briefly discuss about 15N labeling method. So in yeast or bacterial cultures which are grown in 2 separate media, one containing 15 nitrogen. The cells are pulled together, proteins can be extracted from these pulled cells and quantified by using mass spec.

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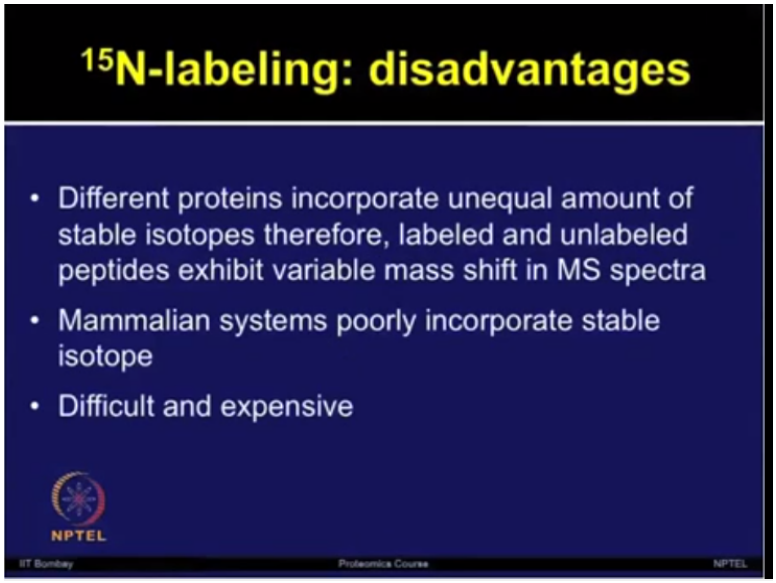

All the 15N labeling is easy method, but there are various disadvantages. The protein incorporation in your control and treatment could be unequal due to these stable isotopes. Therefore, the labeled and unlabeled peptides exhibit variable mass shift in the MS spectrum. The Mammalian systems very poorly incorporate these stable isotopes. Furthermore, this method is difficult and expensive.

Due to these limitations, researchers have tried other labeling methods as well.

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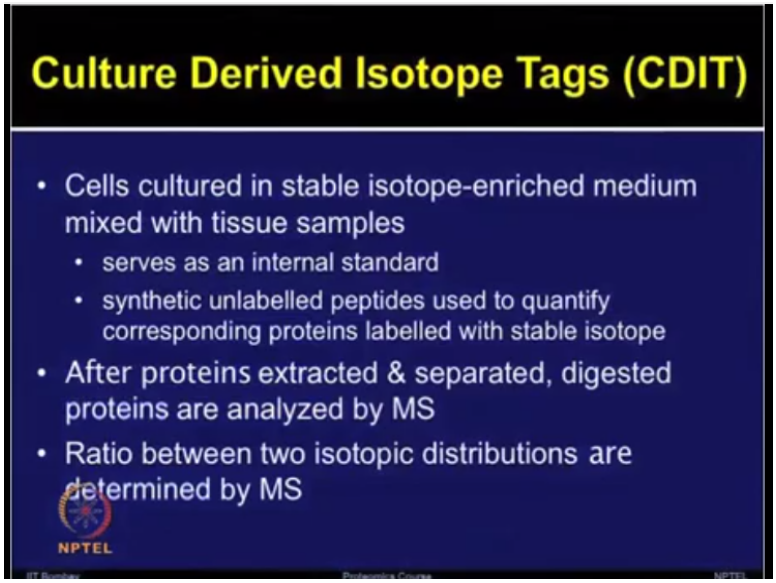

Now let us discuss about culture derived isotope tags or CDIT. In this method, the cells which are cultured in the stable isotope enriched medium, they are mixed with the tissue samples, which serves as an internal standard. The synthetic unlabeled peptides can be used for the

quantification of corresponding proteins, which are labeled with stable isotopes. The proteins can be extracted and digested prior to the MS analysis.

The ratio between 2 isotopic distributions can be determined by mass spec.

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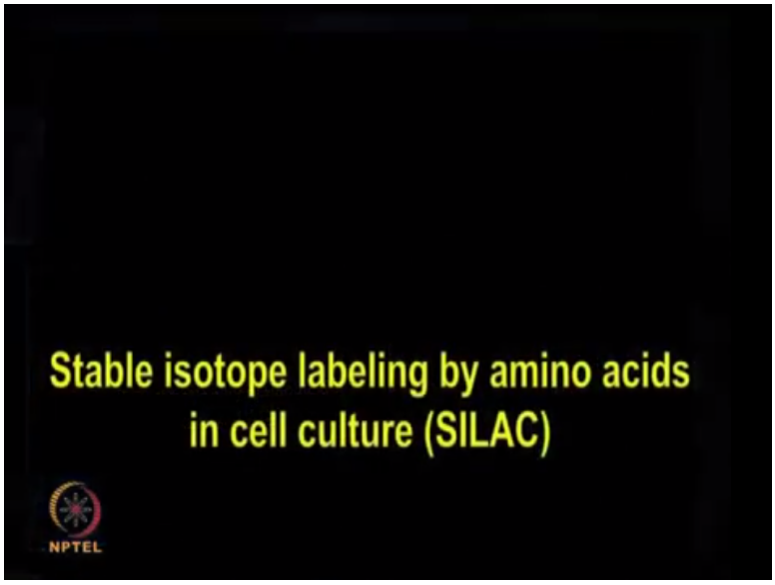

After having discussed some of the less commonly used methods such as 15N media and CDIT now let us discuss about stable isotope labeling by amino acid and cell culture SILAC. So the MS based quantitative proteomics is an increasingly popular approach to study changes in the protein abundance in various biological samples.

SILAC which is a metabolic labeling strategy to encode whole cellular proteome is one of the very widely used method for the quantitative proteomics. In SILAC method, the cells are grown in a culture medium where natural form of an amino acid is replaced with a stable isotopic form such as arginine bearing 13 carbon atoms. Incorporation of the heavy amino acid occurs through the cells grown protein synthesis and turnover.

The SILAC method allows for light and heavy proteomes to be differentiated by the mass spec while avoiding any chemical derivatization and associated purification. Stable isotope labeling by amino acids in cell culture or SILAC, which is a metabolic labeling strategy to encode whole cellular proteome, is widely used method for quantitative proteomics.

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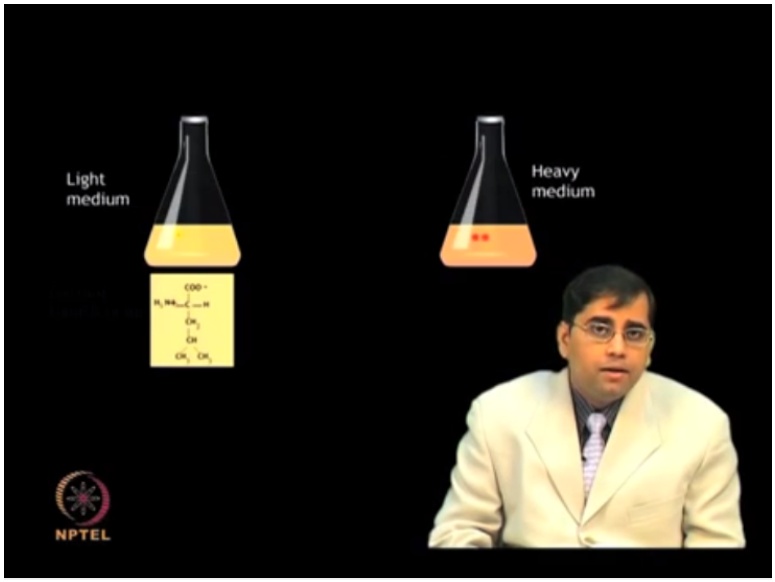

In SILAC, 2 group of cells are cultured in media that are identical in all their aspects except that one contains a heavy isotopic analogue often essential amino acid while the other contains the normal light amino acid. After a number of cell divisions, the grown cells are combined and digested using trypsin.

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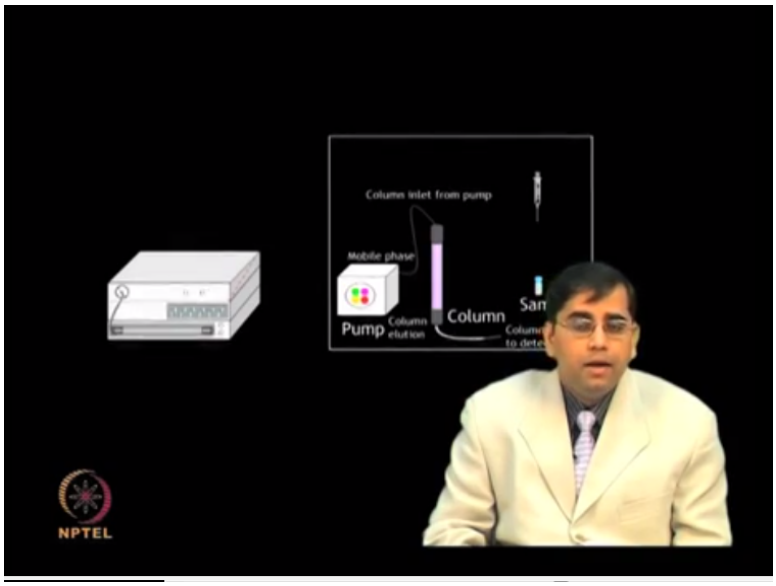

The complex protein mixture is further separated by SDS-PAGE to simplify the analysis. Further application is carried out by liquid chromatography and purified peptide fragments are analyzed by MS/MS.

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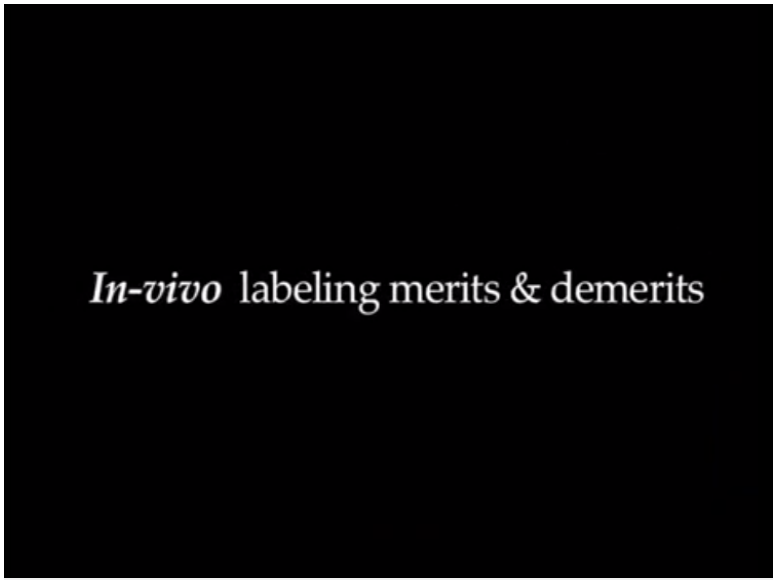

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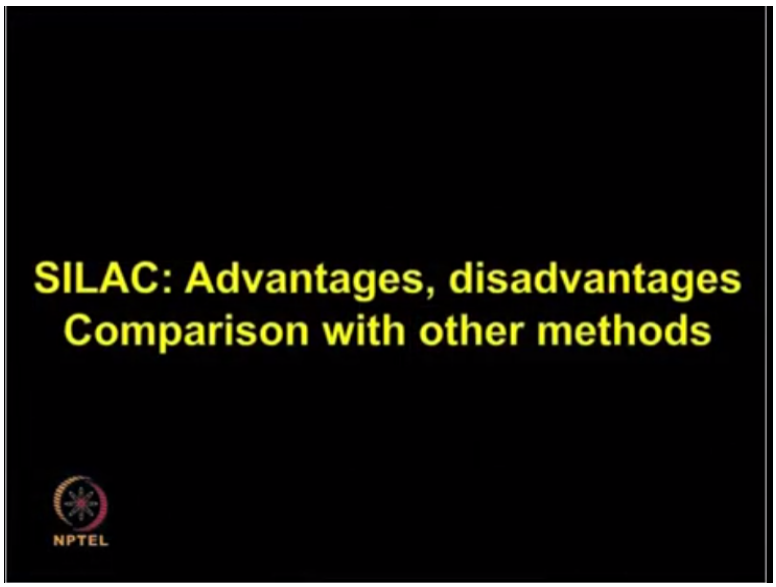

So after discussing the SILAC technique and watching this animation of this entire process now let us discuss about advantages and disadvantages of SILAC method and compare it with some other tagging methods. So as you have discussed and realized that SILAC method is very simple and robust and it labels the entire proteome without chemical derivatization and less sample handling steps.

The labeled samples are mixed at the stage of whole cells, which make SILAC approach ideally suited for the quantitative proteomic experiment because there will be less handling variation, manual artifacts for each population separately. Studies such as the sub-proteome analysis, for example the cellular organelles or complex purification protocols. These can also be performed with very good quantitative accuracy by using these methods.

So let us discuss about some of the advantages of using SILAC method. In SILAC, no chemical difference is observed between the labeled and natural amino acid isotopes.

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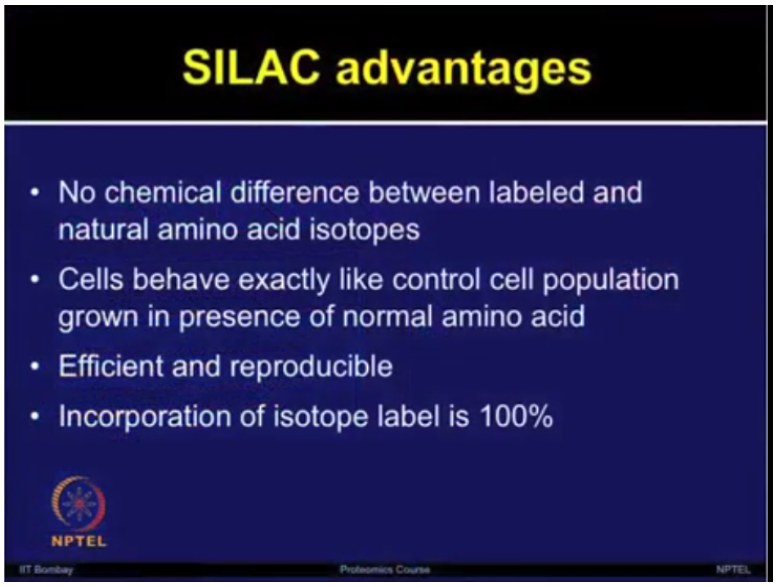

The cells are grown in the tissue culture medium and they behave exactly like the control cell population grown in presence of the normal amino acids. So this method is very efficient and very reproducible. It has been observed that the isotope labels are 100% incorporated and one need to ensure that by doing the doubling for different generation and after 5 or 6 generation it has been observed that it is 100% incorporated.

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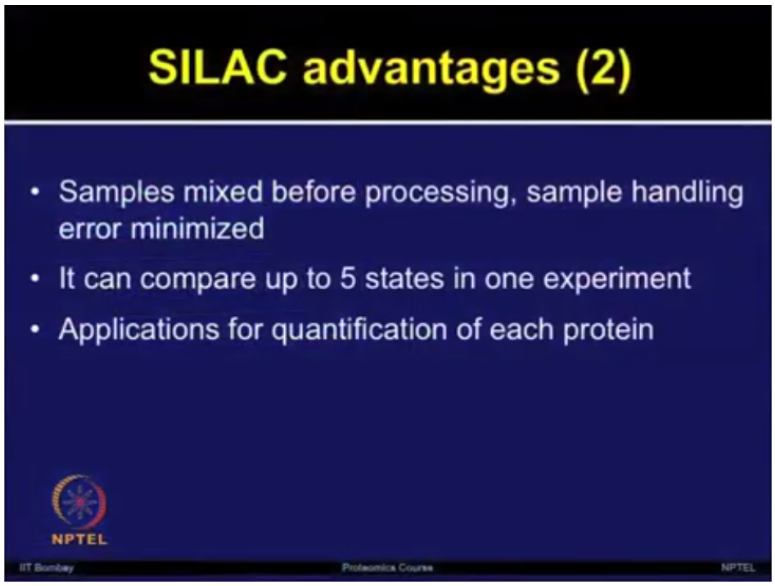

So samples can be mixed prior to the processing before doing the mass spec analysis. So this method eliminates some of the handling errors. Now later SILAC experiments have

demonstrated that it can provide the 5 plexing capability to compare 5 different states in 1 experiment. So there are various applications of quantification of proteins by using SILAC method, which we will discuss in next few slides.

Although, there are many advantages of using SILAC, but there are some disadvantages due to the inherent nature of this method.

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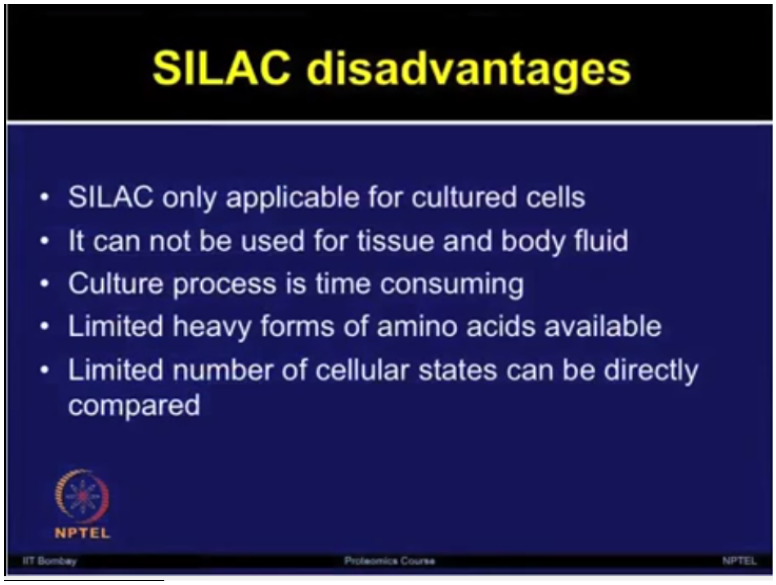

SILAC is applicable for only culture cells. It cannot be used for tissue or body fluids so that is one of the major limitation of this method. The tissue culture process is always very time consuming and it requires very meticulous and efficient work. Then there are few forms of available heavy isotopes. The heavy forms of amino acids which are available. These are commercially available, but there are very limited forms of these heavy amino acids.

Due to this only limited states can be compared by using SILAC.

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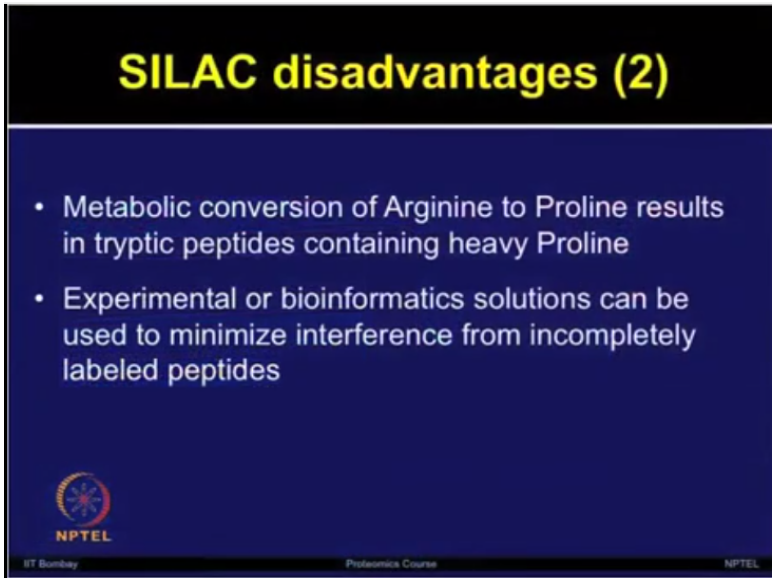

The metabolic conversion of arginine to proline is one of the commonly observed issue in the SILAC experiments, which results in the tryptic peptides containing heavy prolines. Now there are various experimental or bioinformatic solutions in place, which can be used to reduce the interference from incompletely labeled peptides. So let us discuss about how SILAC compares with the radioactivity labeling methods.

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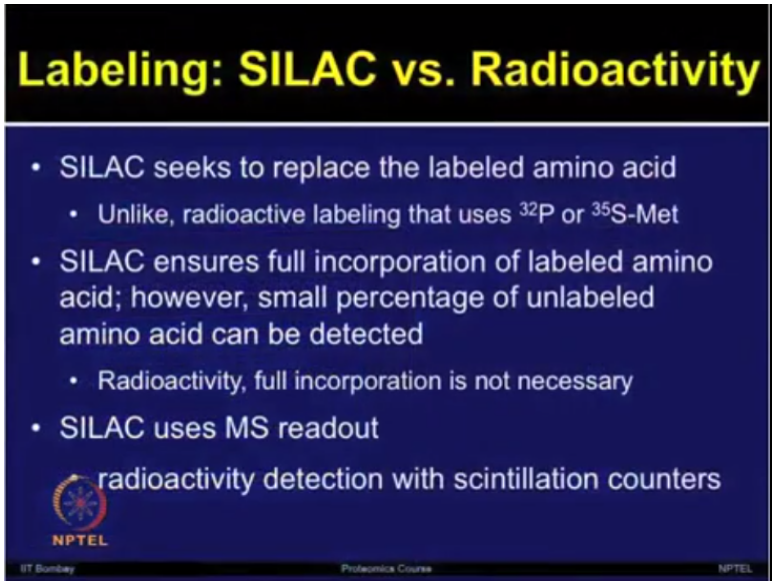

The SILAC method seeks to replace the labeled amino acid, which is unlike the radioactive labeling which uses 32P or 35S methionine. The SILAC method ensures that labeled amino acids are fully incorporated in the cells; however, the small percentage of unlabeled amino acids can be deducted. In the radioactivity based labeling the full incorporation is not necessary.

In SILAC, one can read out the signals by using mass spectrometers whereas the radioactivity detection is possible by using scintillation counters or by registering the films for the signal intensity.

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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 label and these strategies will discuss in the next slide.

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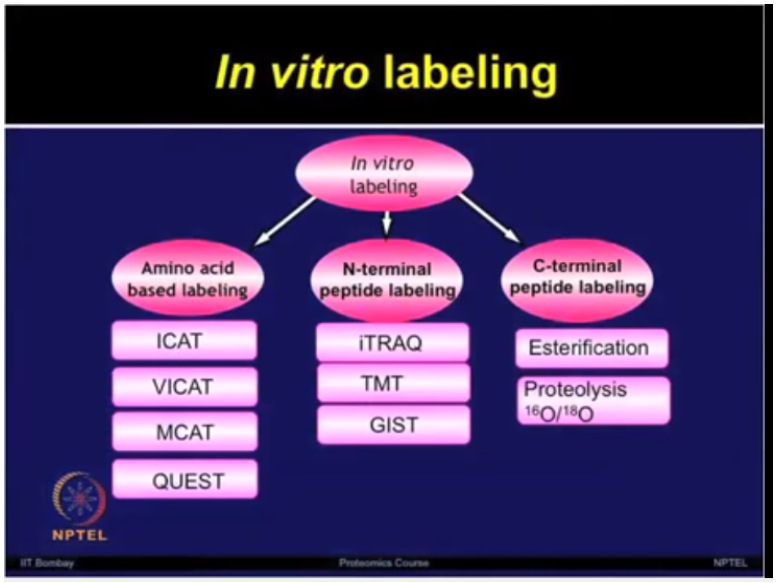

So in vitro labeling can be 3 different types, amino acid based labeling and terminal peptide labeling, C-terminal peptide labeling. In the amino acid based labeling, such as ICAT or isotope coded affinity tag. There are other amino acid based labeling methods as well such as

VICAT or visible isotope coded affinity tag, MCAT or mass-coded abundance tagging, QUEST or quantitation using enhanced signal tags.

Then there are different types 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. 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 possibility of using different type of (()) (18:53) strategies. In our today's discussion, will mainly focus on iTRAQ and TMTs.

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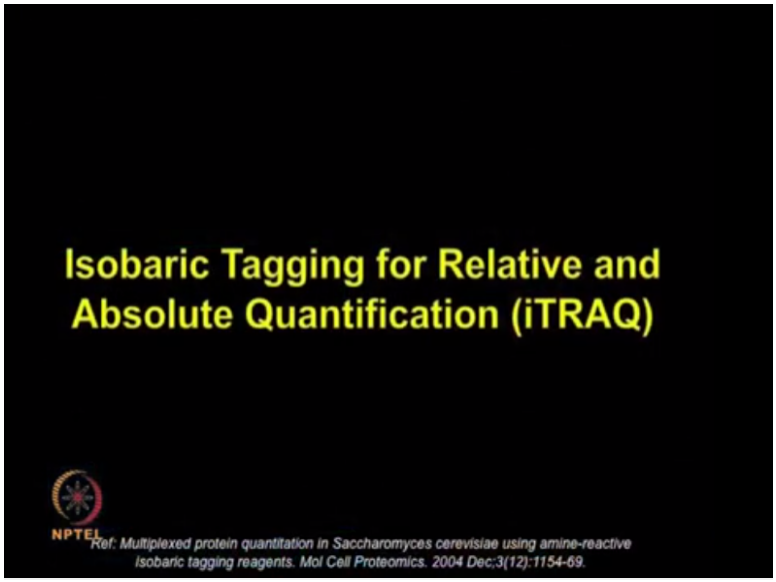

So as I discussed that mass spectrometry has played a very major role in proteomics and now it is becoming a very essential tool to study the complex biological system in various diseases. ITRAQ is a mass spec based technique for relative and absolute quantitation of proteins present in up to 4 samples or up to 8 samples depending upon the type of iTRAQ tags and these labels can be provided in the proteins where the N-terminal MS mode.

The iTRAQ labels available from the applied biosystems and tandem mass tags available from the Thermo Fisher or currently the only tagging technologies, which are commercially available where quantitation is carried out in 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 the applied biosystems.

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ITRAQ, it is a MS based technique for relative and absolute quantitation of protein.

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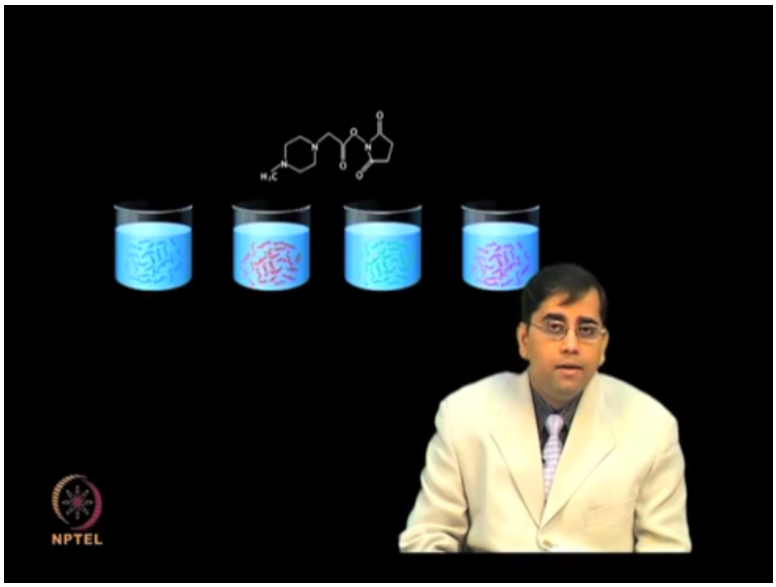

ITRAQ reagents are a set of 4 isomeric amine specific labeling reagents 114, 115, 116 and 117.

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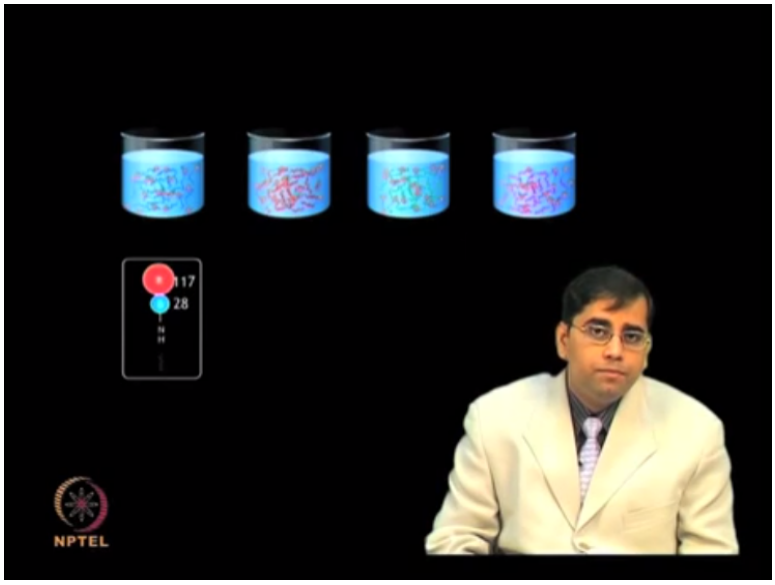

An iTRAQ reagent consist of a reporter group, a balancer group and a peptide reactive group.

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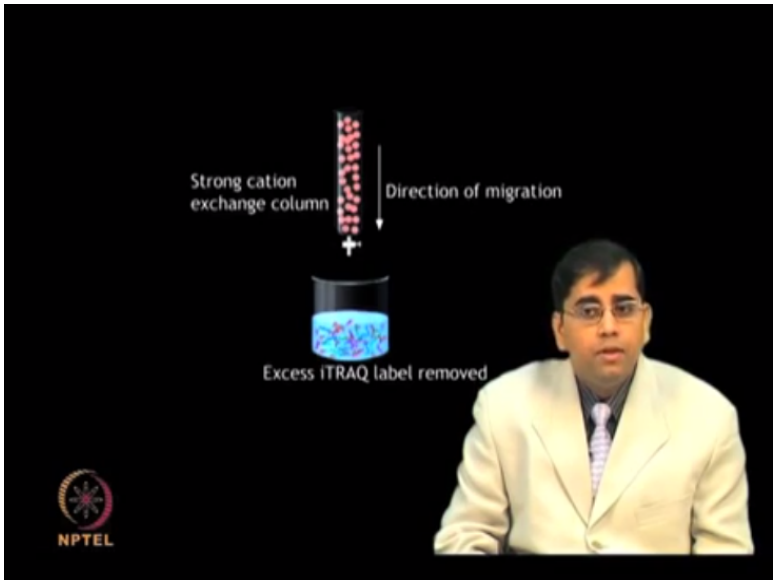

Pulled samples are purified on a strong cation exchange as SCX column to remove the excess unbound reagent.

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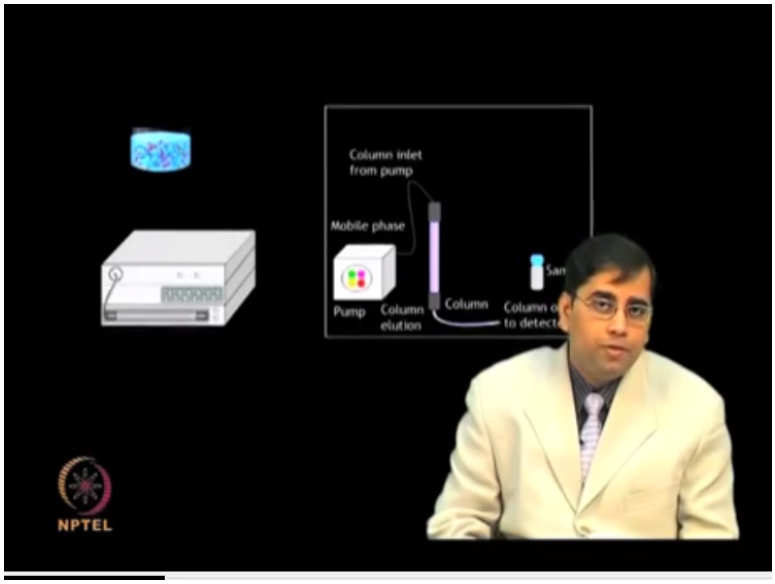

These isobaric labels are detected upon fragmentation and release in mass spectrometry. The data obtained from mass spectrometry can be analyzed by using search engines such as Mascot. The analysis requires inputs regarding the experimental parameters such as enzyme cleavage, modifications, instruments used, peptide tolerance etc.

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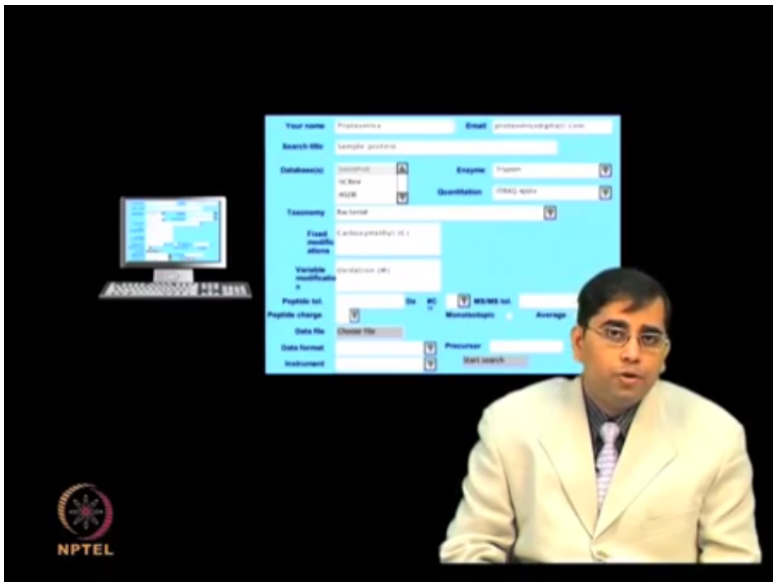

The data files generated from MS is uploaded and the search carried out by employing data bases such as NCBI, MSDB and SysProt.

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Okay 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.

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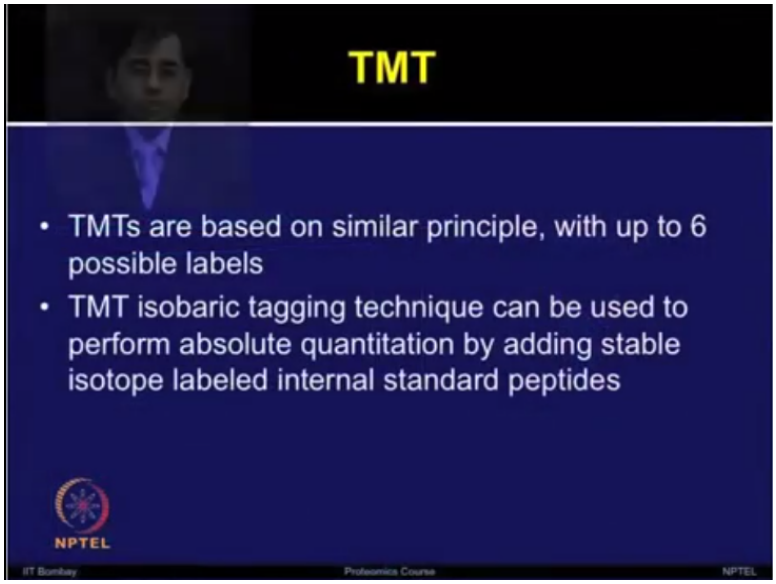

I have given you the reference for the original study on tandem mass tag in the slide. So these tandem mass tags they are based on the similar principle of iTRAQ. Here the possibility for multiplexing is up to 6 possible labels. The TMT isobaric tagging technique can be used to perform absolute quantification by adding a 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 spike into a sample. In that way, the absolute quantification can be obtained.

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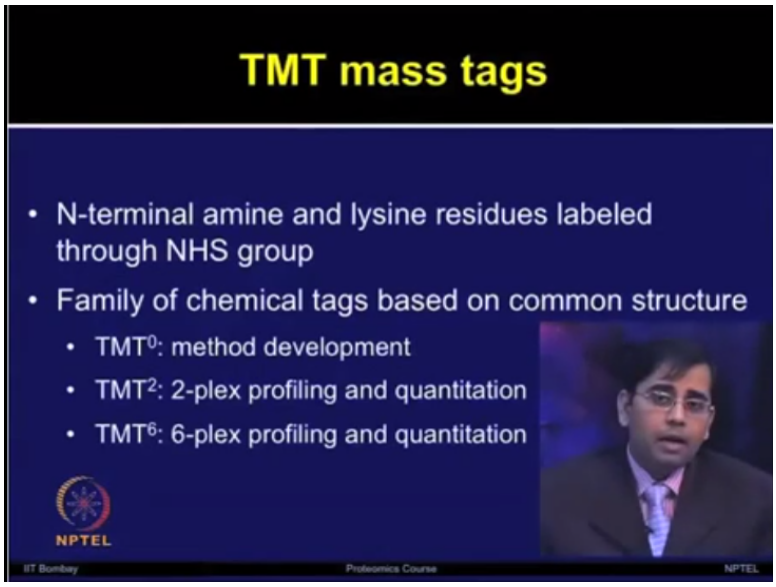

The N-terminal amine and lysine residues are labeled through the NHS group. They are family of chemical tags, which are based on the common structures. The series of TMT tags available, TMT 0, TMT 2-plex, TMT-6 plex. So these TMTs are an innovative set of isobaric mass tags for labeling the proteins and peptides at amine functions and mixing of up to 6 different protein samples are possible.

While duplex and six plex labels TMT differ by the number of isotopic substitutions. The TMT 0 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 for 126 to 131 Dalton. Therefore, it allows for the relative quantitation. The TMT 6-plex, each tag adds a mass of 229 Daltons per labeled amine to the protein.

The TMT duplex and TMT 0 share the TMT complex structures. Let us look at the TMT 0 label structure in more detail.

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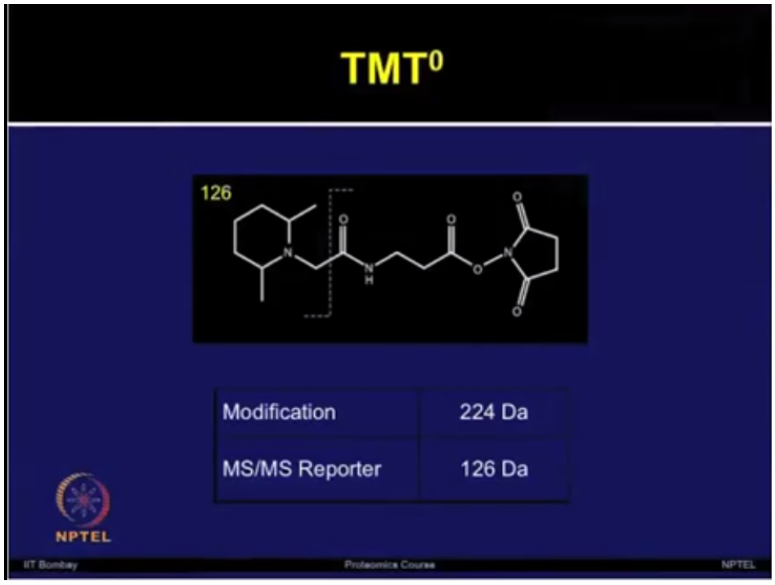

The TMT 0 tag is used for testing and optimization of sample preparation, labeling, fractionation and MS fragmentation for peptide identification and reporter detection. The modification is 224 Daltons and MS/MS reporter ion is 126 Daltons.

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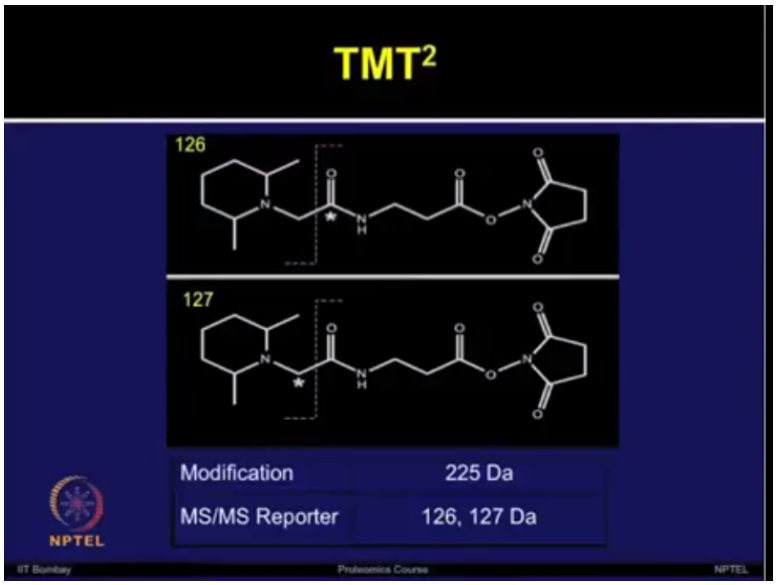

Now let us look at TMT duplex. The TMT duplex reagent allows for the comparison of 2 samples, 126 and 127. These are 2 different MS/MS reporter ions available and modification is 225 Daltons.

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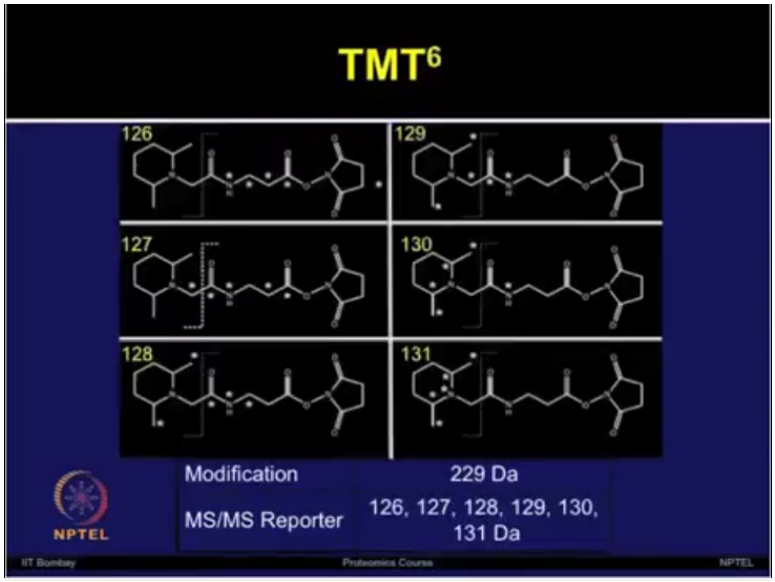

Let us now look at the TMT 6-plex reagent. It allows the comparison of up to 6 conditions. The MS/MS reporter ions as you can see in the structure are from 126, 127, 128, 129, 130 and 131 Daltons. So the TMT 6-plex reagent allows comparison of up to 6 conditions. It could be useful for studying about time codes, drug dose responses, replicates or looking for multiple sample disease comparison, the modification is 229 Daltons.

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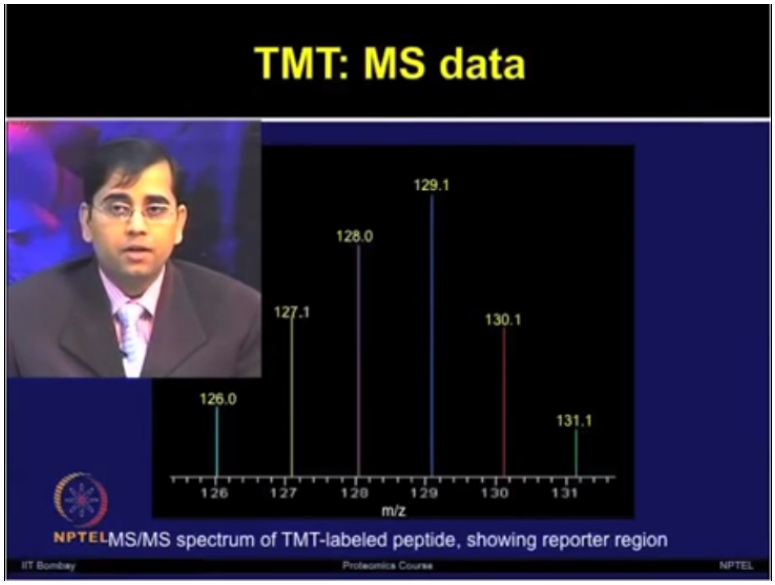

I am sure (()) (26:29) representative MS/MS spectrum of TMT labeled peptide, which is showing a reporter region. The relative abundance of target protein or peptide fragment in such different samples can be easily measured by comparing these signature mass peaks, which are generated by the different mass tags.

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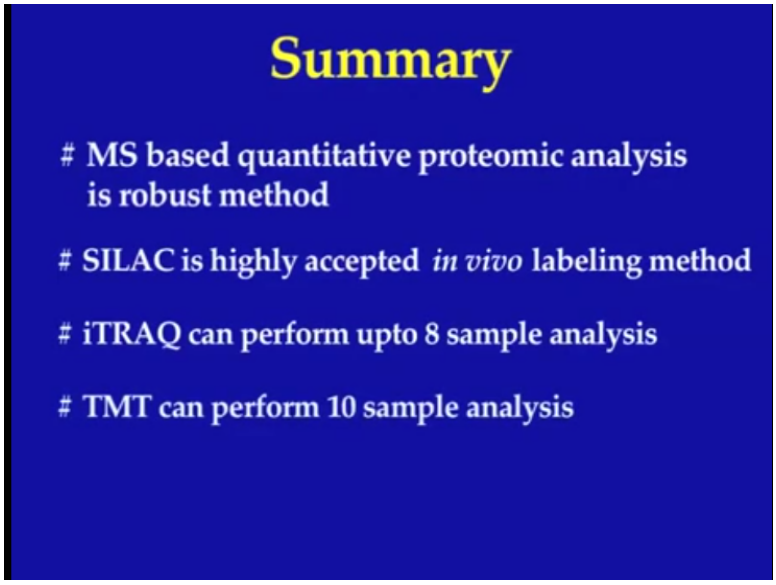

(Refer Slide Time: 27:03)

(Refer Slide Time: 27:09)

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