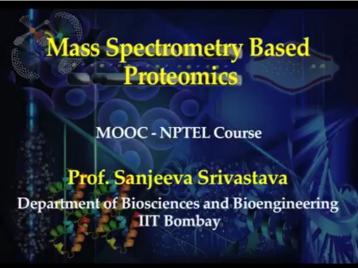
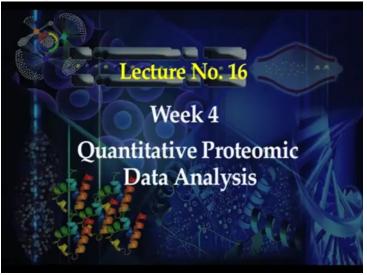
Mass Spectrometry Based Proteomics Professor Sanjeeva Srivastava Department of Biosciences and Bioengineering Indian Institute of Technology, Bombay Mod 04 Lecture Number 16

(Refer Slide Time 00:10)



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Topics to be discussed

- # Review of the quantitative proteomic methods
- # Qualitative proteomic data analysis
- # iTRAQ quantitative proteomic data analysis

(Refer Slide Time 00:23)

Section I Review of the quantitative proteomic methods (Refer Slide Time 00:27)

Stable Isotope Labeling by Amino acids in Cell culture (SILAC)

(Refer Slide Time 00:31)

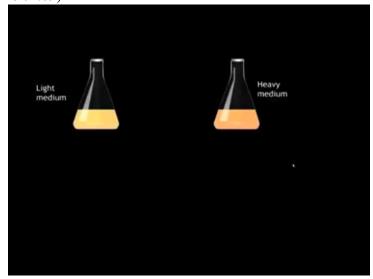
Let's discuss the concepts in SILAC method

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Protein labeling with stable isotopes are effective methods for quantitative proteome profiling using Mass Spectrometry.

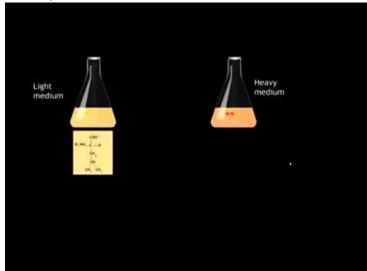
Stable Isotope Labeling by Amino acids in Cell culture or SILAC which is a metabolic labeling strategy to encode the whole cellular proteome is widely used method for the quantitative proteomics.



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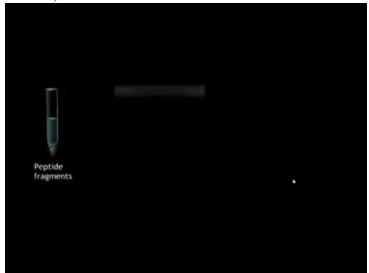
In the SILAC

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... two group of cells are cultured in media two groups 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

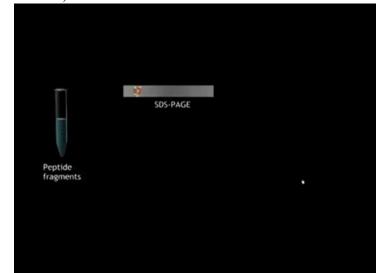
After a number of cell divisions, the grown cells are combined and digested using Trypsin.



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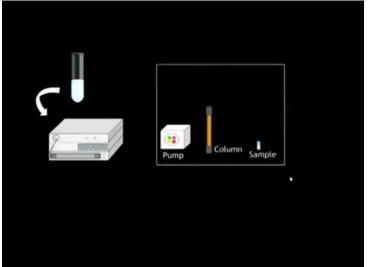
The complex protein mixture is further separated

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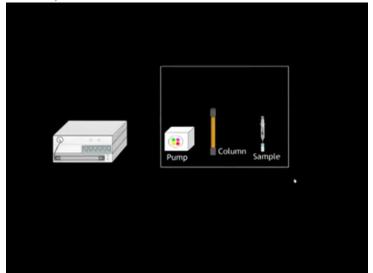
... by SDS PAGE to simplify the analysis

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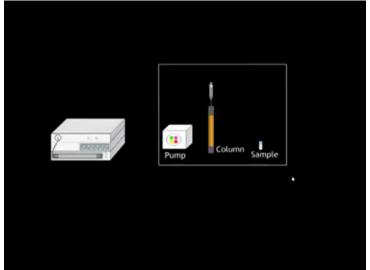
Further application is carried out by liquid chromatography

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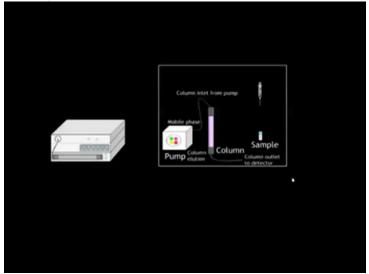
and purified peptide fragments are analyzed

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by MS/MS.

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(Refer Slide Time 02:03)



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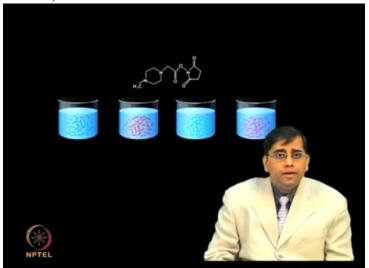


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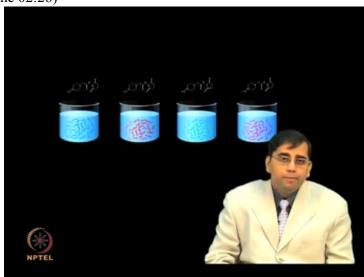


iTRAQ it is a MS based technique for relative and absolute quantitation of protein.

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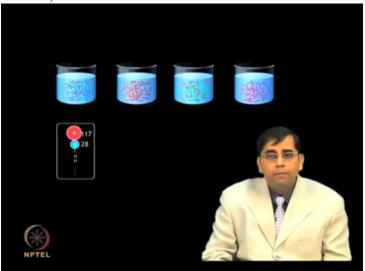
iTRAQ reagents are a set of 4 isobaric amine-specific



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labeling reagents; 114, 115, 116 and 117. An iTRAQ reagent consists of a reporter group

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a balancer group and a peptide reactor group

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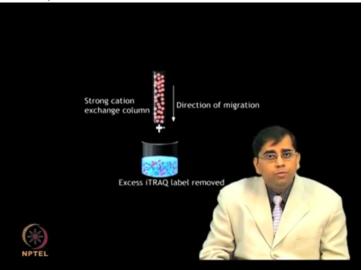
Pooled samples are purified on Strong Cation eXchange SCX column

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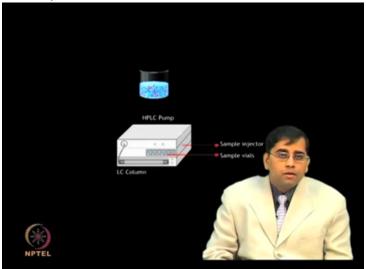
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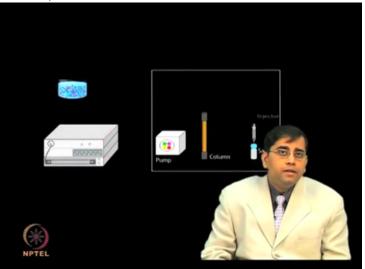
the excess unbound reagent ...

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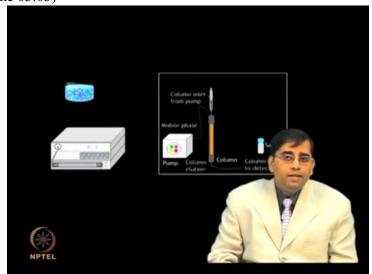
These isobaric labels are detected

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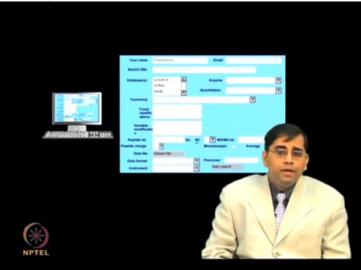
... upon fragmentation and release ...

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... in mass spectrometry.

(Refer Slide Time 03:07)



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.

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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(Refer Slide Time 03:53)

Let's discuss the concepts in TMT method

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Now let us talk about Tandem Mass Tag or TMT. This method is similar to iTRAQ which we just discussed. TMT is also MS/MS based

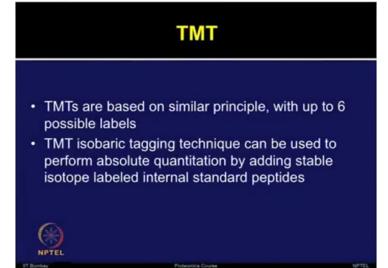


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quantitative technique which uses the isotopomer labels referred as tandem mass tags.

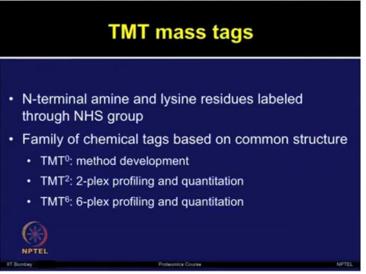
It also provides the accurate quantification of peptides and proteins. The Tandem Mass Tags have been developed by the Proteome Sciences and currently commercialized by Thermo Fisher. I have given you the reference for the original study on Tandem Mass Tag in the slide.

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So these Tandem Mass Tags they are based on 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 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.



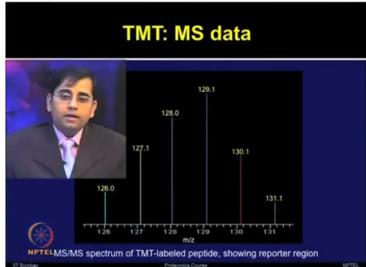
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The N-terminal amine and lysine residues are labeled though NHS group. There are family of chemical tags which are based on the common structures. The series of TMT tags available TMT 0, TMT 2-plex, TMT 6-plex.

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.

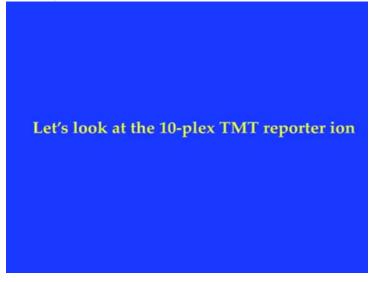
While duplex and 6-plex labels TMT differ by the number of isotopic substitutions, the TMT 0 is non-isotopically substituted structure that has been produced for only method development.

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

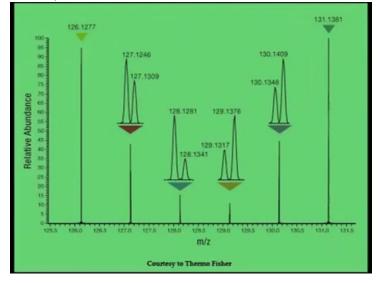


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I am showing you one representative MS/MS spectrum of TMT labeled peptide which is showing a reporter region. The relative abundance of target protein or peptide fragment in 6 different samples can be easily measured by comparing these signature mass peaks which are generated by the different mass tags. (Refer Slide Time 06:53)



(Refer Slide Time 06:58)



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Points to ponder

SILAC is used for labeling of proteins *in vivo*, whereas iTRAQ and TMT methods are used for *in vitro* labeling of proteins

Based on the ionization source, charge on the peptides varies (e.g. MALDI gives singly charged peptides and ESI generates multiply charged peptides)

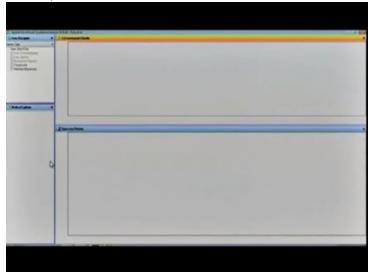
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Let's discuss the qualitative data analysis

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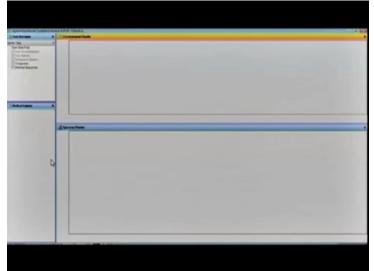


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Data Visualization and qualitative analysis; Qualitative analysis, Click on

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Agilent Qualitative Analysis software icon which will pop up a new window

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clicking on open a folder or file option we can open a Mass spec

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the intensity of eluted peptide ions on the y axis

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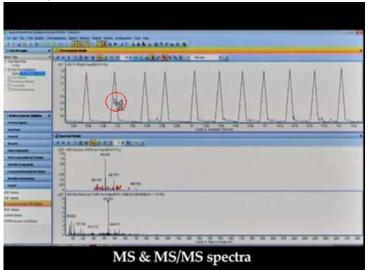
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use right click of the mouse to zoom in the chromatogram

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another window which shows different spectral peaks representing the precursor ions with the red color annotation

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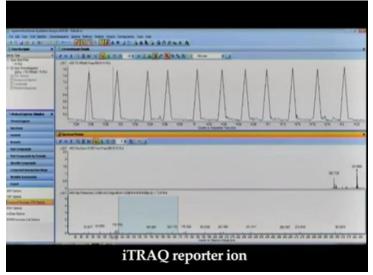
if the peptides are labeled with iTRAQ reagent

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we can see the reporter ions in this MS/MS spectral view window

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Points to ponder

SILAC is used for labeling of proteins *in vivo*, whereas iTRAQ and TMT methods are used for *in vitro* labeling of proteins

Based on the ionization source, charge on the peptides varies (e.g. MALDI gives singly charged peptides and ESI generates multiply charged peptides)

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Points to ponder

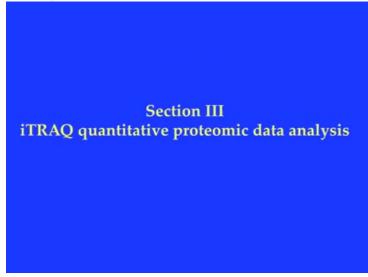
You can visualize the total ion current chromatogram (TIC), which gives idea about peptide elution at different time points

You should overlap the peak areas of two different samples having same retention time

The MS and MS/MS spectra for different peptides can be visualized

TIC, MS and MS/MS spectra, all together provide an idea about the quality of the mass spectrometry data

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S. No	Software/d atabase	Description	URL site
1	MASCOT	Search engine for protein identification using mass spectrometry data	http://www.matrixscience.co m/
2	MS-Fit	Used for mining the sequence of the protein from MS data	prospector.ucsf.edu
3	SEQUEST	Used for interpretation of tandem mass spectra data for protein identification and amino acid sequence	http://fields.scripps.edu/sequ est/
4	X!Tandem	Used for protein identification using tandem mass spectra data	http://www.thegpm.org/tand em/index.html
5	Sequit!	De novo sequencing of protein using tandem mass spectrum	http://www.sequit.org/
6	MSQuant	Quantitative proteomic information from MS and LC data	http://msquant.sourceforge. net/

Mass spectrometry has succeeded in identifying proteins based on the amino acid sequence information derived from the Tryptic digested peptides.

To analyze the data obtained from the mass spectrometry, sophisticated databases for protein identification were built and PMF, Peptide Mass Fingerprinting, PFF, Peptide Fragmenting Fingerprinting and MS/MS Ion Search are the foremost.

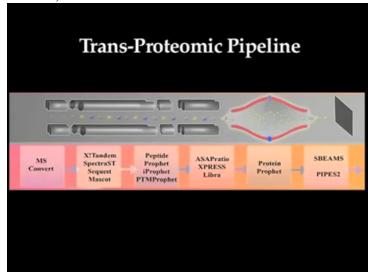
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S. No	Software	Description	URL site
1	MapQuant	Used for MS quantification after making two dimensional map	http://arep.med.harvard.edu/ MapQuant/
2	XCMS	Used for LC-MS data handling for relative quantization, visualization.	http://metlin.scripps.edu/xcm s/
3	MsInspect	Used to combine the LC-MS and LC- MS/MS peptide data and also for peptide array generation	http://proteomics.fhcrc.org/C PL/msinspect/index.html
4	Mzmine	Mainly used for MS and LC-MS data processing purpose	http://mzmine.sourceforge.n et/
5	Pep3D	Convert LC-MS or LC-MS/MS data into 2D map as m/z vs time	
6	SpecArray	Used for generation of expression peptide arrays from LC-MS data	tools.proteomecenter.org/Sp ecArray.php
7	Msight	It represent the data from both MS and separation steps such as chromatography, 2De etc	http://web.expasy.org/MSigh t/

In the last 2 decades, many algorithms, new tools were developed to process the large datasets.

Mascot is the widely used and trusted search engine for mass spectrometry data analysis.

It has comprehensive database covering various organisms and is compatible with all existing mass spectrometry softwares.



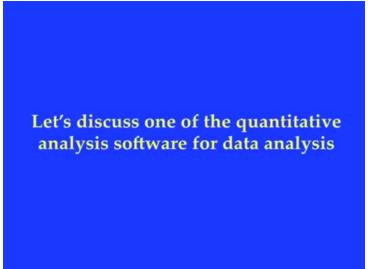
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Besides SEQUEST, X! Tandem is also used for MS/MS data analysis.

In addition to vendor-specific tools and databases, there are many online open source data analysis software.

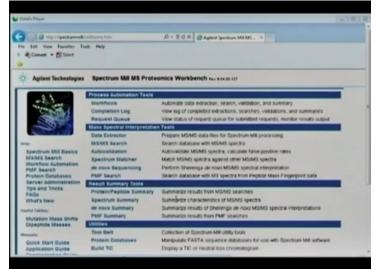
Apart from the softwares mentioned in the table, many open source software were introduced in the recent years such as Trans-Proteomic Pipeline, TPP, OpenMS, MaxQuant and many more.

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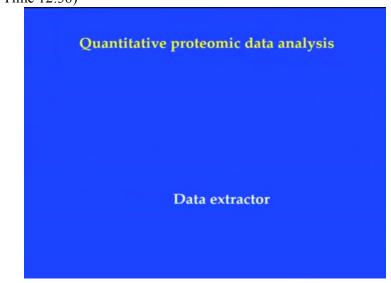
For demonstration we have used Spectrum Mill software (Refer Slide Time 12:13)



We will focus on the iTRAQ data analysis using Spectrum Mill software.

Spectrum Mill software, Spectrum Mill software is stand alone software used for LC MS/MS data analysis generated using Agilent LC MS/MS mass spectrometry. Here we can see the home page of Spectrum Mill having different tools for data analysis.

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Data extraction

LC MS/MS data analysis can be started using data extractor tool where we can load our data files into the Spectrum Mill and prepare files for MS/MS analysis

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And upload the selected file into the software. As we are analyzing the iTRAQ data analysis

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so before extracting the MS/MS data, we need to assign the modifications on the peptide.

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Further merge the MS2 and MS3 spectra from the same precursor.

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Keep the remaining parameters as default and save the method for future analysis.

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The extraction of MS/MS files

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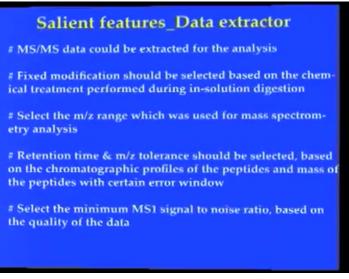
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the extraction is done then we can proceed for MS/MS search.

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Quantitative proteomic data analysis

MS/MS ion search

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MS/MS ion search is the tool used for data search against the database few important parameters has to be assigned for the analysis such as database, species,

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Once the MS/MS search is done, we can validate the data for statistically analysis using

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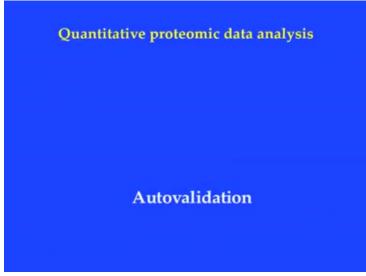
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Auto-validation tab

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Salient features_MS/MS search # Taxonomy should be selected
Enzyme used for digestion should be selected
Missed cleavages should be selected either 1 or 2
MS and MS/MS tolerance should be selected depending upon instrument used
Fixed and variable modifications on peptide should be assigned
iTRAQ should be selected as fixed modification when you a performing iTRAQ quantitation experiment

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Auto-validation, in Auto-validation both peptide and protein validation is needed to be performed

First we will do the peptide validation by selecting peptide in mode tab

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Click on Validate the files

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Similarly protein validation is done by selecting protein polishing in mode tab

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1% FDR is assigned and remaining parameters are set as default.

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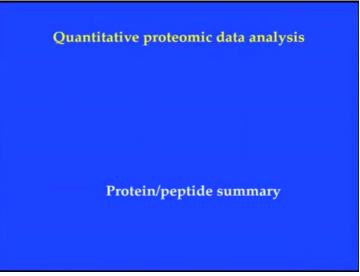
Now validate files

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Salient features_Auto-validation

- # Statistical analysis of MS data is essential for the proteomics experiment
- # 1% FDR is the minimum statistical analysis for MS data
- # Validate the peptides by applying 1% FDR
- # Validate the proteins by applying 1% FDR

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Protein peptide summary

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Protein peptide summary is the tool to visualize the results obtained from the MS/MS analysis.

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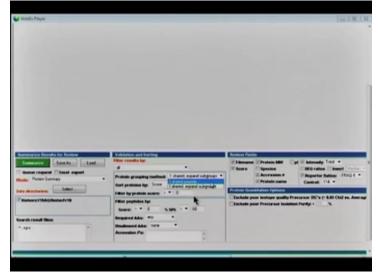
In the mode tab, we have different options to display the results

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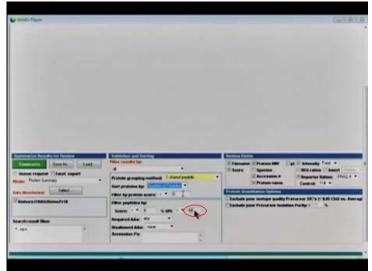
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We need to assign few parameters prior to displaying the results such as Click valid in filter result tabs to display only the statistically significant protein list.

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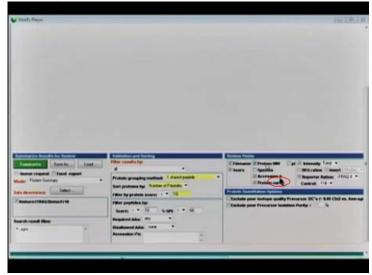


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SPI was assigned as 60 which is an optimum value for MS/MS analysis

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In the review field we can select species, molecular weight, score, peptide information, Reporter ion density, session number and many other parameters (Refer Slide Time 23:34)

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Keep remaining parameters as default

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and summarize the results. It will show the number of proteins identified or quantified from the analyzed sample

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in the coverage tab the highlighted sequence will represent the peptides detected in mass spectrometry and remaining sequences will appear in black color.

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It also displays the results with all the selected parameters

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such as protein name, species, unique peptides, score and intensity of the reporter ion, accession number and many more. Further we can summarize the results based on the peptides and we can export or save the results for further analysis.

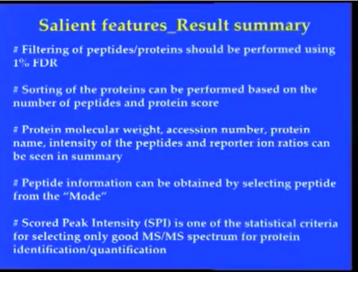
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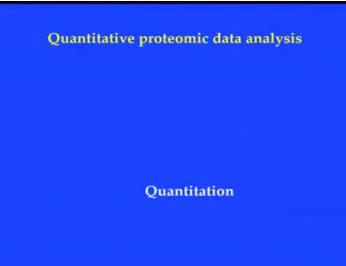
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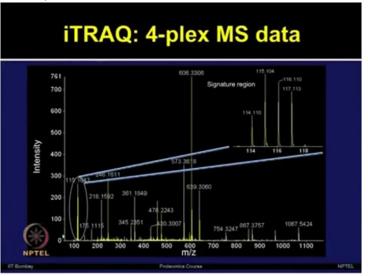
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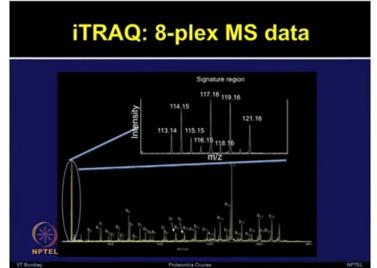


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This is the representative spectra for the 4-plex iTRAQ experiment. The MS data is shown and MS/MS spectrum is showing the reporter region, the signature of these 4-flex iTRAQ labeled peptides; 114, 115, 116 and 17

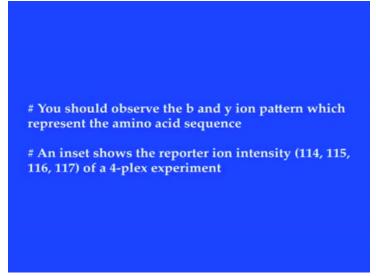
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Now we will have a look on the 8-Plex MS data

So in MS/MS spectrum, now here we are showing the reporter region signature of 8-plex iTRAQ region showing 113.14, 114.15, 115.15, 116.15, 117.16, 118.16, 119.16 and 121.16 reporter ions.

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Points to Ponder

- # SILAC, iTRAQ and TMT are used for labeling of proteins for quantification
- # Total ion chromatogram (TIC) gives the basic idea about the peptides separation on LC
- # Based on the ionization source charge on the peptides varies (eg. MALDI gives singly charged peptides and ESI generates multiply charged peptides)
- # Validation of the MS data is compulsory
- # No. of unique peptides, sequence coverage and 1% FDR is considered for data robustness

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Summary

Gel-free MS methods provide much robust platform for the quantitative analysis as compared to the gelbased platforms like 2-DE and 2D-DIGE

SILAC, iTRAQ and TMT are widely used MS-based quantitation methods

These quantitative methods allow simultaneous quantification and identification

For accurate quantification, stringent criteria such as 1%FDR, >1 peptide should be used (Refer Slide Time 27:34)

