**Mass Spectrometry Based Proteomics**
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**Department of Biosciences and Bioengineering**
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**Mod 03 Lecture Number 11**

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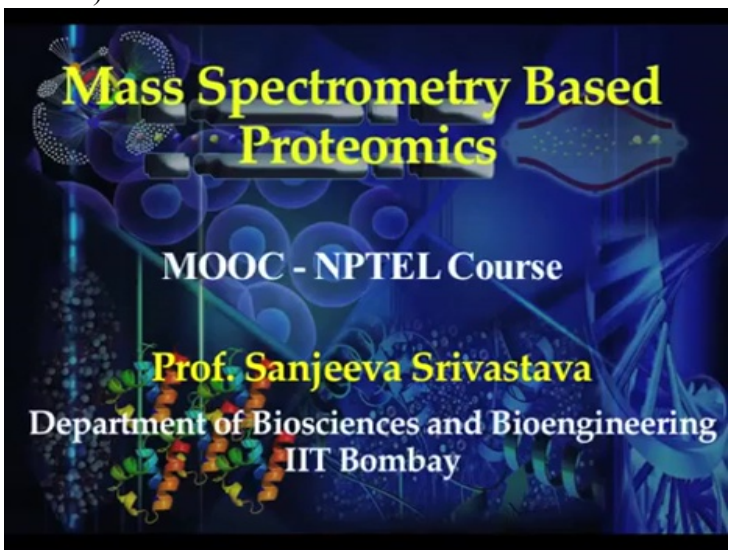


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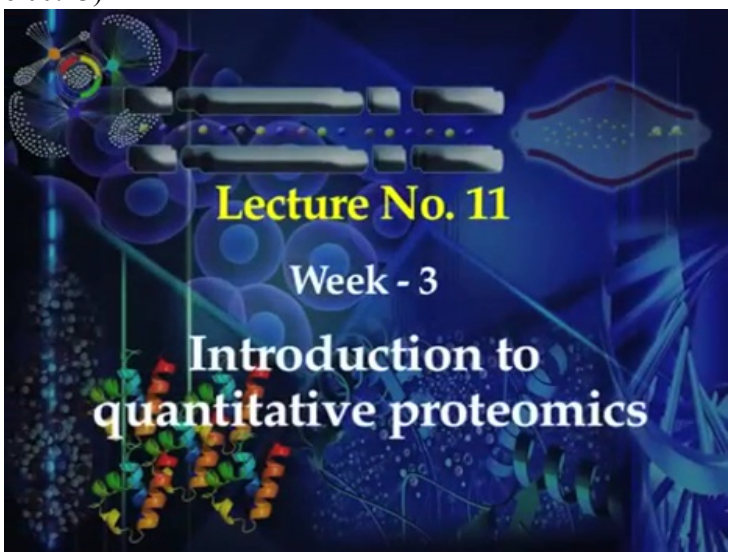

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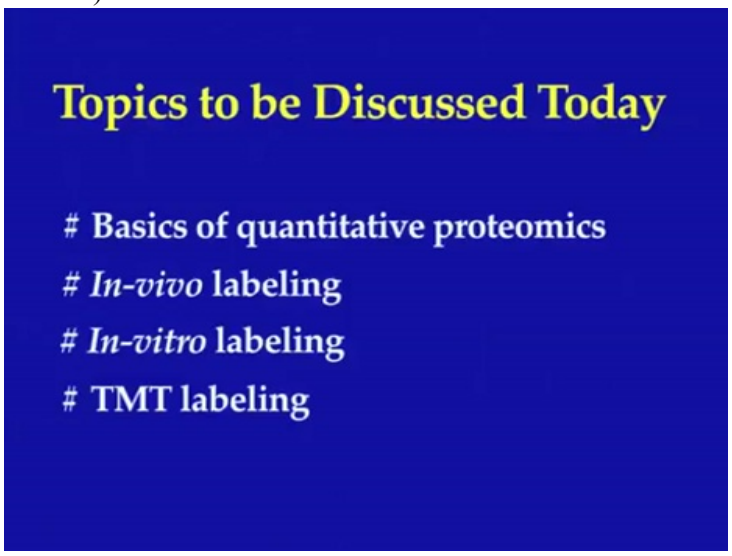


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The complexity and dynamic nature of proteome present major technological challenges. Mass spectrometry advancements have improved the high throughput identification and quantification of proteins. The mass spectrometers have advanced significantly and now offering opportunities to understand the human diseases and discover biomarkers.

MS has been 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 quantitative purpose remains limited.

Many advancement in MS during the last decade have provided new ways for protein analysis and facilitated the study of proteomic analysis of various biological systems.

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

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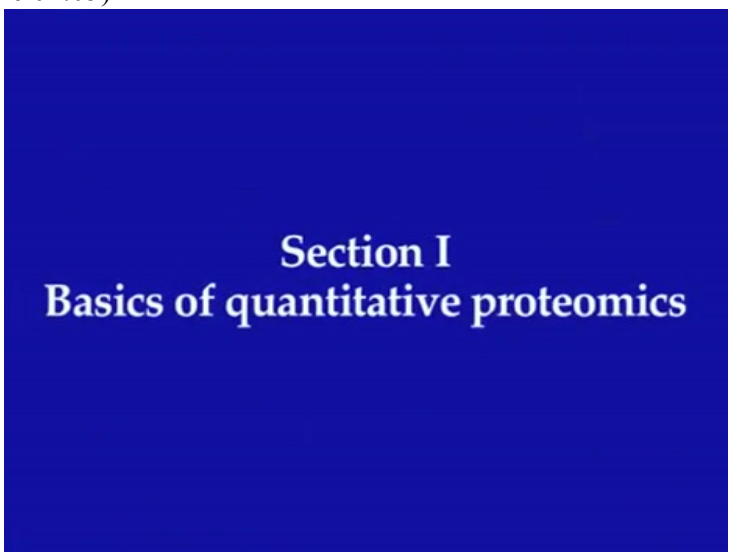


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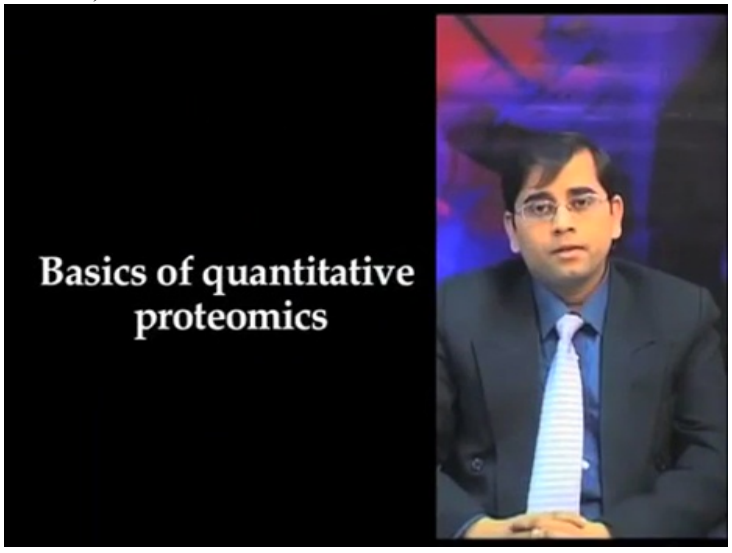


Protein labeling with stable isotopes are new and effective methods for quantitative proteome profiling using Mass Spec. These isotopic labels can be introduced in vivo or in vitro and provide relative abundance of proteins for proteomic analysis.

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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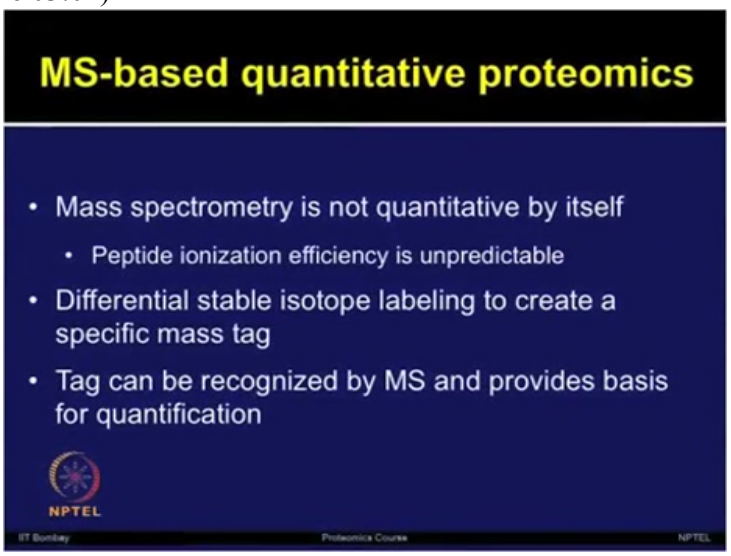


So as I discussed mass spectrometer is not quantitative technique by itself due to the peptide ionization efficiency which is unpredictable. So the differential stable isotope labeling is used to create a specific mass tag.

There are different types of mass tags which are currently very promising for various quantitative proteomic applications including ICAT, ITRAQ, SILAC as well as various other tags. So, these tags can be recognized by MS and provide the basis for quantification.

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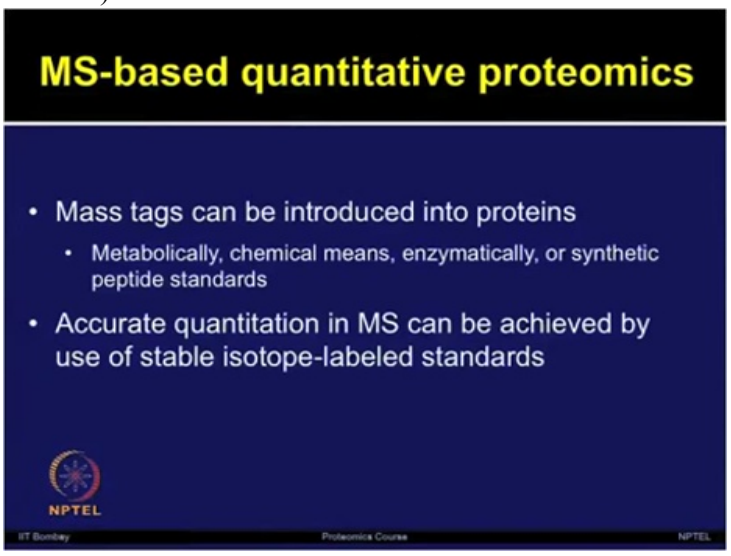


These mass tags can be introduced by various methods into the proteins such as metabolic labeling, chemical means of labeling, enzymatic methods or by using synthetic peptide standards. The accurate quantification in Mass spec can be achieved by the use of stable isotope-labeled standards.

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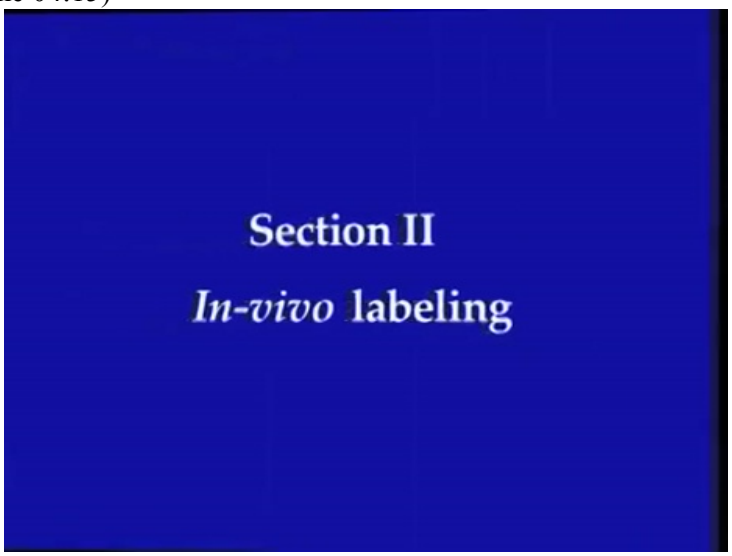

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So there are different ways of quantitative proteomic analysis, in vitro and in vivo labeling methods. In today's lecture we will focus on in vivo labeling methods. So, most of the quantitative proteomics approaches by MS utilize isotopic labels as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

The 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.

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

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There are different ways of in vivo labeling such as enrichment of 15 N media, Culture Derived Isotope Tags or CDIT, Stable Isotope Labeling by Amino acids in Cell Culture or SILAC. Although we will discuss briefly about 15 N media method and Culture Derived Isotope Tags and then we will focus on mainly SILAC method for rest of the lecture.

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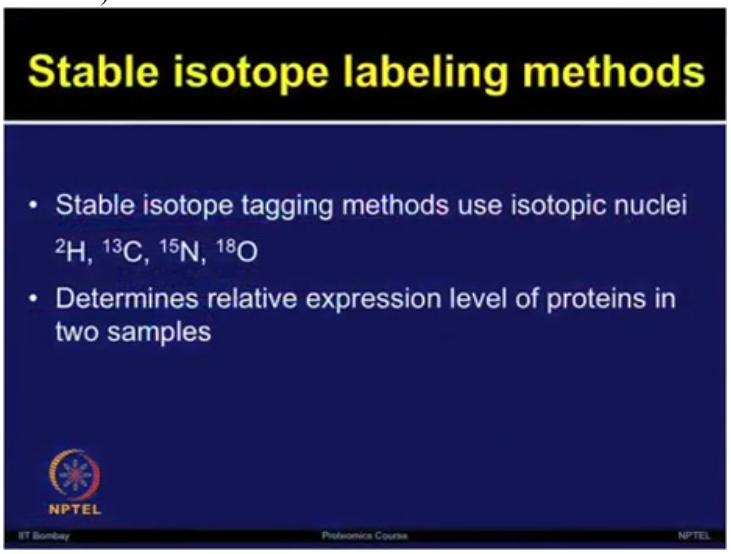


So the stable isotope tagging methods use isotopic nuclei such as 2 Hydrogen, 13 Carbon, 15 Nitrogen and 18 Oxygen. These stable isotopes are incorporated in place of natural abundance isotopes in heavy standard.

By using the stable isotope labeling methods one can determine the relative expression level of proteins in two samples.

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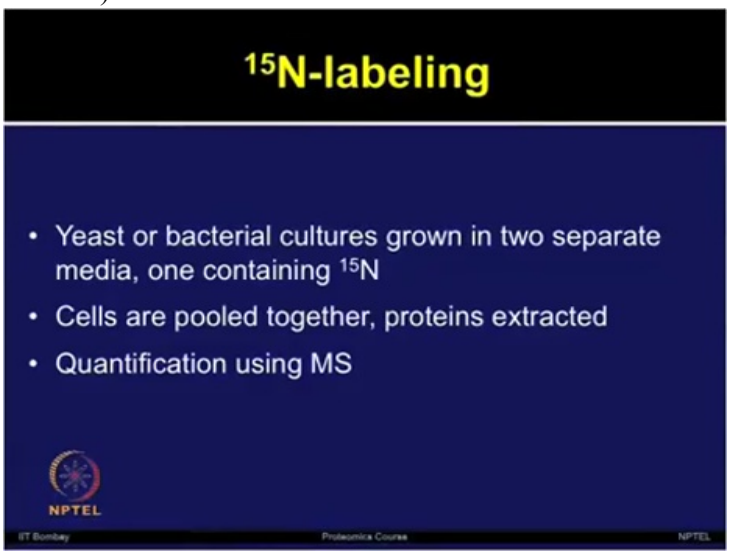


Let's briefly discuss about 15N labeling methods.

Yeast or bacterial cultures which are grown in two separate media; one containing 15 Nitrogen. The cells are pooled together. Proteins can be extracted from these pooled cells and quantified by using Mass Spec.

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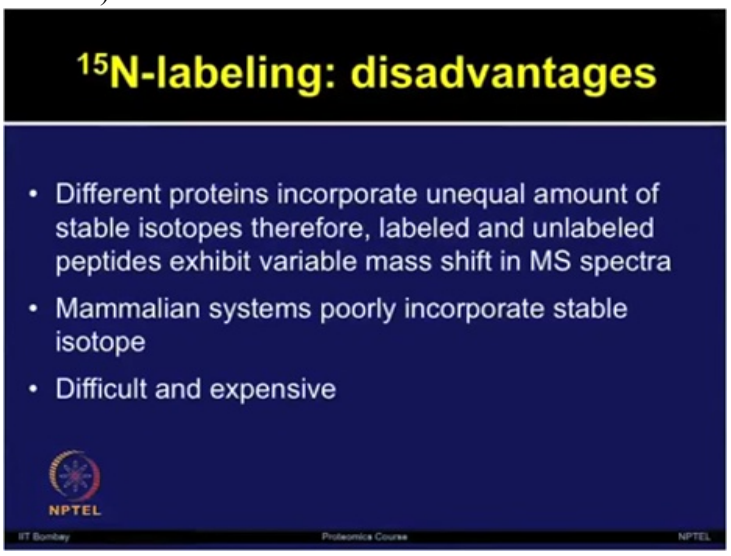


Although 15N labeling is an easy method but there are various disadvantages.

The protein incorporation in your control and the 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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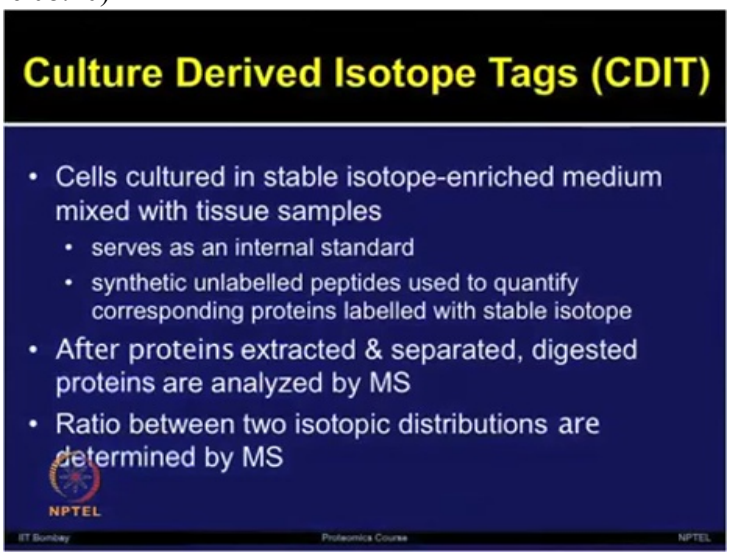


Now let's discuss about Culture Derived Isotope Tags or CDIT.

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 the two isotopic distributions can be determined by Mass Spec.

After having discussed some of the less commonly used such as 15N media and CDIT, now let's discuss about…

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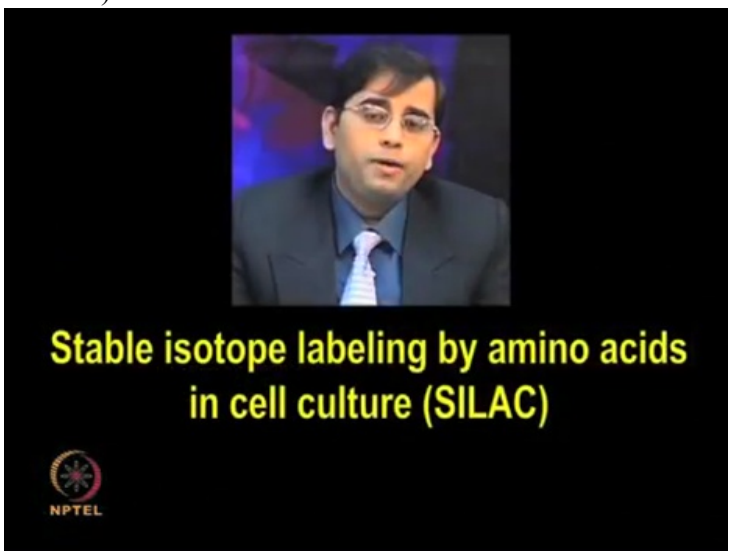


… Stable Isotope Labeling by Amino acids in Cell Culture, SILAC; so, the MS-based quantitative proteomics is an increasingly popular approach to study changes in the protein abundance in various biological samples.

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 derivatisation and associated purification.

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Stable Isotope Labeling by Amino acids in Cell Culture or SILAC, which is a metabolic labeling strategy to encode whole cellular proteome. It is widely used method for the quantitative proteomics.

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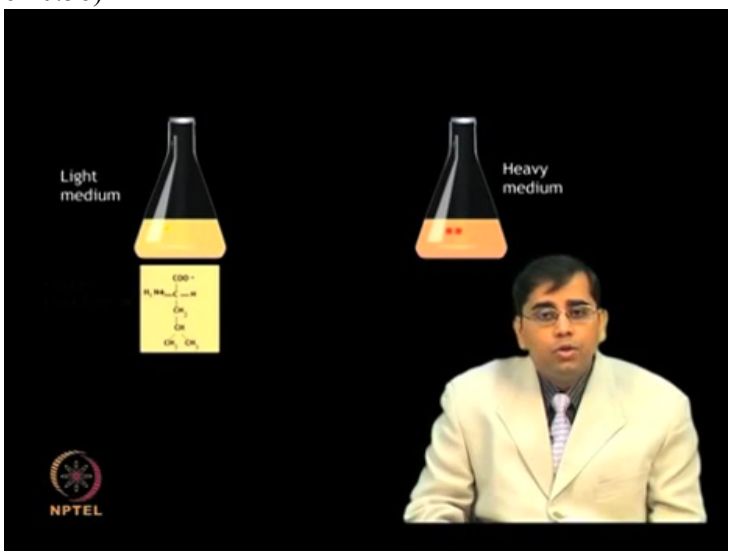


In SILAC method two group of cells are cultured in media that are identical in all the respects except that one contains a heavy, isotopic analog of an essential amino acid while the other contains the normal light amino acid. After a number of cell divisions, the grown cells are combined and digested using Trypsin.

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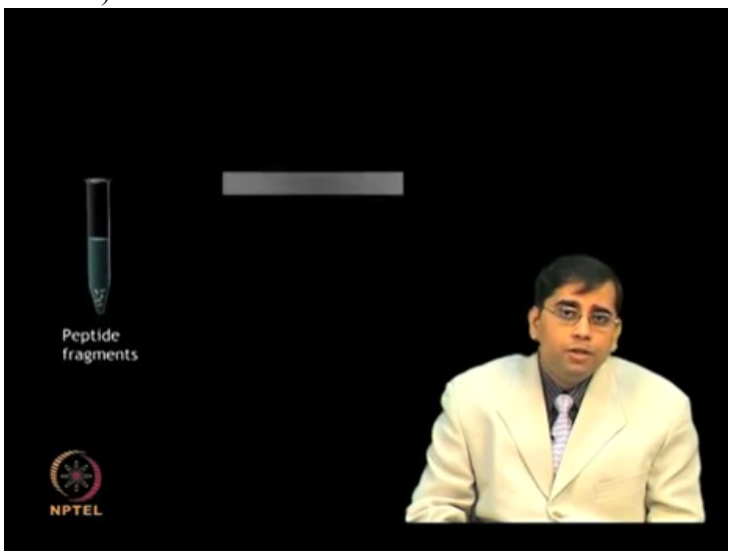


The complex protein mixture is further separated

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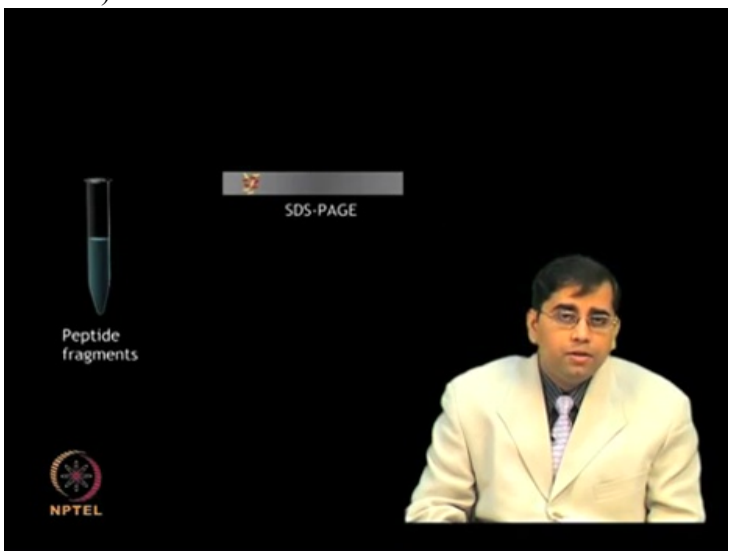


….by SDS page to simplify this analysis.

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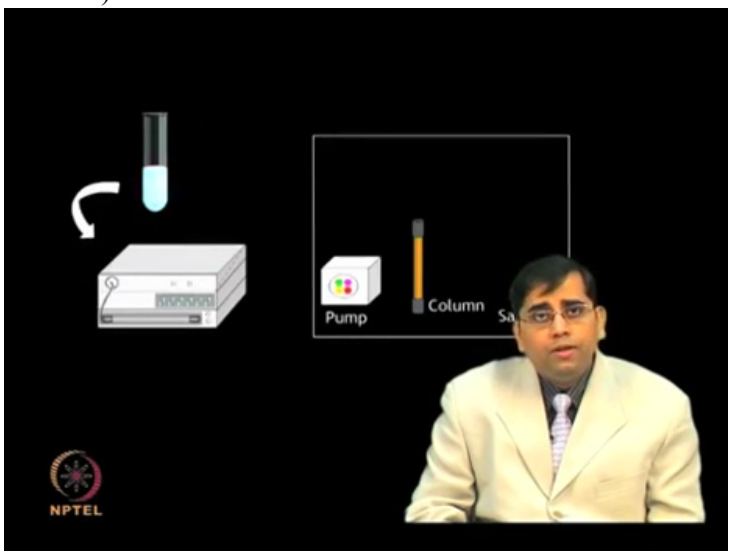


Further application is carried out by liquid chromatography

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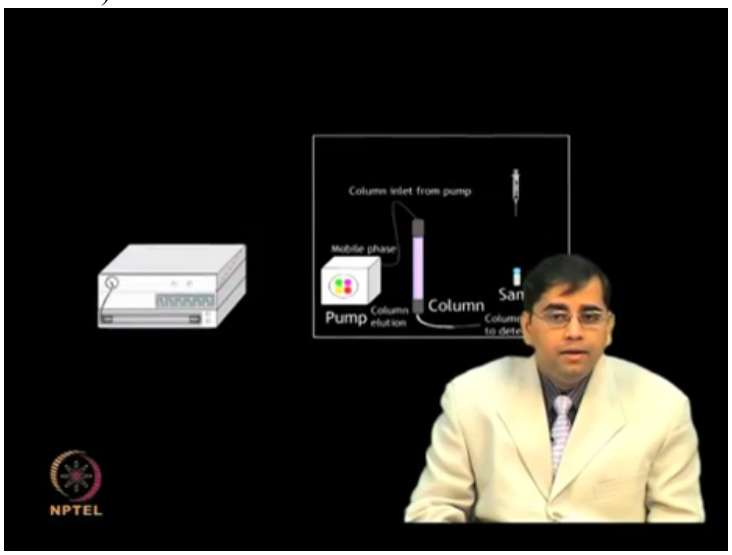


… and purified peptide fragments are analyzed by MS/MS.

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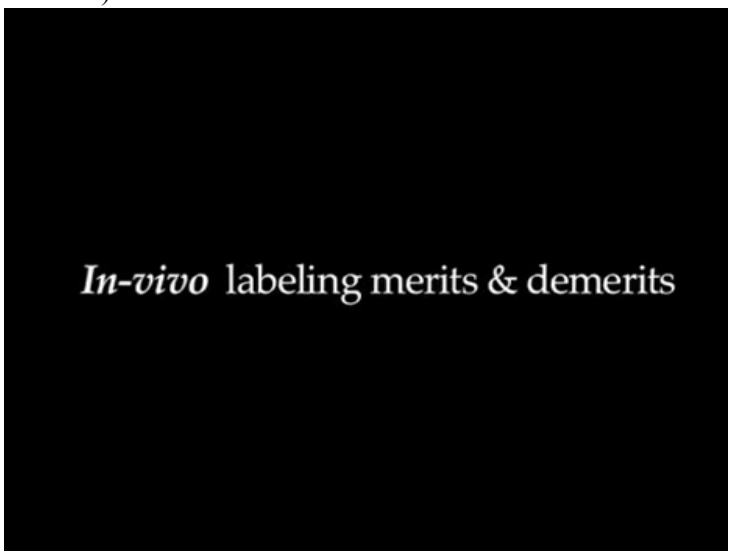


So after discussing the SILAC technique and watching this animation of this entire process, now let's discuss about …

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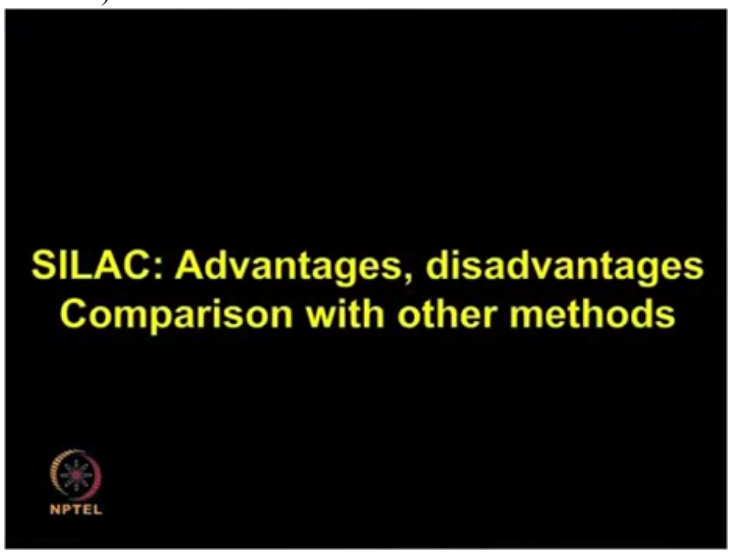


advantages and disadvantages of SILAC method and compare it with some other tagging methods.

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 label samples are mixed at the stage of whole cells which make SILAC approach ideally suited for the quantitative proteomics 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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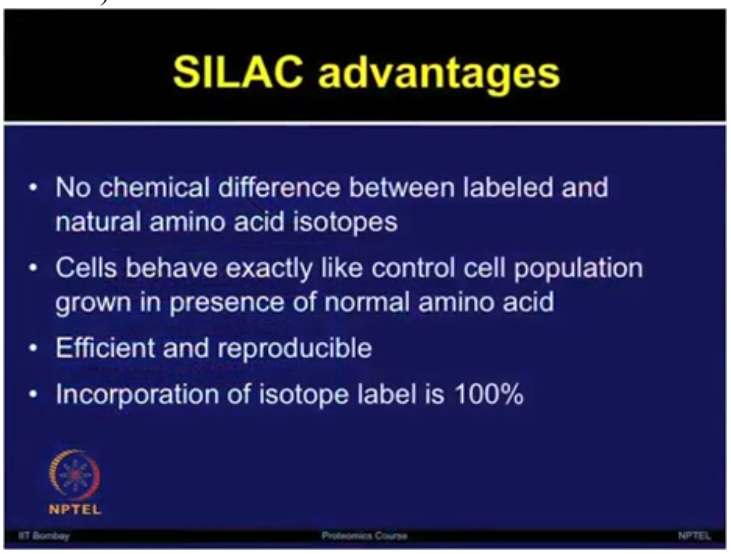


The cells are grown in the tissue culture medium and they behave exactly like the control cell population grown in presence of the normal amino acids. So, this method is very efficient and very reproducible.

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. So, samples can be mixed prior to the processing before doing the Mass Spec analysis.

So, this method eliminates some of the handling errors.

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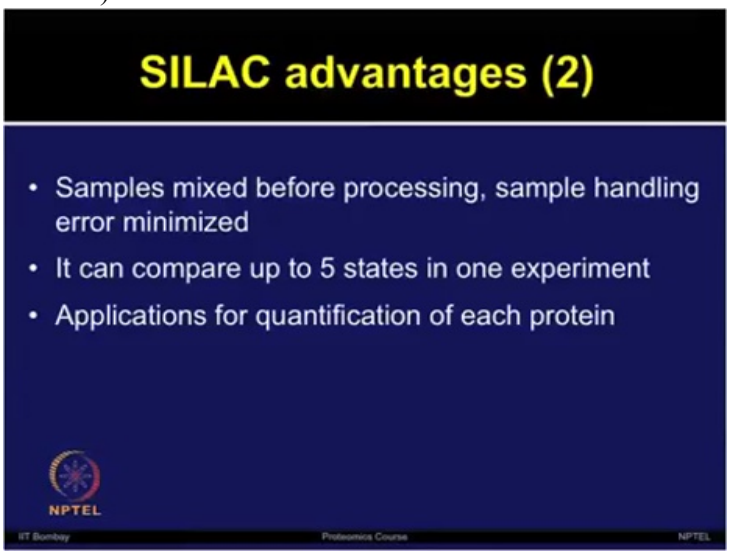


Now, let us... SILAC experiments have demonstrated that it can provide the 5 flexing capability to compare 5 different states in one experiment. So there are various applications of quantification of proteins by using SILAC method which we will discuss in next few slides.

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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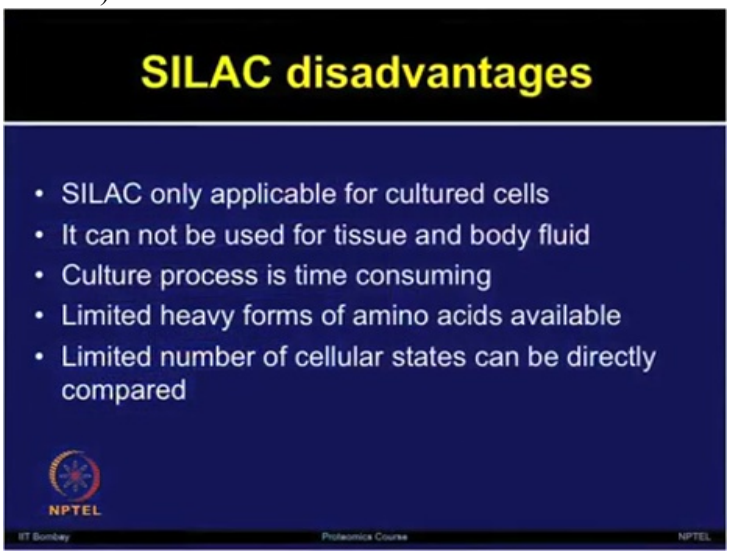


SILAC is applicable for only culture cells. It cannot be use for tissue or body fluids.

So, that is one of the major limitations 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.

The metabolic conversion of arginine to proline is one of the commonly observed issue in the SILAC experiments which results…

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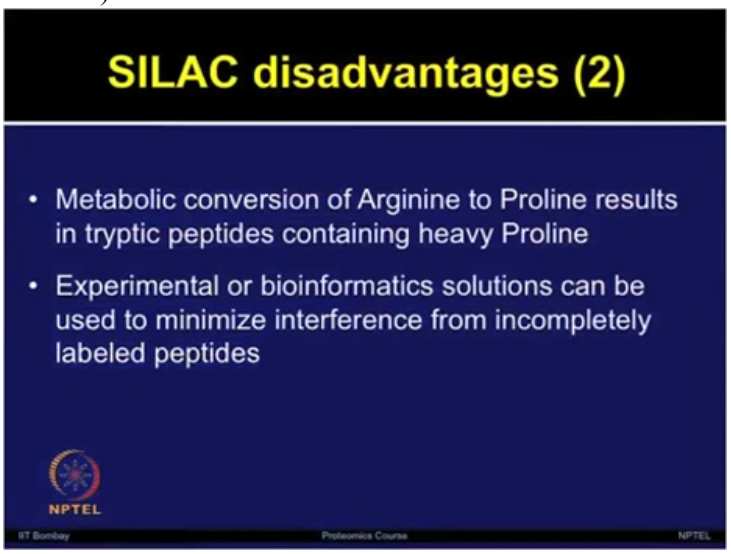


… in the tryptic peptides containing heavy prolines

Now there are various experimental or bio-informatics solutions in place which can be used to reduce the interference from incompletely labeled peptides.

We will discuss about how SILAC compares with the radioactivity labeling methods.
The SILAC method seeks to replace the labeled amino acid which is unlike

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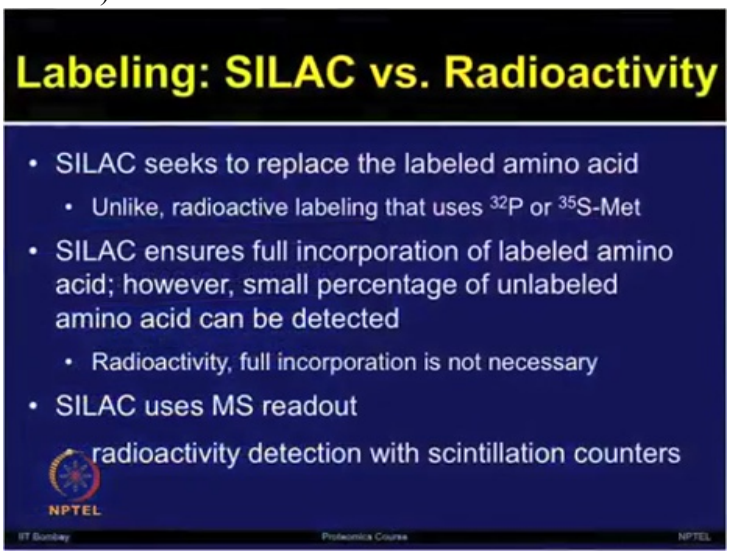


the radioactive labeling; which uses 32 P or 35 S methionine.

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 base 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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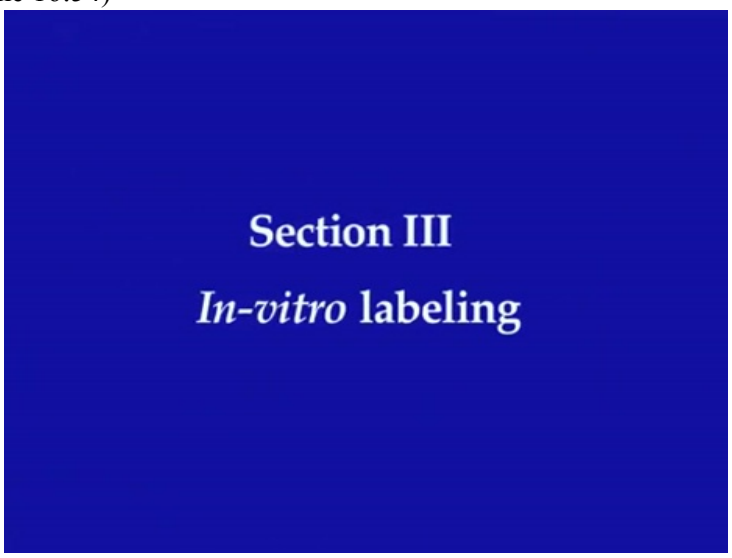

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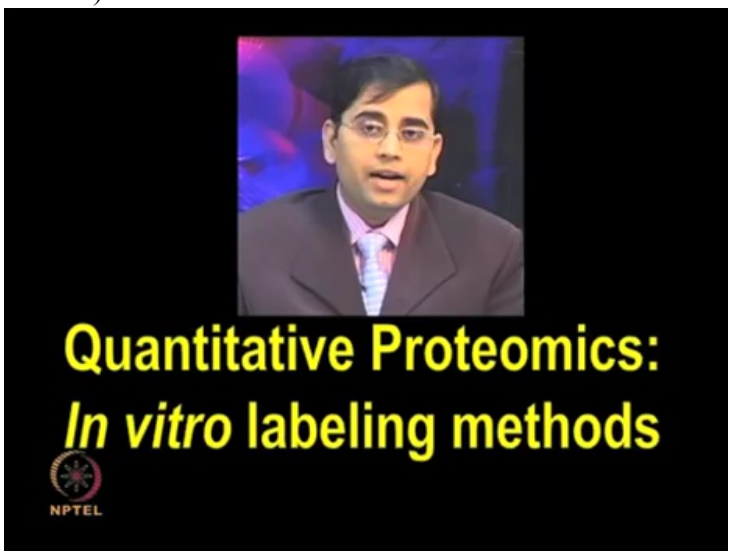


In vitro labeling methods rely on use of labeling reactions at a specific site in proteins or peptides. Based on various labeling chemistry, different types of strategies have been developed to introduce isotopes at either protein or peptide level. And these strategies, we will discuss in the next slides.

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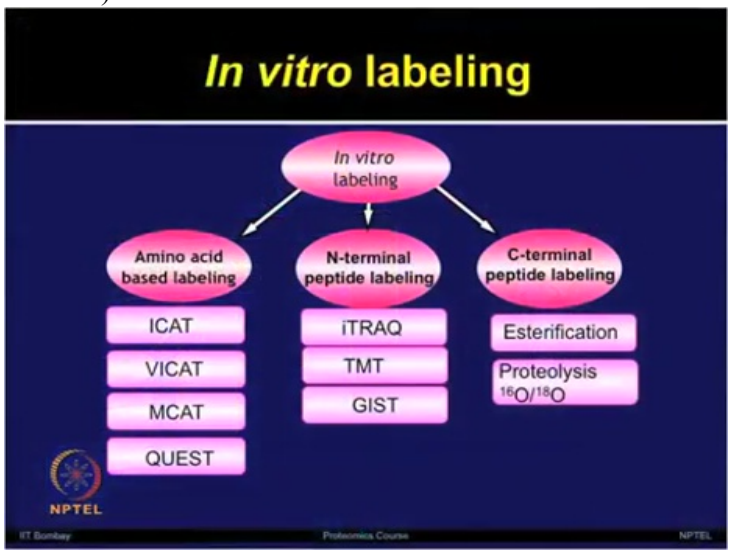


So, in vitro labeling can be 3 different types; amino acid based labeling, N-terminal peptide labeling and C-terminal peptide labeling.

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.

Then there are C-terminal 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 based labeling approaches available and depending upon your quantitative application there is a possibility of using different type of tagging strategies.

In our today's discussion we will mainly focus on iTRAQ and TMTs. So as I discussed that Mass Spectrometry has played a very major role in proteomics

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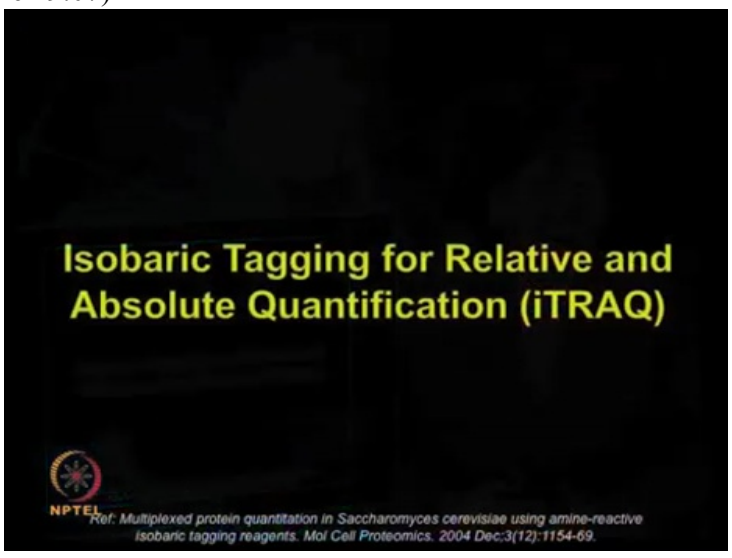


.. and now it is becoming a very essential tool to study the complex biological system in various diseases.

iTRAQ is a Mass Spec -based technique for relative and absolute quantitation of proteins present in up to four samples or up to eight samples depending upon the type of iTRAQ tags and these labels can be provided in the proteins where there are N-terminal MS mode.

The iTRAQ labels are available from Applied Biosystems and Tandem Mass Tags available from Thermo Fisher are currently the only tagging technologies which are commercially available, where quantitation can 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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iTRAQ, it is MS based technique for relative and absolute quantitation of protein.

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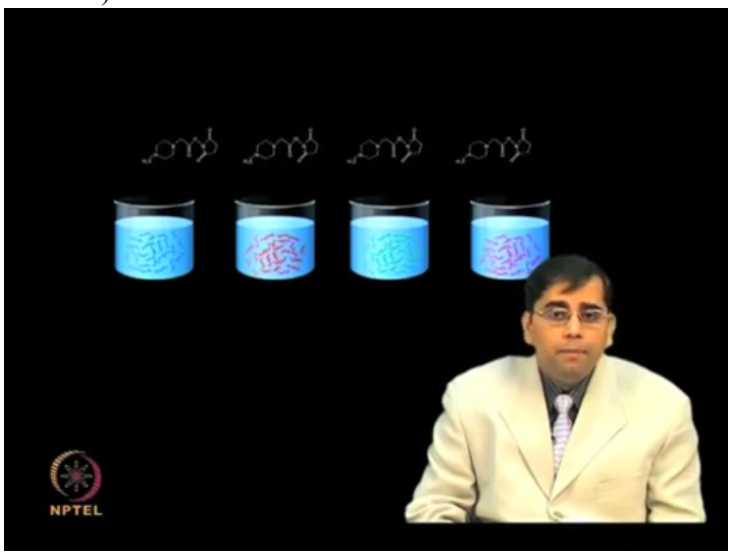


iTRAQ reagents are set of 4 isobaric amine specific labeling reagents, 114, 115, 116 or 117.

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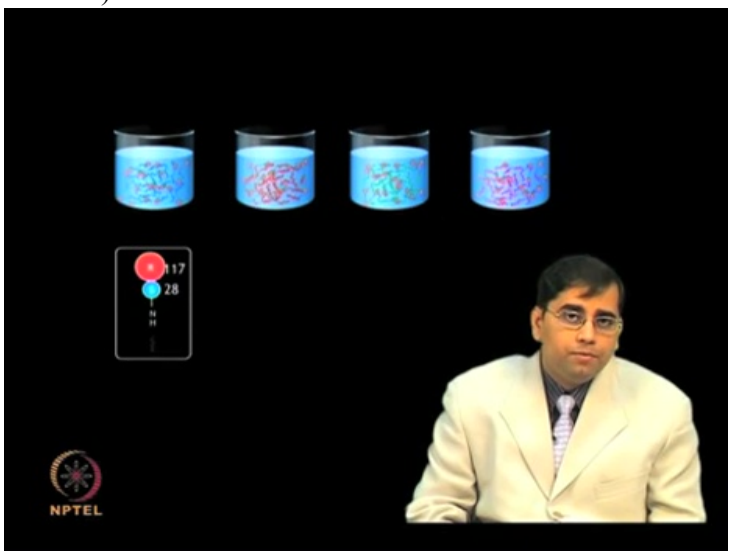


An iTRAQ reagent consists of a reporter group, a balancer group and a peptide reactive group.

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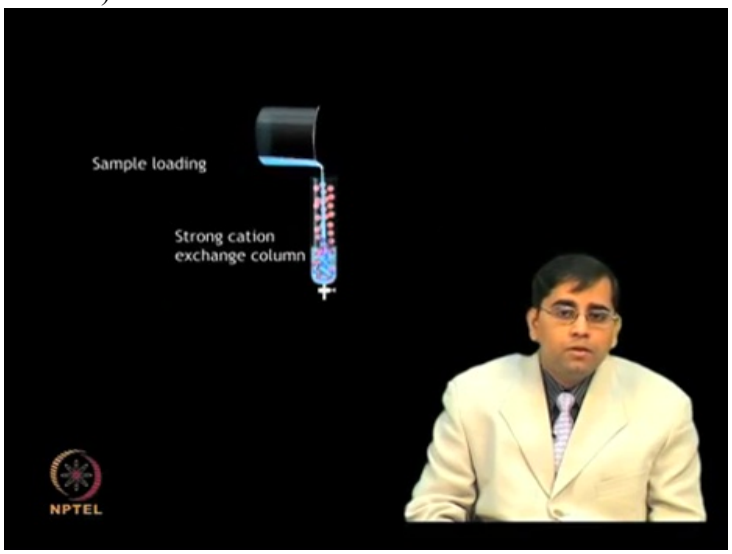


Pooled samples are purified on a Strong Cation eXchange

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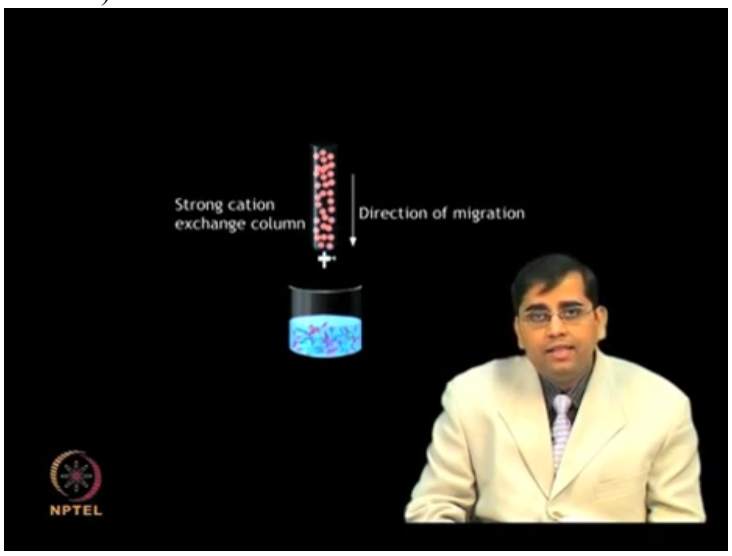


SCX column to remove

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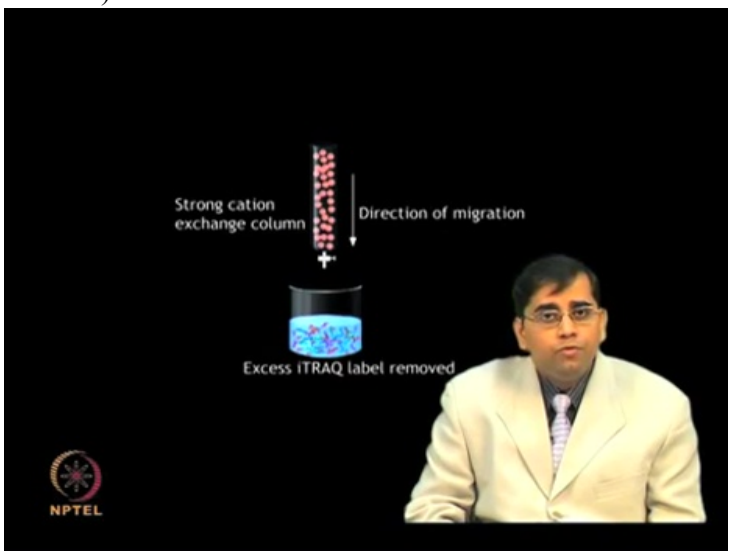


the excess unbound reagent.

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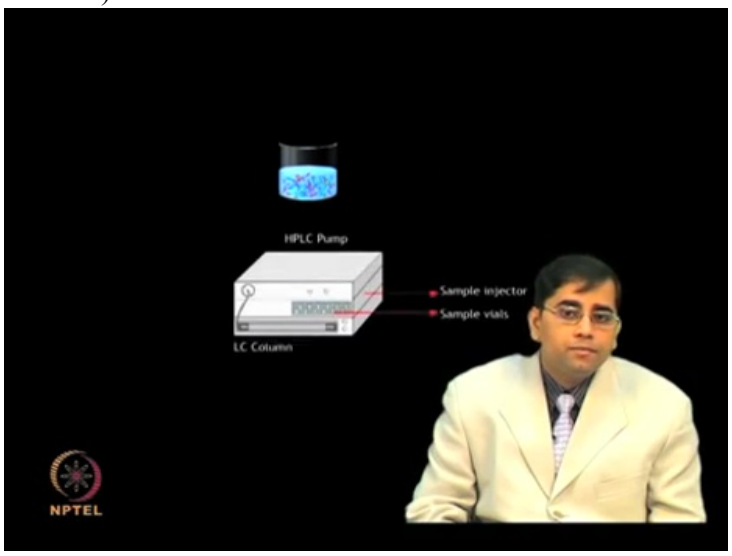


These isobaric labels are detected upon fragmentation and release

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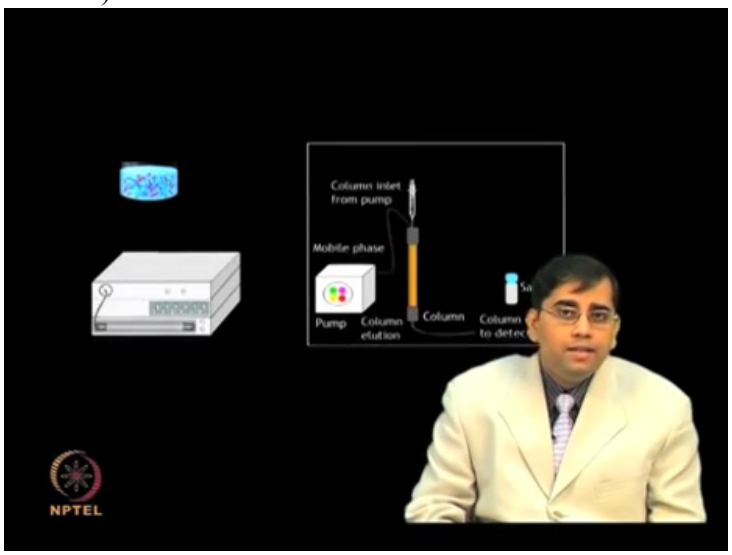


… in mass spectrometry

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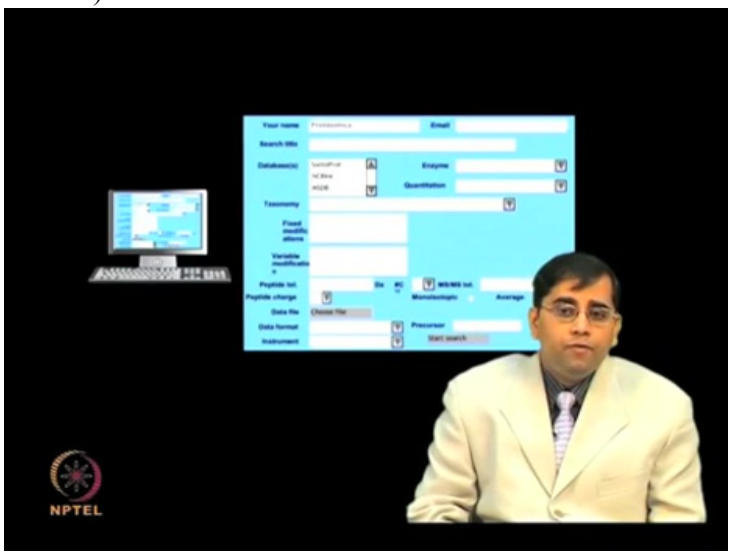

Data obtained from mass spectrometry can be analyzed using search engines such as MASCOT. The analysis requires inputs regarding the experimental parameters used such as enzyme cleavage, modifications, instruments used, peptide tolerance etc.

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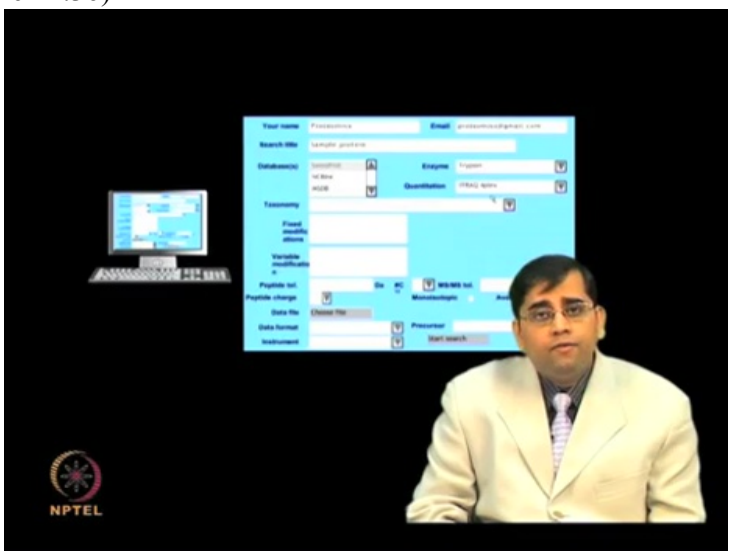

The data files generated from MS is uploaded and the search carried out by employing databases such as NCBI, MSDB and Swiss Prot.

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Ok so now let's talk about Tandem Mass Tag or TMT. This method is similar to iTRAQ which we just discussed. TMT is also MS/MS based

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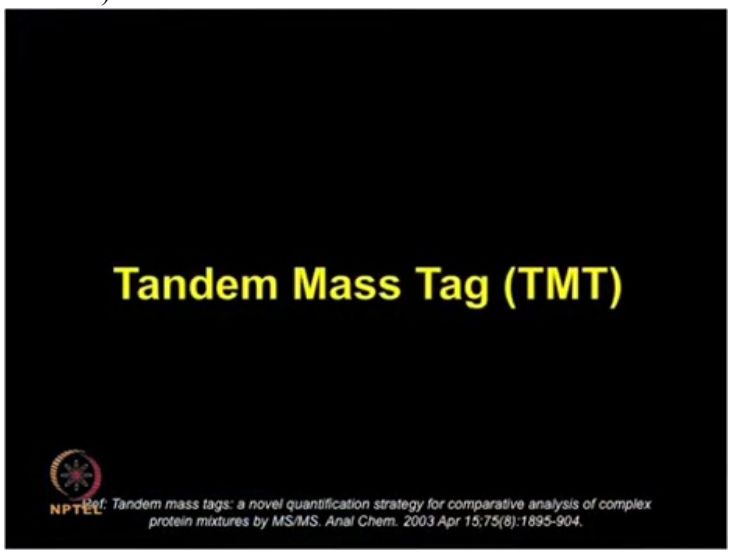


… quantitative technique which uses the isotopomer labels referred as tandem mass tags. It also provides the accurate quantification of peptides and proteins. Tandem Mass Tags have been developed by the Proteome Sciences and currently commercialized by Thermo Fisher. I have given you the reference for the original study on Tandem Mass Tag in the slide.

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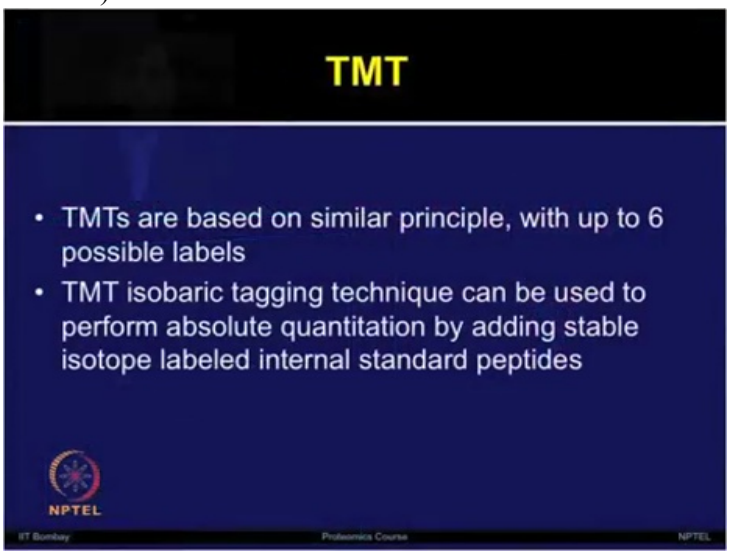


So these Tandem Mass Tags, they are based on similar principle of iTRAQ. Here the possibility for multiplexing is up to 6 possible labels.

The TMT isobaring 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 spiked into a sample. In that way absolute quantification can be obtained.

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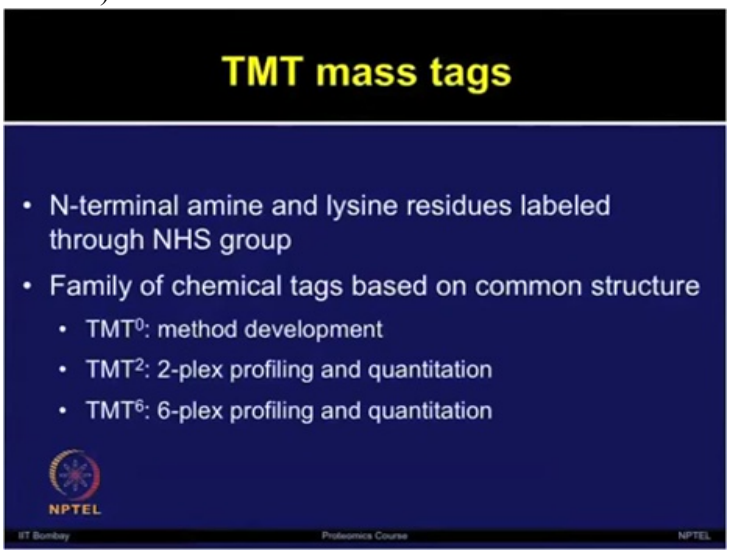


The N-terminal amine and lysine residues are labeled though NHS group. There are family of chemical tags which are based on the common structures. The series of TMT tags available TMT 0, TMT 2-plex, TMT 6-plex.

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

While duplex and 6-plex labels TMT differ by the number of isotopic substitutions, TMT 0 is non-isotopically substituted structure that has been produced for only method development.

During the MS/MS analysis the TMT tags give rise to 6 reporter ions from 126 to 131 Dalton, therefore it allows for the relative quantitation. 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's look at TMT 0 label structure in more detail.

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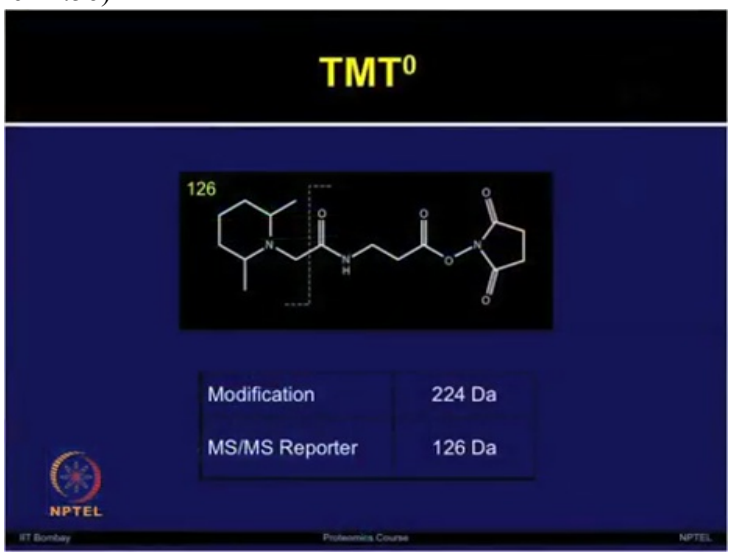


The TMT 0 tag is used for testing and optimization of sample preparation, labeling, fractionation and MS fragmentation for peptide identification and reporter detection. The modification is 224 Daltons and MS/MS reporter ion is 126 Daltons.

Now let's look at TMT duplex.

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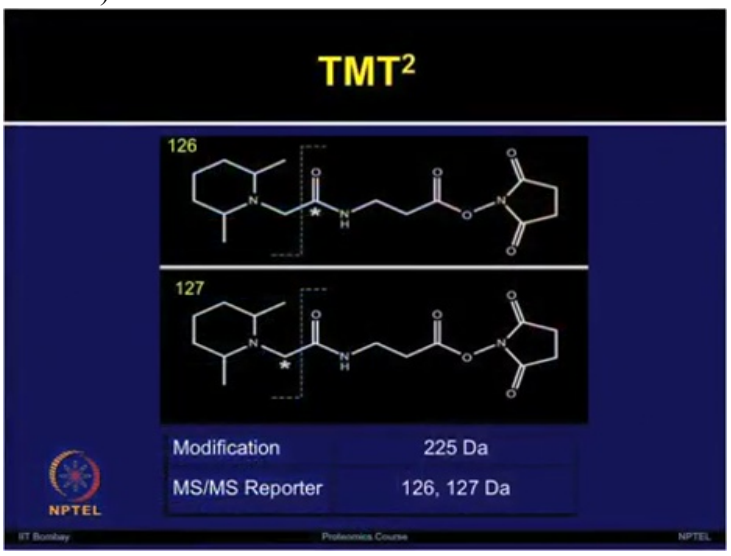


The TMT duplex reagent allows for the comparison of two samples. 126 and 127, these are two different MS/MS reporter ions available and modification is 225 Daltons.

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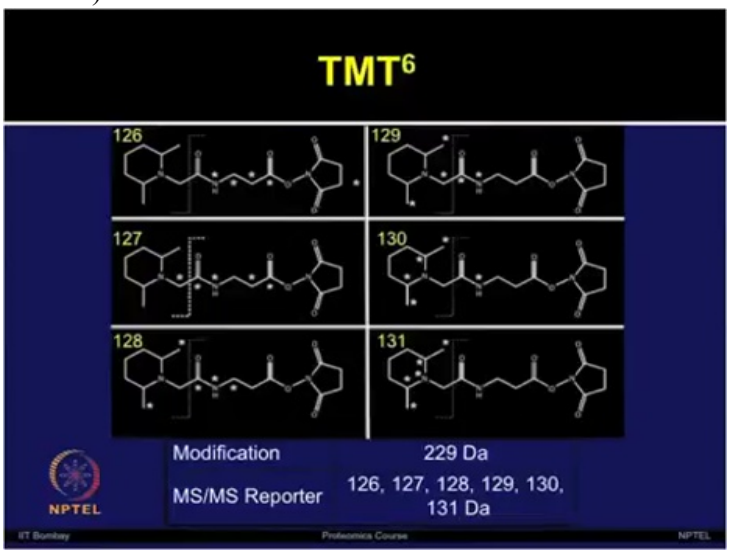


Let's now look at TMT 6-plex reagent. It allows the comparison of up to 6 conditions. The MS/MS reporter ions, as you can see in the structure are from 126, 127, 128, 129, 130 and 131 Daltons.

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

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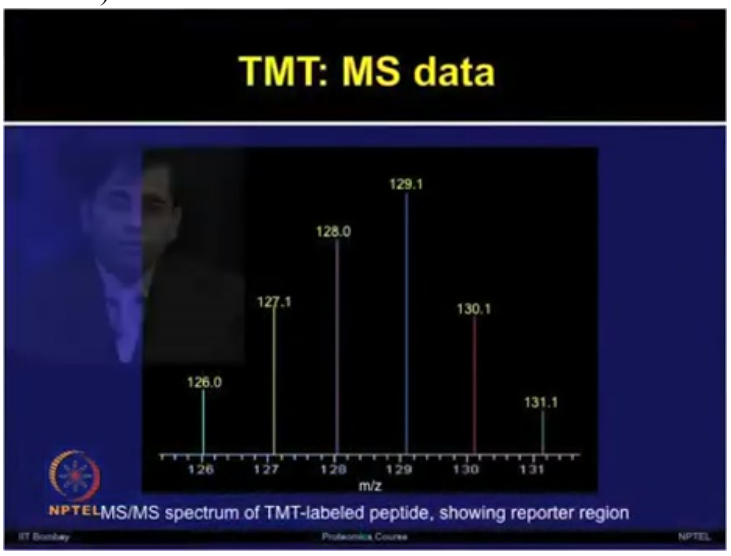


I am showing you one representative MS/MS spectrum of TMT labeled peptide which is showing a reporter region. The relative abundance of target protein or peptide fragment in 6 different samples can be easily measured by comparing these signature mass peaks which are generated by the different mass tags.

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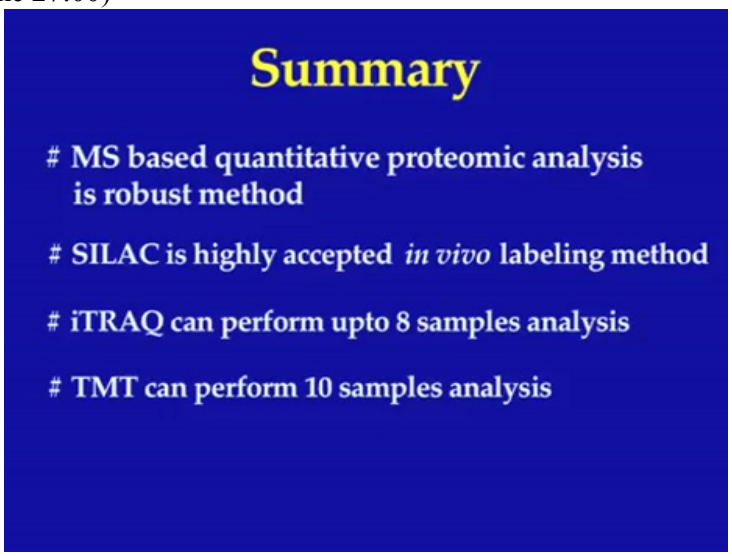

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