LECTURE-14

In vitro Quantitative Proteomics using iTRAQ

HANDOUT

PREAMBLE

One of the major advantages of mass spectrometry over conventional gel-based proteomics, apart from deciphering the identity of the protein, is the ability to quantify the proteins under consideration. The conventional mass spectrometry provides an idea about the relative abundance of the ions in question, but using different isotopic or isobaric tags, it is now possible to actually determine the relative abundance. Although there are many quantitative proteomic approaches using mass spectrometry but iTRAQ, ICAT and SILAC have become most popular. However, these techniques have their own pros and cons and that's why these techniques are selected based on the type of applications. Amongst all these techniques, iTRAQ is currently one of the most popular techniques because of its versatility and multiplexing ability. This lecture mainly discusses the principle, working protocol and some advantages and disadvantages of iTRAQ based quantitative proteomics.

OUTLINE OF LECTURE

1. Quantitative proteomic approaches
2. Principle of iTRAQ
3. iTRAQ reagent
4. Working protocol

PROF. SANJEEVA SRIVASTAVA
DEPARTMENT OF BIOSCIENCES & BIOENGINEERING
IIT BOMBAY, MUMBAI, INDIA

5. Data Analysis
6. Advantages and limitations of iTRAQ

1. QUANTITATIVE PROTEOMIC APPROACHES

Proteins are the most dynamic entities that govern the cellular activities. Hence it becomes necessary to study the dynamic change in protein concentration, subject to change in various environmental, physiological or medical processes. Conventional techniques like ELISA and RIA can be used to quantitatively study the dynamic changes in protein concentration, but these techniques are limited by the study of only one protein at a time and also with the availability of pure antibodies against these proteins. Gel-based proteomic analysis has some disadvantages of reproducibility, membrane protein analysis, abundant protein representation, etc. Advanced gel based proteomic analysis like DIGE give a qualitative and a semi-quantitative analysis of change in proteome level, subject to various external stimuli.

In clinical proteomic studies, it becomes necessary to know the absolute difference in the levels of proteins subject to two different conditions, ideally, a normal/healthy against treated/pathological states. Also, the number of samples to be analyzed becomes extremely large to negate any false positive results, especially, when biomarker discovery is a question. For this high throughput requirement, mass spectrometry based quantitative proteomics has emerged as an essential tool in clinical proteomics. Mass spectrometric approaches include ICAT, iTRAQ and SILAC, which can quantitatively determine the difference in proteome level, subject to two or more conditions. The control and treated samples are labeled with different reporter tags. The difference in light and heavier reporter ions provides quantitative differences and protein identity is also established simultaneously. The major advantage of iTRAQ technique is the availability of multiplexing, i.e., studying the difference in the samples of more than

two patients (4 or 8). This multiplexing helps in high-throughput analysis, reduces manual artifacts and accelerates the proteomic analysis.

2. PRINCIPLE OF iTRAQ

Isobaric tag for relative and absolute quantitation (iTRAQ) is an *in vitro* labeling technique, where the peptides are labeled with specific tags, which is used for quantitation during MS analysis. Since MS is capable of measuring the mass (or m/z) of the peptide, the tags are so designed that for each sample the tag remains unique and at the same time for all the samples pooled together, the overall mass of various tags remain the same. The iTRAQ reagent contains a reporter group, which is lost during fragmentation at MS/MS level. This reporter group is different for different iTRAQ tags and they differ from each other by mass of 1 Da. Thus, when the peptide identified by the first mass analyzer, is moved into the second mass analyzer for further fragmentation, the reporter ion is released and is measured by the detector. According to the number of samples, reporter ions are generated and hence their absolute quantitation is a measure of quantitation of peptide and hence protein in various samples. The advantage of iTRAQ is the ability to perform multiplexing, i.e. analyzing more than two samples at the same time. Currently, iTRAQ reagents are available with four or eight reporter groups and hence, at a time four (4-plex) or eight (8-plex) samples can be analyzed and quantified. Proteins from different samples are digested with trypsin, and peptides so obtained are differentially labeled with the iTRAQ reagents. The iTRAQ reagents interact with the primary amine or the N-terminal region of the protein and thus there is no biasness for any particular amino acid, which is a limitation in some quantitative proteomic techniques (Fig 1).

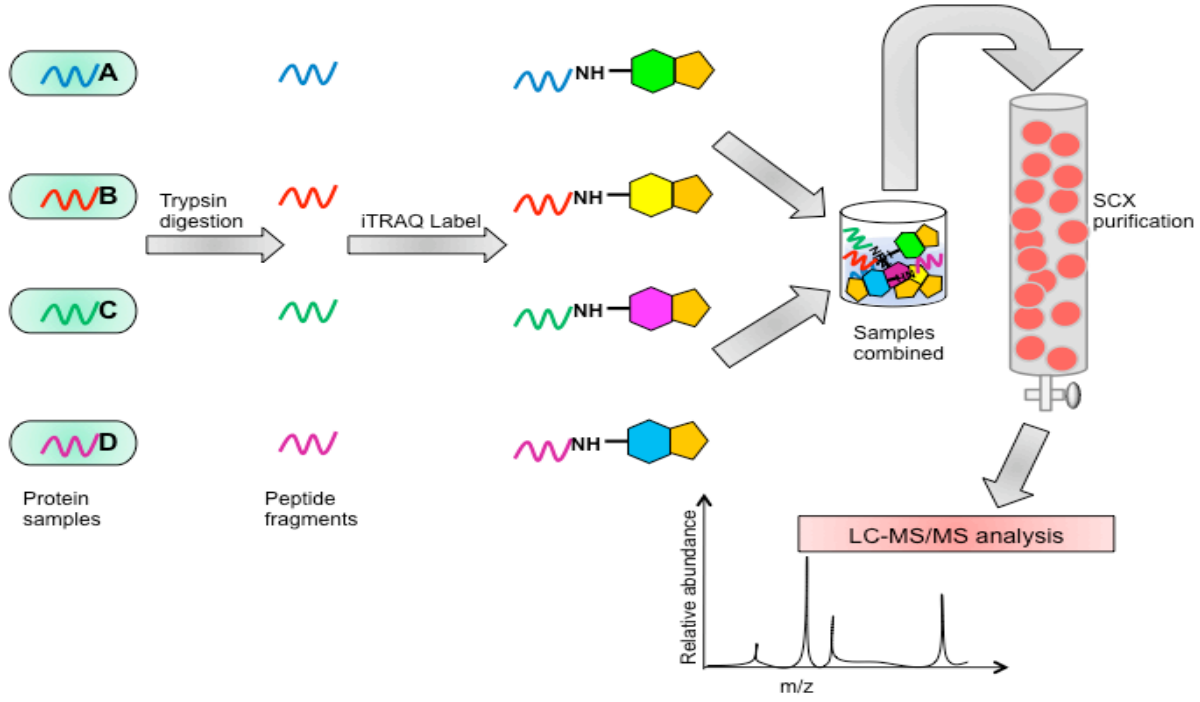

Fig 1. Principle of isobaric tag for relative and absolute quantitation (iTRAQ)

3. iTRAQ REAGENT

As explained earlier, iTRAQ is based on the labeling of peptides with isobaric tags, i.e., tags having same molecular weights, but producing different ions during fragmentation, due to their inherent property. iTRAQ-based labeling depends on the availability of a primary amine and hence it is also called N-terminal labeling. The iTRAQ reagent consists of three regions (Fig 2):

a) A reactive group, which specifically binds to the primary amine group of the peptides. Chemically it is an ester of N-hydroxy-succinamide.
b) A balance group, which is chemically a carbonyl group.
c) A reporter group, which is chemically dimethyl piperazine.

The reporter group differs in their weight but the overall weight of the tag remains constant due to the balance group. The mass is kept constant at 145 Da. In a 4-plex experiment, the reporter group has mass ranging from 114 to 117 Da and accordingly balancer group having mass ranging from 31 to 28, i.e., a reporter group having mass 114 Da has a balancer group of mass 31 Da, while a reporter group of mass 117 Da has a balancer group of mass 28 Da. The reactive group interacts with the primary amine of the peptide and forms a strong amide linkage, which retains even during fragmentation.

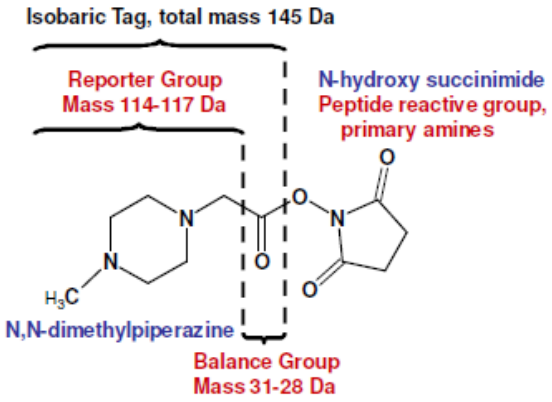

Fig 2. Structure of iTRAQ reagent

The reporter group produces excellent signature ion and b and y ion series. It also maintains the ionization state of the peptide and hence better ion capture.

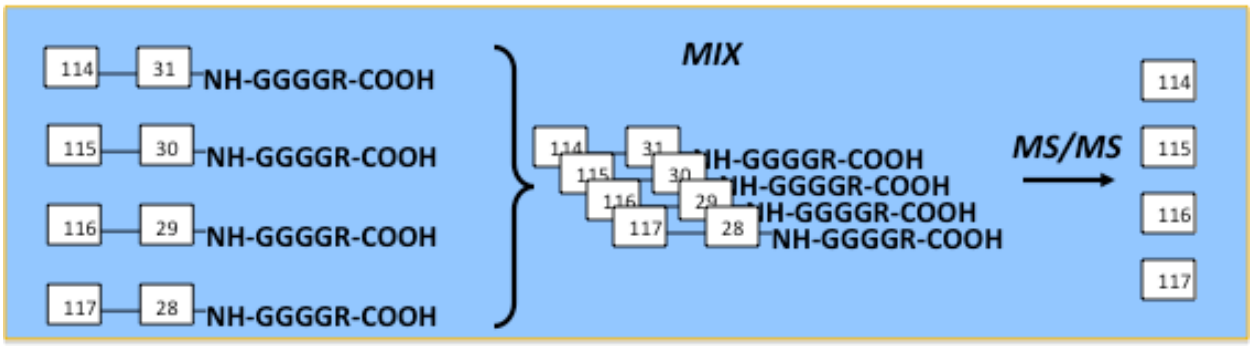

Fig 3. A typical 4-plexing iTRAQ experiment

The limitations of iCAT regarding multiplexing were addressed by iTRAQ, where multiplexing (4-plex) could be done (Fig 3). This multiplexing was further increased to 8-plex, thus increasing the high throughput requirement in clinical proteomic studies.

Illustration: iTRAQ labeling

The identification and quantitation of complex protein mixtures has been facilitated by MS-based quantitative proteomic techniques. Isobaric tag for relative and absolute quantification (iTRAQ) consists of amine-specific, stable isotope reagents that can label peptides from eight different biological samples. The protein samples to be analyzed are first digested with trypsin into smaller peptide fragments. The trypsin cleaves the proteins at the C-terminal of lysine and arginine residues unless they are followed by a proline residue.

The peptide fragments generated are separated by SDS- to simplify the mixture and then tagged with the iTRAQ label. The iTRAQ reagent consists of a reporter group, a balance portion and a peptide reactive group that interacts with the N-terminus of peptide or free amino group of Lysine residues, giving it an overall mass of 145. The reporter group used to label each peptide sample is unique, with mass varying between 114-117, thereby enabling the labeling and quantification of four samples simultaneously. This has been further improved to allow labeling of eight samples simultaneously. The labeled samples are then pooled together. The pooled samples are purified on a strong cation exchange column to remove any excess unbound iTRAQ reagent.

4. WORKING PROTOCOL

Mass spectrometry has its own limitations in terms of sensitivity. Mass spectrometry is also sensitive to salt contamination and hence samples like cerebrospinal fluid or serum, containing high concentration of salt need to be processed before MS analysis (Fig 4 and 7).

The protein is dissolved in mass spectrometry compatible solvent, ammonium bicarbonate, pH 8.5 and then reduced to all the disulfide bonds using either dithio threitol (DTT) or β-mercaptoethanol. To reduce, abnormal fragmentation from amino acids like cysteine, which give out hydrogen sulfide ions, cystein blocking reagents like methyl methane thiosulfonate (MMTS) is also added. The reduced protein is then trypsinized and salt contamination is removed by zip-tipping. In Zip tipping process protein is passed through microtip columns containing C-18 backbone, which hydrophobically interacts with the protein and trap them, eluting out the salts and other contaminants. The protein is eluted back using acetonitrile and formic acid.

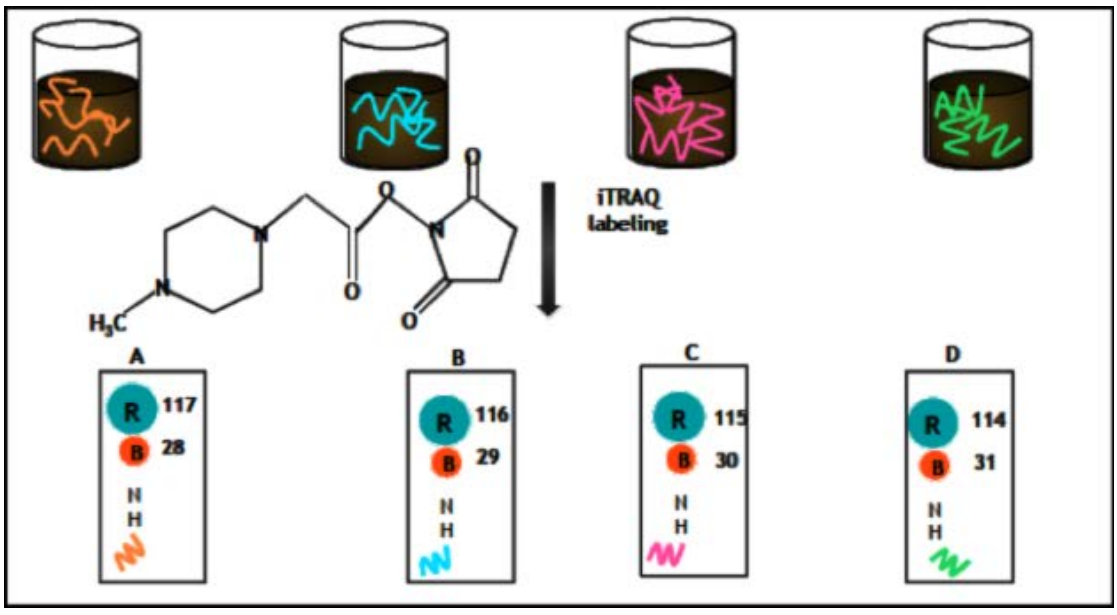

Fig 4. Peptide labeling by iTRAQ reagent

The peptides so obtained are labeled with iTRAQ reagents and then pooled. The pooled samples are passed through strong cation exchange columns to remove excess iTRAQ reagent and obtain specifically labeled peptides prior to MS analysis is performed (Fig 5 and 6).

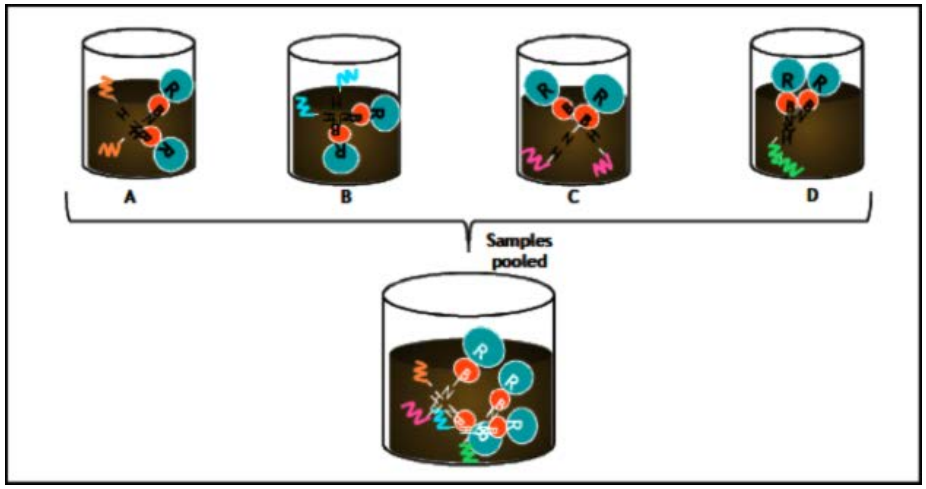

Fig 5. Pooling samples labeled from different iTRAQ reagents

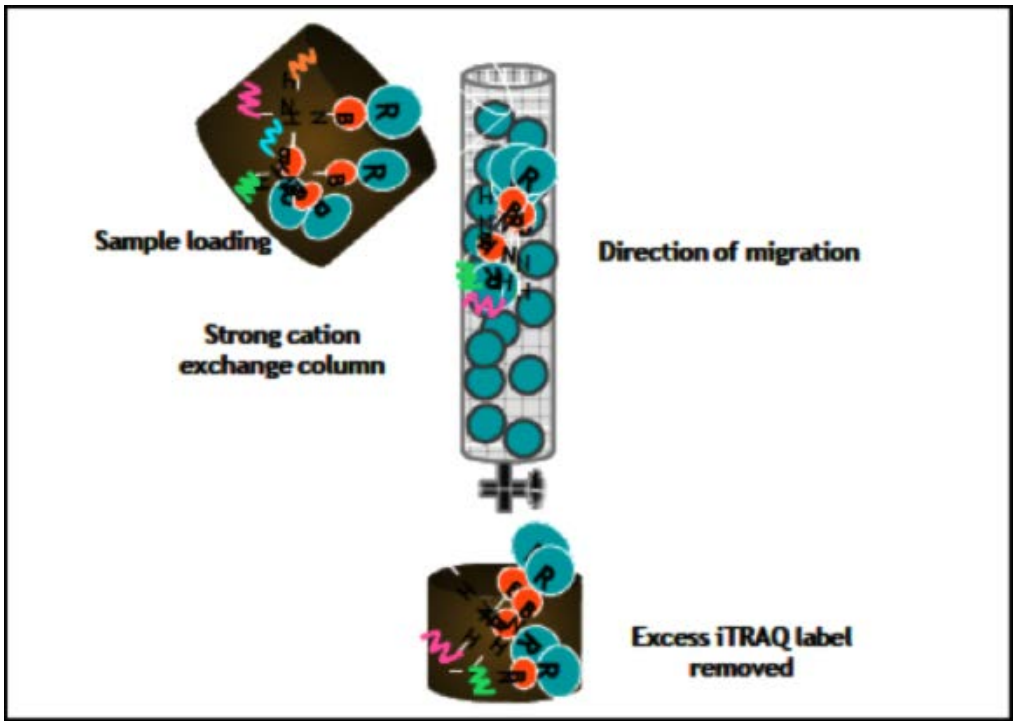

Fig 6. Peptide purification

Illustration: iTRAQ protocol: chromatography for clean-up and purification

This step facilitates sample clean up prior to further finer separation and purification using reverse phase chromatography. Further purification of the SCX purified peptides is carried out by reverse phase liquid chromatography, wherein the sample is passed through a column containing a packed stationary phase matrix that selectively adsorbs only certain analyte molecules. The eluted fractions are further analysis by MS. The purified labeled peptide fragments are then analyzed by MS/MS.

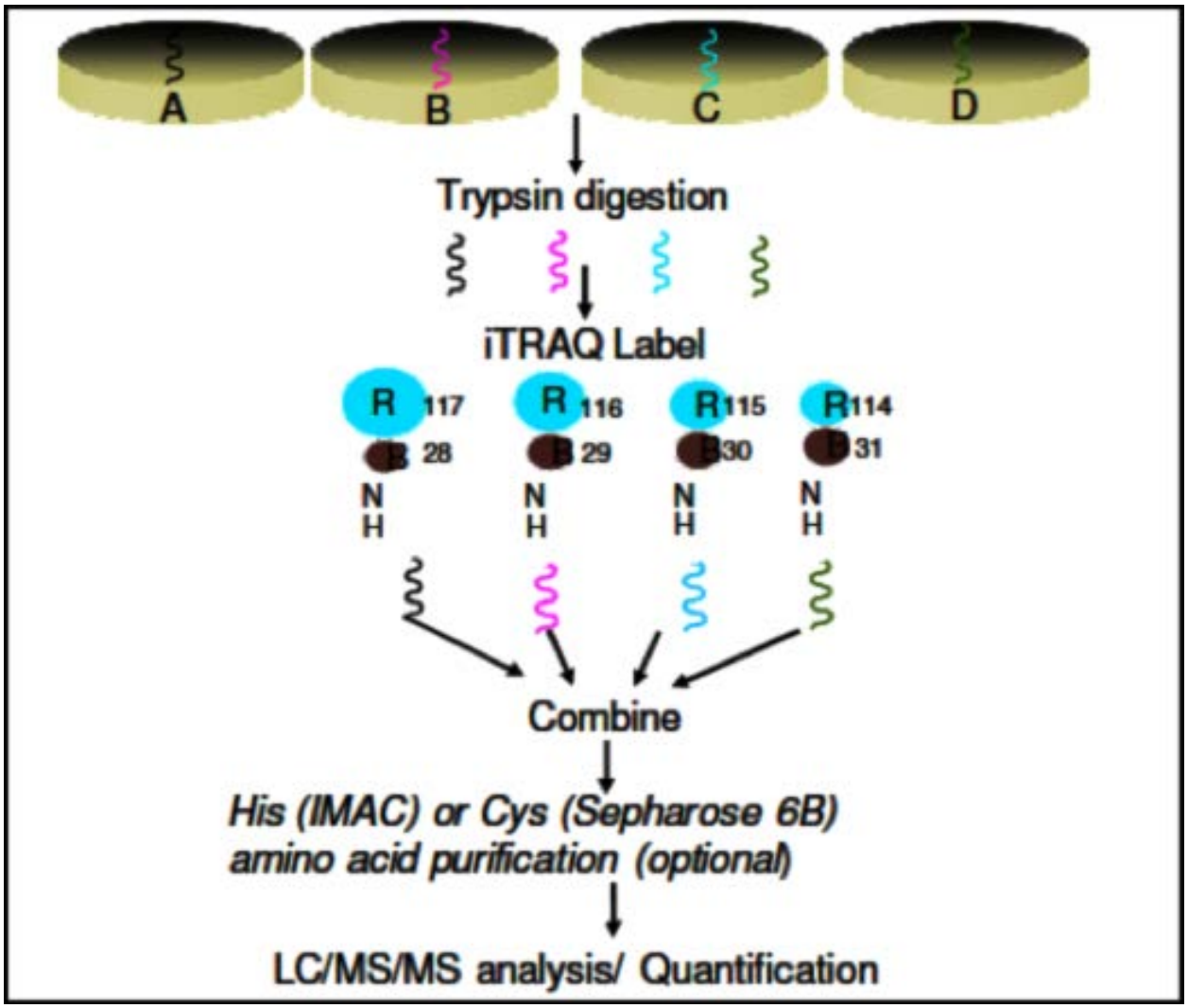

Fig 7. An overview of iTRAQ protocol

5. DATA ANALYSIS

iTRAQ enables simultaneous identification and quantification of peptides and hence proteins (Fig 8). This can be illustrated with the following examples.

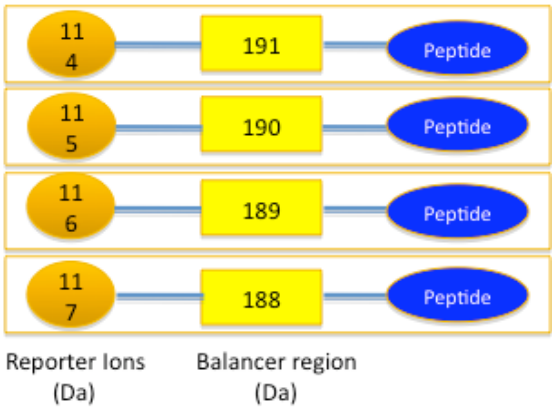

Fig 8. iTRAQ labeling for 4-plex experiment

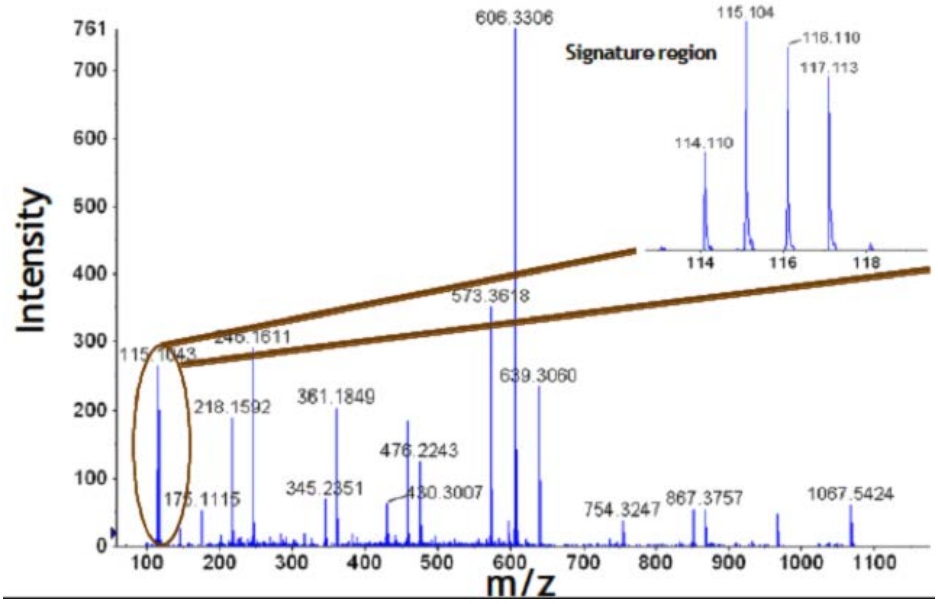

Fig 9. iTRAQ 4-plex experiment MS spectrum

In the above figure, the lower spectrum is the data obtained from the second MS analyzer, which identifies the protein and also quantifies it. The peak at 115.1643 is our peak of interest where absolute quantification data of our protein, obtained from four different samples, lies. Zooming into the spectrum at m/z 115.1643 provides finer details about the quantity of reporter ions generated from the peptide (shown in the top spectrum). The reporter ion corresponding to m/z 115.104 shows maximum intensity and that corresponding to m/z 114.110 shows the least (Fig 9). This quantitative information becomes important for the validation of biomarkers, for example, in glioblastoma. Certain biomarkers like haptoglobin or retinol binding protein have varying concentration at different stages of glioblastoma. Quantifying the amount of the biomarker provides an idea about the grade of glioblastoma the patient is suffering from and hence can be diagnosed accordingly.

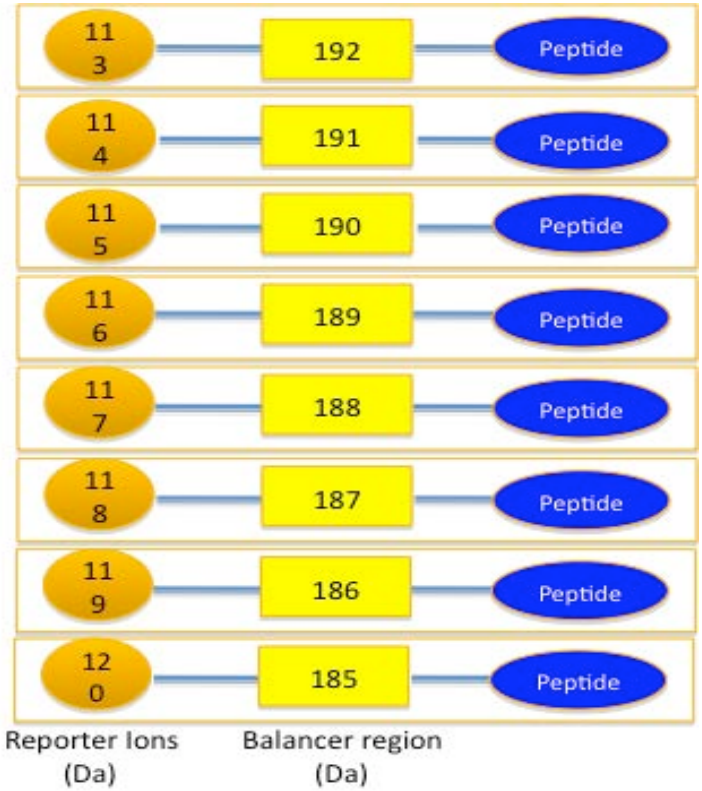

Fig 10. iTRAQ labeling for 4-plex experiment

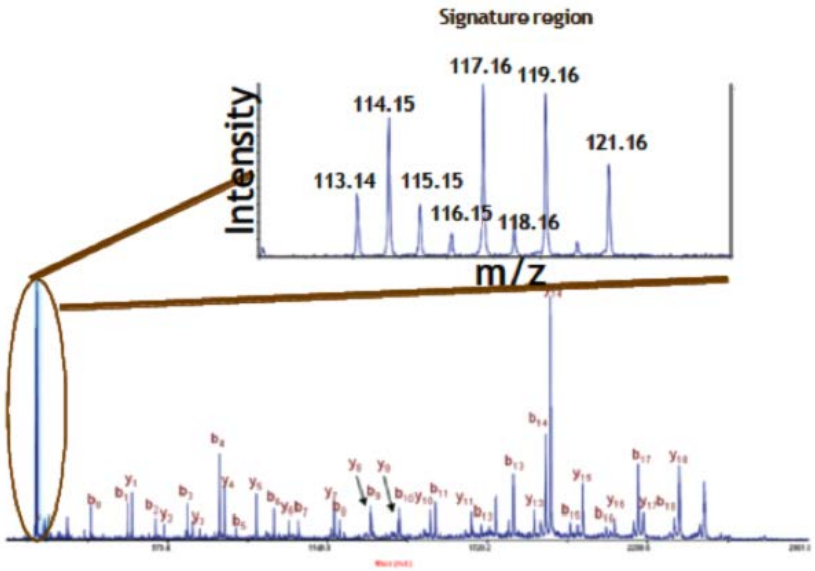

Fig 11. An eight plexing MS spectrum from iTRAQ experiment

Similarly, in 8-plexing, the reporter ion reports from 113.14 Da to 120.14 Da and accordingly the data is analyzed (Fig 10 and 11).

Illustration: Data analysis

The purified labeled peptide fragments are analyzed by MS/MS. The different masse of the reporter groups allows the peptide fragments to be identified. The reporter group is lost during fragmentation. Relative quantification of up to eight samples can now be performed using iTRAQ. The MS/MS data analysis shareware has some extra inputs such as Quantitation, MS/MS tolerance, peptide charge, instrument etc. in addition to the fields for PMF. They require inputs from the user regarding the experimental parameters used such as enzyme cleavage, protein name, modifications etc. and the desired search criteria taxonomy, peptide tolerance etc. Commonly used protein databases against which the MS information is processed to retrieve sequence data include NCBI, MSDB and SwissProt. The data file generated from MS is uploaded and the search carried out.

6. ADVANTAGES AND LIMITATIONS OF iTRAQ

Quantitative proteomics using mass spectrometry based approach is one of the most important advantages of iTRAQ, especially in the field of clinical proteomics. The progress of metabolism of a particular drug or the progressive concentration of certain biomarkers at different stages of a particular disease can easily be quantified using iTRAQ-based studies. Another great advantage of iTRAQ over iCAT is the independence on specific amino acids, like iCAT depends on cysteine labeling. Since the reactive group of iTRAQ reagent only interacts with primary amine, there appears no dependence on any specific amino acid and hence no biasness occurs. This increases the amount of proteome covered by the technique, unlike iCAT.

Multiplexing ability is another great advantage of iTRAQ. Since the detection is based on the reporter ion solely, the greater the number of different types of reporter ions, greater is the number of samples that can be analyzed in one experiment. Currently, number of reporter ion available is eight and hence, multiplexing at the level of eight samples is achievable. This becomes important in discovery of biomarkers for clinical diagnosis, because, a large number of samples need to be validated simultaneously.

However, iTRAQ reagents are extremely costly and also very sensitive to contamination from salts. Thus extensive zip tipping and peptide enrichment needs to be performed. Also, there is a requirement of sophisticated software for analyzing iTRAQ data. Another disadvantage of the entire protocol and not of iTRAQ in general, is

the variability arising due to the inefficient enzymatic digestion. Nonetheless, iTRAQ is a very powerful tool for absolute quantification of multiple samples in an efficient manner.

REFERENCE

1. Proteomics – Introduction to Methods and Applications – Edited by Agniezka Kraj and Jerzy Silberring, Wiley Publications
2. Proteomics by Mass Spectrometry: Approaches, Advances, and Applications John R. Yates, Cristian I. Ruse and Aleksey Nakorchevsky - Annu. Rev. Biomed. Eng. 2009. 11:49–79
3. Quantitative Proteomics Mass Spectrometry - Edited by Salvatore Sechi – Humana Press