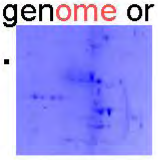
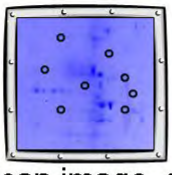
Proteomics

The Proteome indicates the proteins expressed by the genome or tissue

.



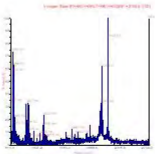
Run gel, stain



Scan image, select spots

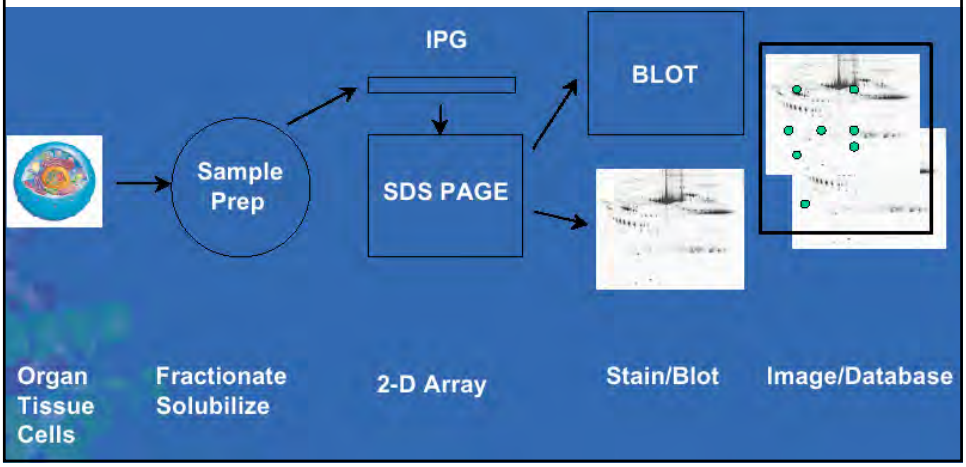
Excise spots,
digest proteins
and extract peptides

Database searches

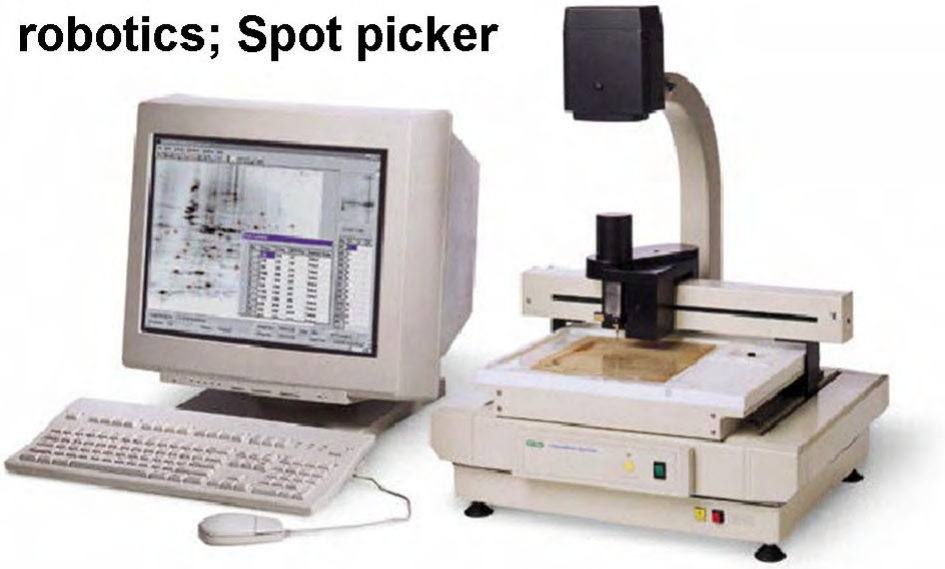


Mass analysis

Thousands of proteins can be reproducibly separated on one 2D gel from ~1mg of tissue/biopsy or biological fluid.

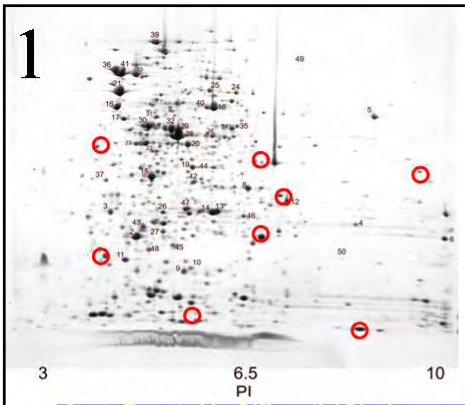


Automation and robotics; Spot picker



Automation of digestion by robotics

1



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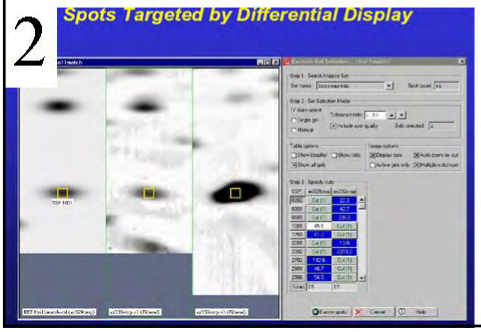
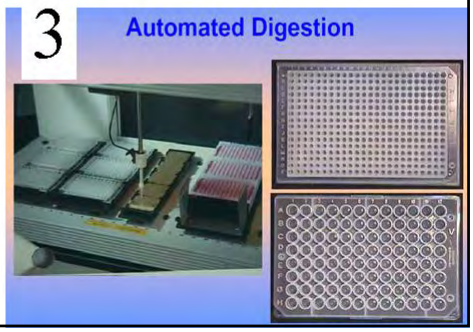
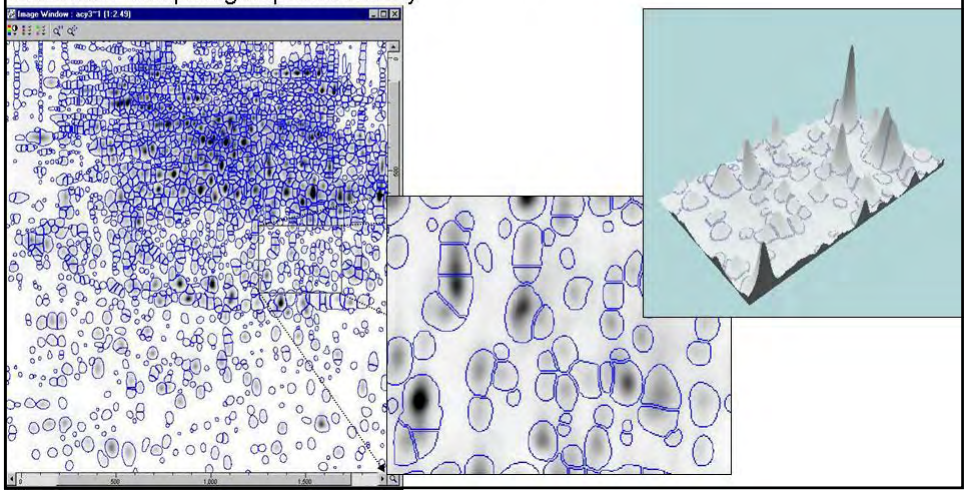


3



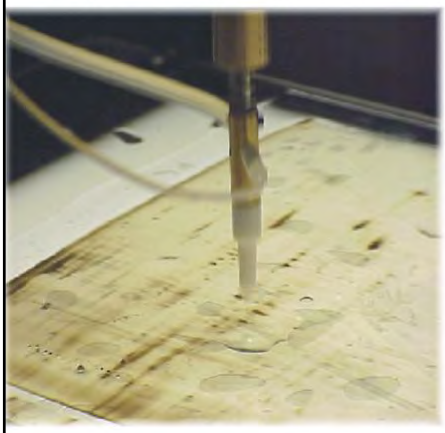
Enhanced spot detection

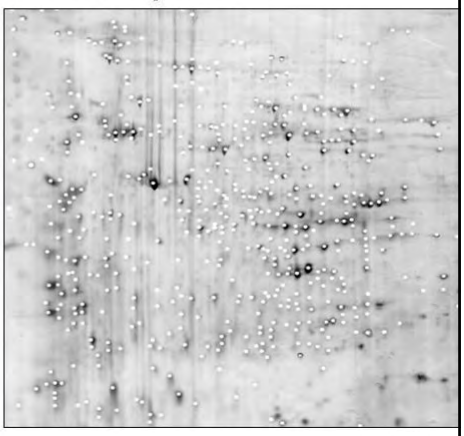
Detects spots on the internal standard
Detects spots unique to rest of multiplex
Propagates this map to all images within the group
Independently grows out spot boundaries
Ensures exact and accurate definition of each spot on each gel
Maintains multiplex group consistency



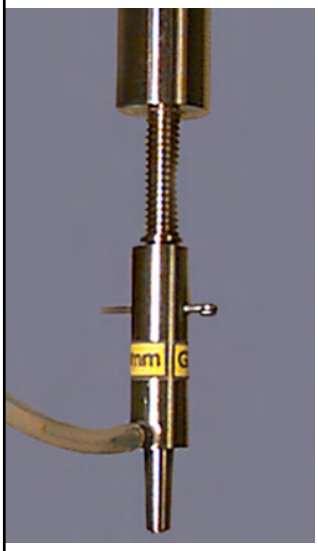
ProPic Spot picking robot

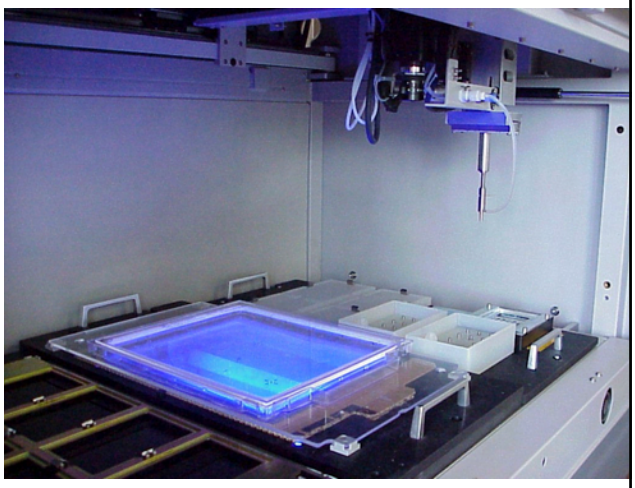
The ProPic cuts protein spots of interest and deposits gel plugs into a 96 well plate

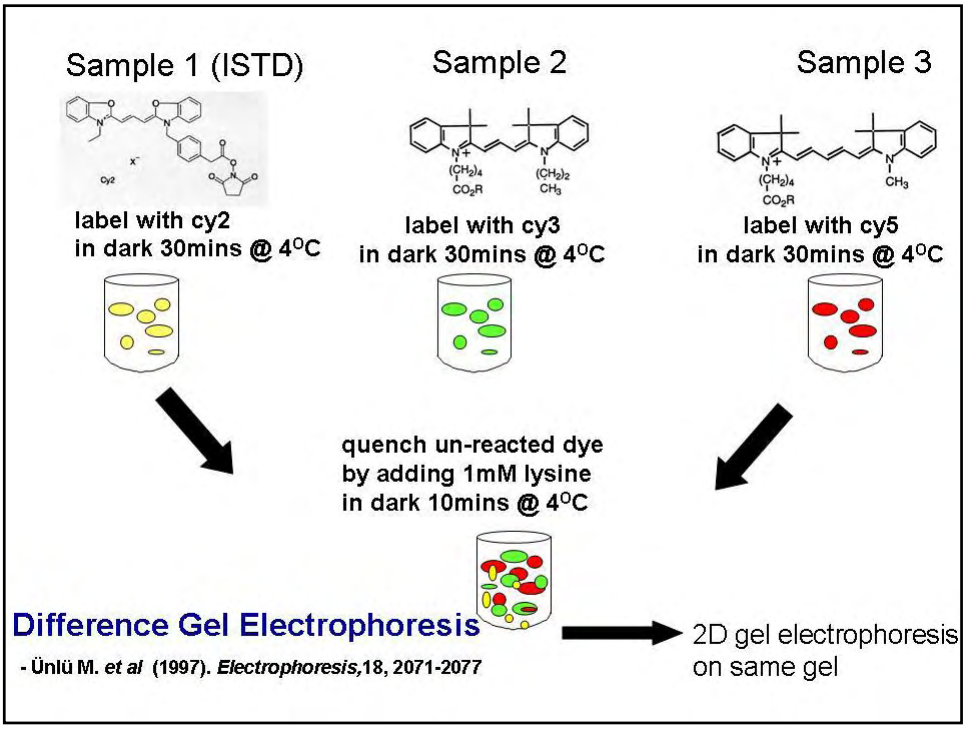




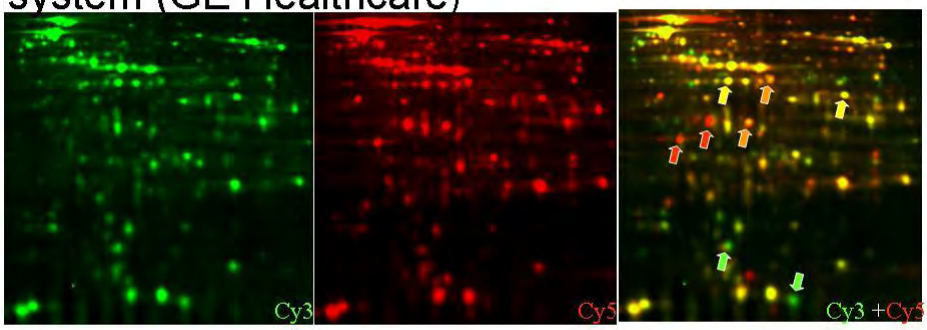
ProPic Spot picking robot







Overlay Analysis -Image at different wavelengths on a Storm or Typhoon imaging system (GE Healthcare)



no difference

presence / absence

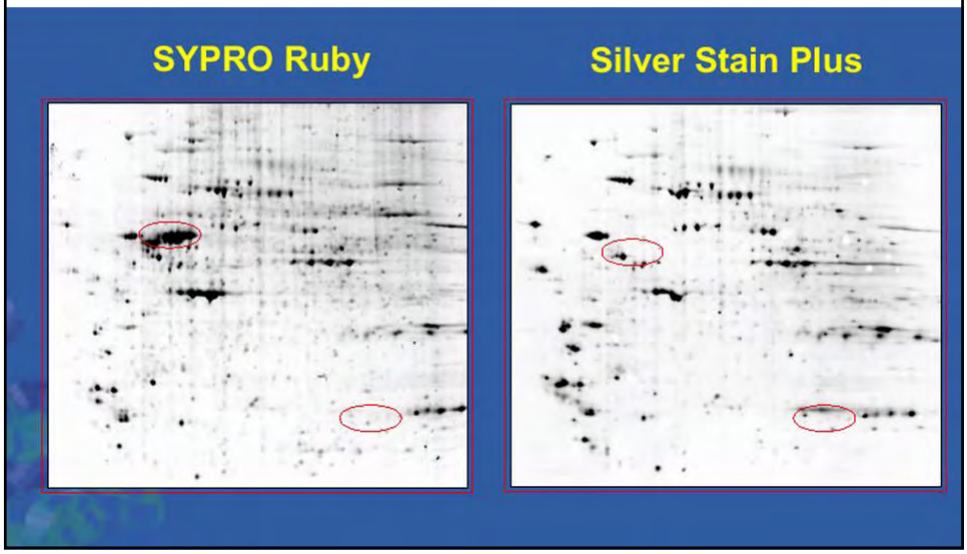
up / down-regulation

Detection sensitivity of gel staining methods

Staining method	Protein detection limit	Compatibility with MS
Silver + glutaraldehyd	1-10 ng	-
Silver	10 ng	+/-
Coomassie R-250	>100 ng	+
Coomassie G-250	>30 ng	+
Negative Zn-imidazole	>100 ng	+
SYPRO Orange	>30 ng	+
SYPRO Ruby	>10 ng	+

WITA GmbH

Some proteins are more sensitive to particular stains



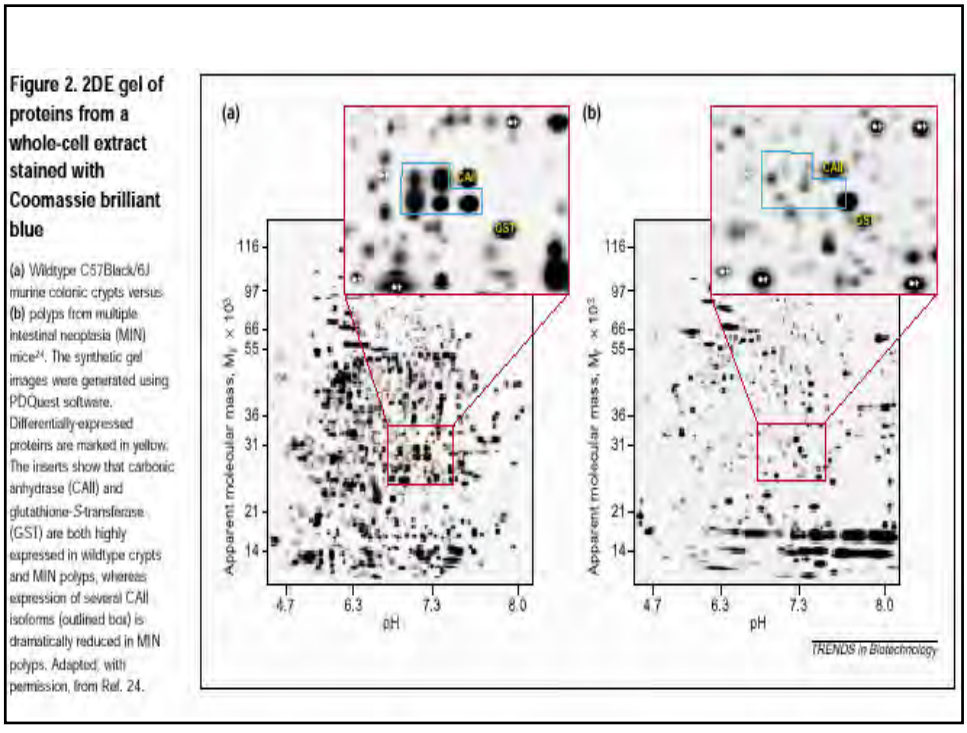
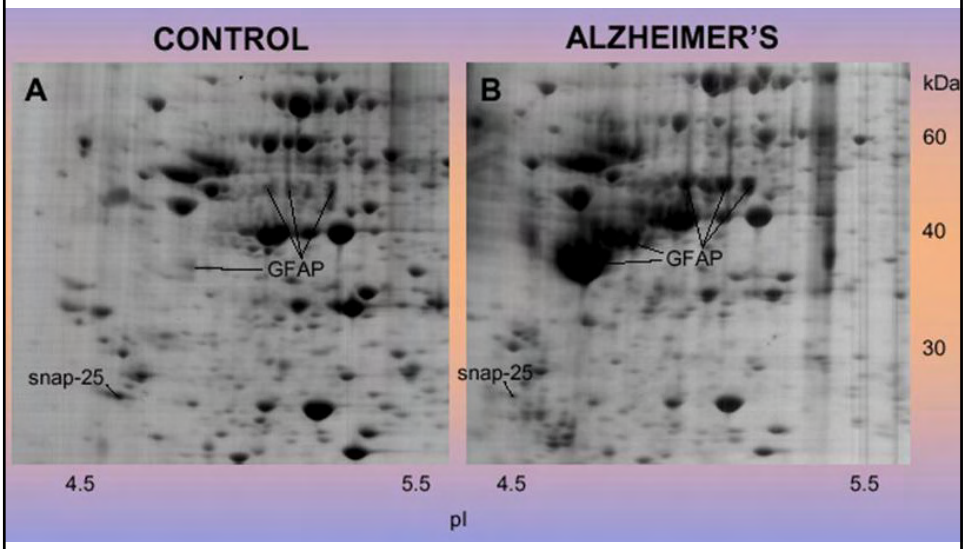


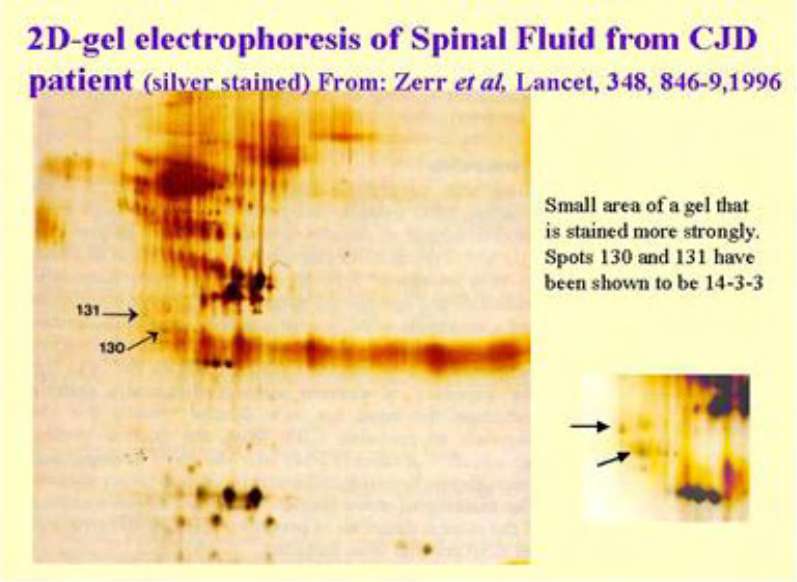
Figure 2. 2DE gel of proteins from a whole-cell extract stained with Coomassie brilliant blue

(a) Wildtype C57Black/6J murine colonic crypts versus (b) polyps from multiple intestinal neoplasia (MIN) mice[24]. The synthetic gel images were generated using PDQuest software. Differentially-expressed proteins are marked in yellow. The inserts show that carbonic anhydrase (CAII) and glutathione-*S*-transferase (GST) are both highly expressed in wildtype crypts and MIN polyps, whereas expression of several CAII isoforms (outlined box) is dramatically reduced in MIN polyps. Adapted, with permission, from Ref. 24.



14-3-3 and neurodegenerative diseases

14-3-3 proteins in neurodegenerative disease



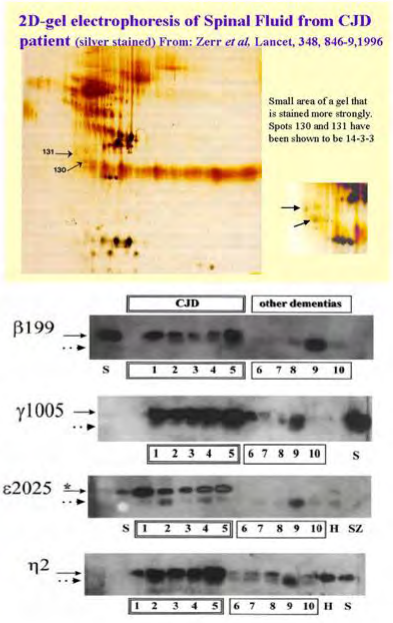
14-3-3 in Neurodegenerative Disease

1. **Prion diseases**: (a) 14-3-3s are novel biochemical markers in cerebrospinal fluid (CSF) of patients with neurodegenerative diseases.

Although six isoforms expressed in brain we showed that only four of these isoforms **(β, ε, γ & η)** are present in CSF of sporadic CJD patients but not in healthy subjects

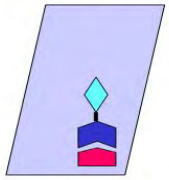
(b) Specific isoforms of 14-3-3 also appear in CSF of sheep with scrapie and cows with BSE. Another subset of isoforms is present in CSF of patients with a range of other neurodegenerative diseases including **14-3-3η** in Alzheimer's disease patients.

(Wiltfang et al. J Neurochem 73 (1999).



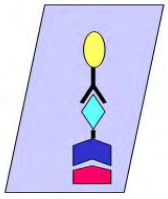
2. Protein-protein interactions and the overlay technique

1. Digoxigenin-labelled 14-3-3γ interacting with PVDF membrane-bound protein



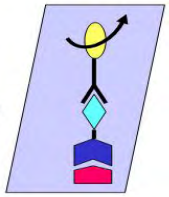
Interacting protein

2. + anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (AP)



Dig-labelled 14-3-3 γ

3. + substrate for the reaction with AP (X-phosphate, NBT)

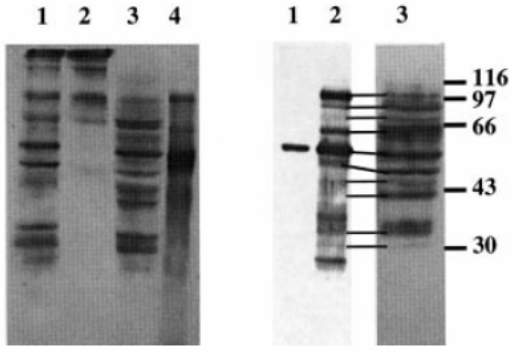


Anti-Dig-AP

Colour reaction

8

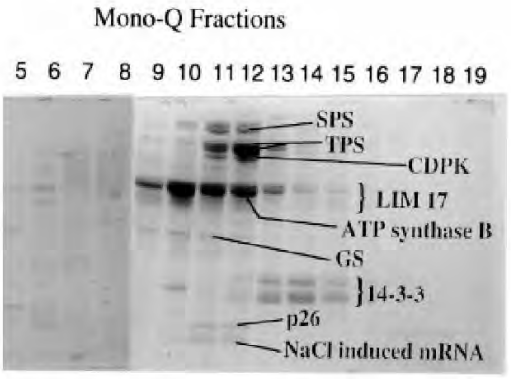
Protein-protein interactions and the overlay technique



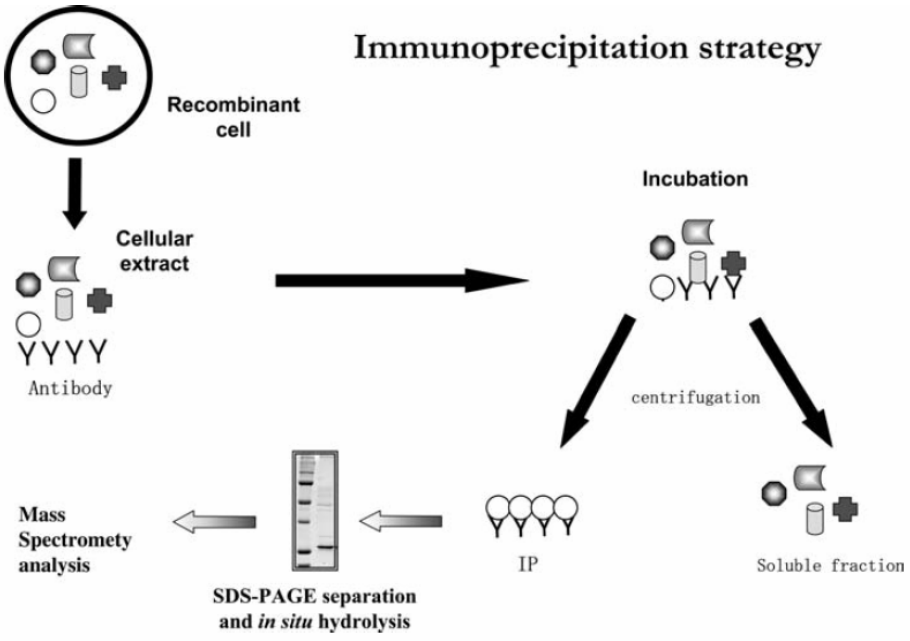
Proteins were run on a 12% (w/v) SDS-polyacrylamide gel, transferred to nitrocellulose and probed with digoxygenin labelled (DIG-) 14-3-3 (c) DIG-14-3-3 overlays of dephosphorylated proteins, indicating alignment of bands in a crude soluble extract and 14-3-3 column eluate. Lane 1, 14-3-3 column eluate (5 µl) was incubated with purified bovine protein phosphatase 2A at 50 mU ml^{-1} for 30 min at 30°C and dephosphorylation stopped with the protein phosphatase inhibitor, microcystin-LR (2 µM) prior to electrophoresis. In lane 2, the microcystin-LR (2 µM final) was added to the protein phosphatase 2A prior to incubation with the 14-3-3 column eluate. The bands in the 14-3-3 column eluate (lane 2) are compared with a crude extract (lane 3). The slight

Protein-protein interactions and the overlay technique

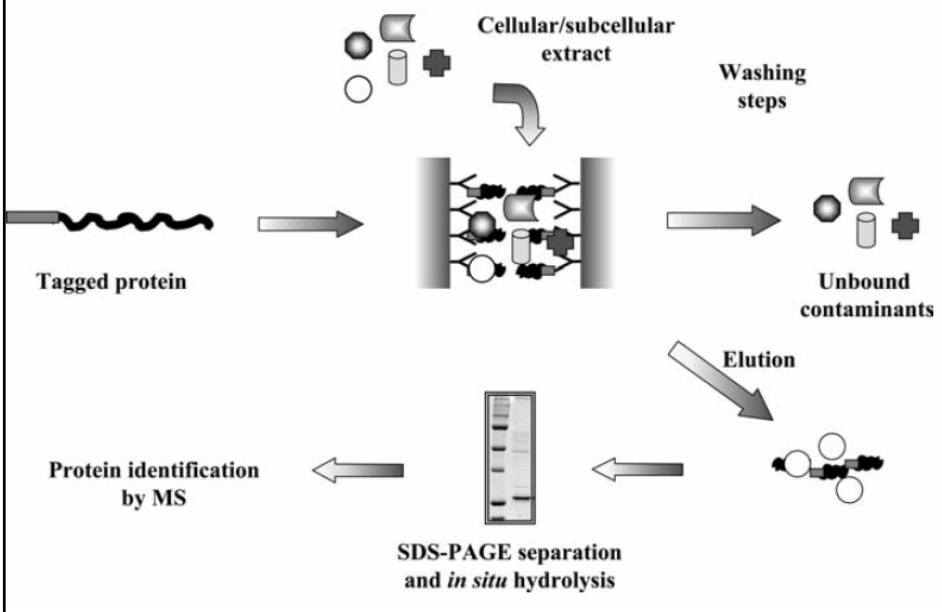
Separation and identification of DIG labelled 14-3-3 interacting proteins

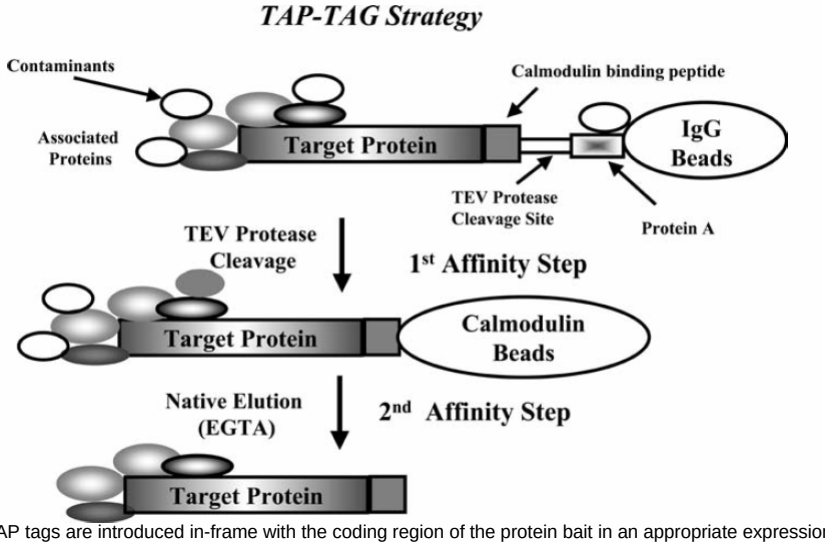


Moorhead et al Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. Plant J. 1999 18, 1-12.



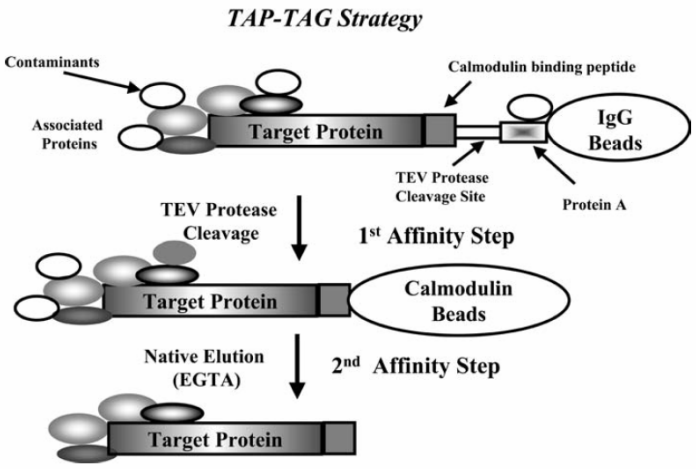
Affinity chromatography





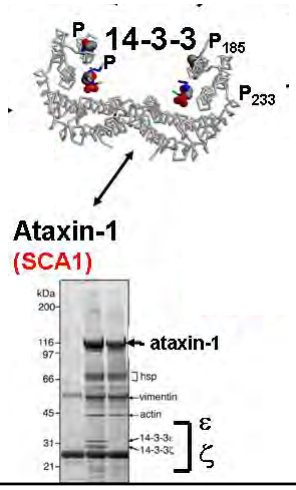
TAP tags are introduced in-frame with the coding region of the protein bait in an appropriate expression vector. The tagged construct can then be transiently or stably introduced into recipient cells or organisms. Optimally, the recombinant vector should replace the endogenous wild-type gene, although this condition might not always be possible. Overexpression of the protein bait is avoided - for recovery of protein complexes expressed at their natural level.

The purification procedure based on a double affinity step significantly reduces occurrence of nonspecific protein contaminants, decreasing both the unspecific background noise on the SDS gel and the possible presence of false positives.

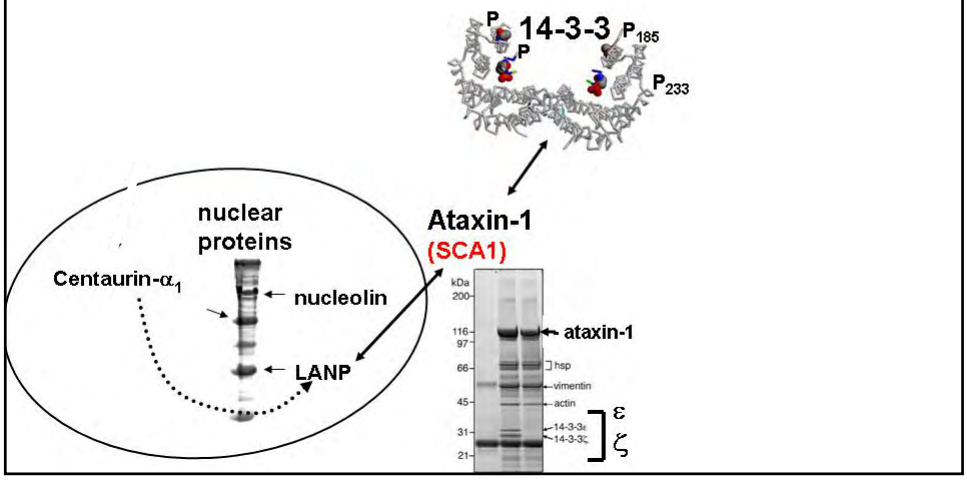


The first affinity purification step of the native complex is binding of Protein A to an IgG matrix. Elution of the bound material under native conditions is achieved by using the TEV protease and the eluate of this first affinity step is then incubated with calmodulin-coated beads in the presence of calcium. Nonspecific protein contaminants and the excess of TEV protease are removed by repetitive washing and the bound material is released under mild conditions by elution with EGTA. Individual protein components of the eluted complex can then be fractionated by SDS-PAGE and identified by different mass spectrometric methodologies.

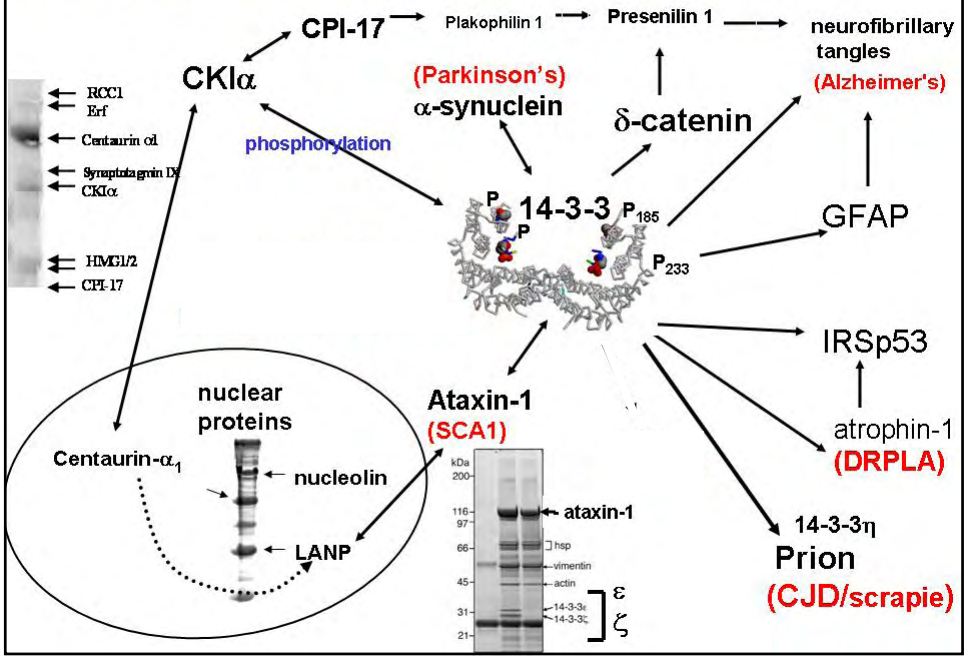
14-3-3 protein complexes in neurodegenerative disease



14-3-3 protein complexes in neurodegenerative disease



14-3-3 protein complexes in neurodegenerative disease



14-3-3 in Neurodegenerative Disease

3. Polyglutamine repeat diseases:

Nine known neurodegenerative diseases caused by CAG repeat expansion resulting in proteins containing a greatly expanded polyglutamine (polyQ) stretch; from approximately 20 glutamines in normal individuals to over 40 in affected individuals.

The mutant proteins are unrelated except for the polyQ tract.

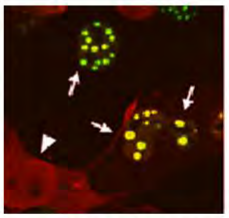
(a) Spinocerebellar ataxia type-1 (SCA1):

14-3-3 ζ and ε stabilize phosphorylated ataxin-1 which accumulates in neurons, inducing neurodegeneration (Chen et al, 2003, Cell 113, 457-468).

(b) Dentatorubral-pallidoluysian atrophy (DRPLA):

atrophin-1, IRSp53 and 14-3-3 interact (Mackie & Aitken, 2005 "Novel brain 14-3-3 interacting proteins involved in neurodegenerative disease". FEBS J, 272, 4202-4210).

(c) Huntington's disease: (others have more recently shown that 14-3-3 associates with phosphorylated huntingtin).



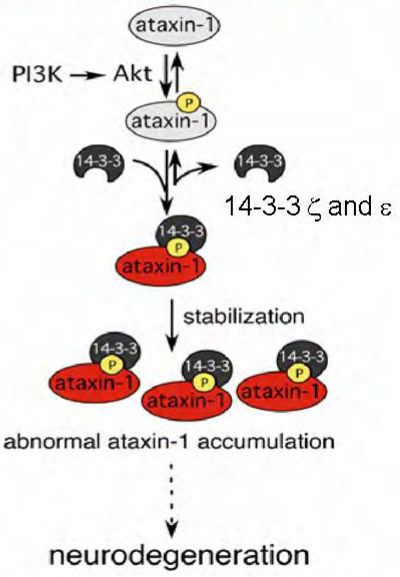
Colocalisation and stabilisation of ataxin-1 with 14-3-3ε

Interaction of Akt-Phosphorylated Ataxin-1 with 14-3-3 Mediates Neurodegeneration in Spinocerebellar Ataxia Type 1: (Chen et al Cell, 2003).

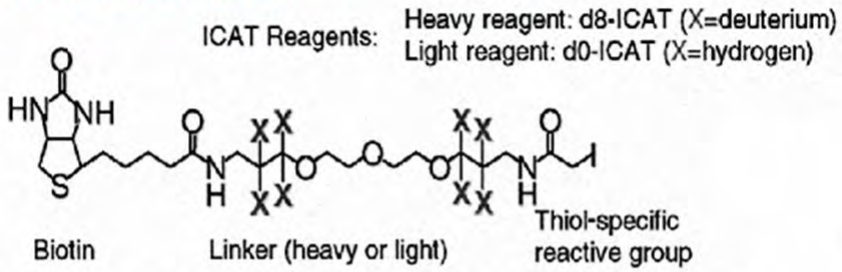
Spinocerebellar ataxia type 1 (SCA1) is one of several neurological disorders caused by a CAG repeat expansion. In SCA1, this produces an abnormally long polyGln tract in ataxin-1.

Mutant polyGln proteins accumulate in neurons, inducing neurodegeneration. 14-3-3 mediates neurotoxicity of ataxin-1 by binding to and stabilizing ataxin-1, thereby slowing its normal degradation.

Association of ataxin-1 with 14-3-3 ζ and ε is regulated by Akt phosphorylation on residue 776: **RRWS^{P}AP**.



The ICAT strategy for quantifying differential protein expression.



Structure of the ICAT reagent, which is in two forms, heavy (8 deuteriums) and light (no deuterium).

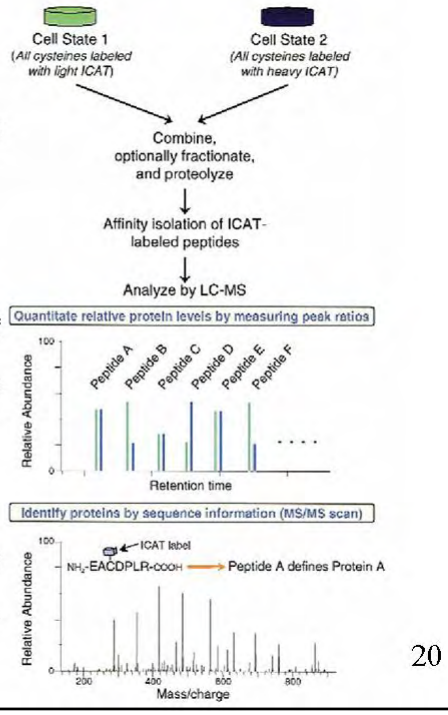
The reagent has three elements:

- an affinity tag (biotin), to isolate ICAT-labeled peptides
- a linker that can incorporate stable isotopes
- a reactive group with specificity toward thiol groups (cysteines)

The ICAT strategy for quantifying differential protein expression.

Two protein mixtures representing two different cell states treated with the isotopically light and heavy ICAT reagents; an ICAT reagent is covalently attached to each cysteinyl residue in every protein. The protein mixtures are combined, proteolyzed and ICAT-labeled peptides are isolated on an avidin column utilizing the biotin tag.

Peptides are separated by HPLC. A pair of ICAT-labeled peptides are chemically identical and are easily visualized because they essentially coelute, and there is an 8 Da mass difference measured in a scanning mass spectrometer (four m/z units difference for a doubly charged ion). The ratios of the original amounts of proteins from the two cell states are strictly maintained in the peptide fragments. The relative quantification is determined by the ratio of the peptide pairs. The protein is identified by database searching with the sequence information.



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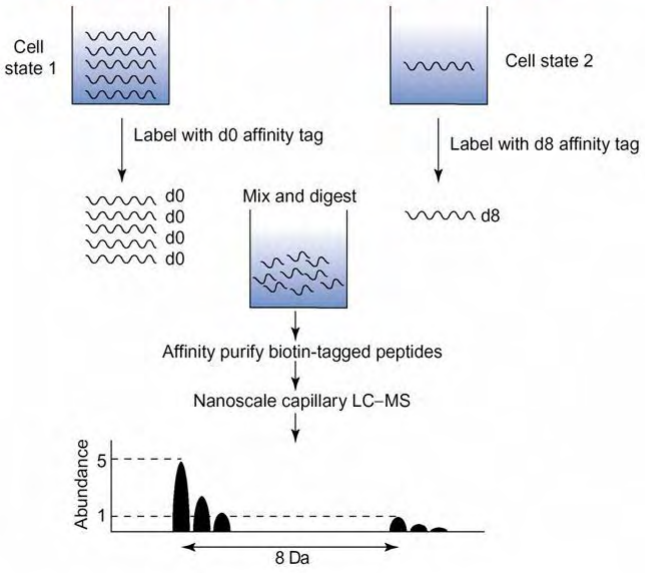
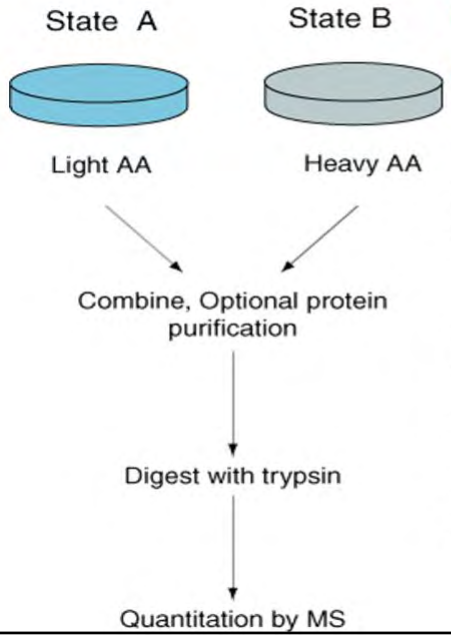
Schematic of ICAT Method



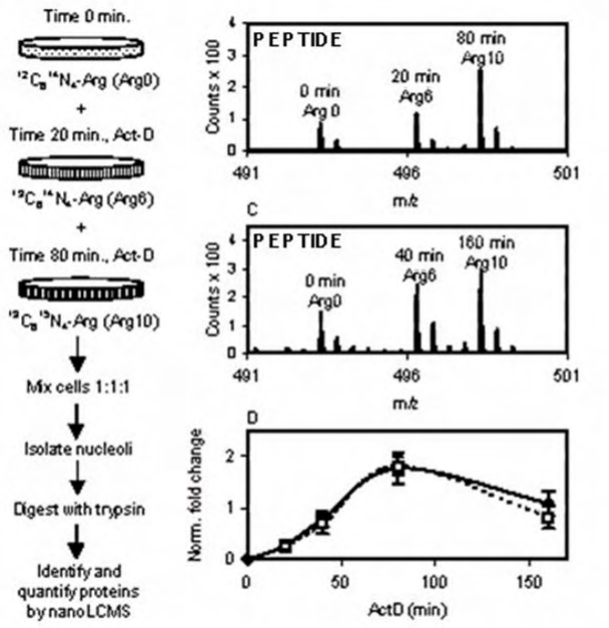
SILAC



Stable isotope labelling with amino acids in cell culture (SILAC) is a simple and straightforward approach for in vivo incorporation of a tag into proteins for relative quantitation by mass spectrometry.

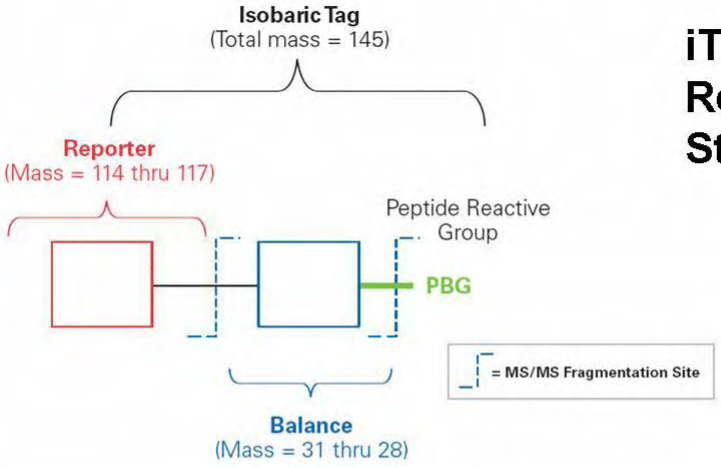
$^{12}C6^{14}N4$-Arg, $^{13}C6^{14}N4$-Arg and $^{13}C6^{15}N4$-Arg isotopically labelled amino acids can be used.

^{12}C- and ^{13}C- labelled leucine also used.



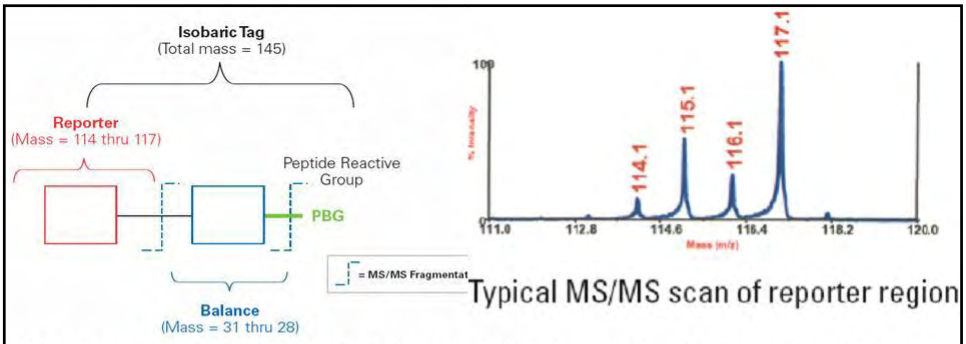
Applied Biosystems iTRAQ Reagents are a set of four, isobaric (same mass) reagents which are amine specific and yield labelled peptides which are identical in mass and hence also identical in single MS mode, but which produce strong, diagnostic, low-mass MS/MS signature ions allowing for quantitation of up to four different samples simultaneously. This gives:

- Analysis of up to four different biological samples in a single experiment (or triplicate analyses of the same sample).
- Absolute quantitation across numerous sample states, for the uniform comparison of normal, diseased and/or drug treated states.
- Information such as post-translational modifications which is not lost using this new chemistry.
- Since all peptides are tagged, proteome coverage is expanded and analysis of multiple peptides per protein improves the confidence and quantitation.
- Protein identification is simplified by improved fragmentation patterns, with no signal splitting in either the MS or MS/MS therefore low-level analysis is enhanced as a result of the signal amplification.
- The complexity of MS and MS/MS data is not increased by mixing multiple proteome samples together.



iTRAQ Reagent Structure

iTRAQ Reagents are isobaric tagging reagents consisting of: a reporter group, a balance group, and a peptide reactive group. The peptide reactive group covalently links an iTRAQ Reagent isobaric tag with each lysine side chain and N-terminus of a peptide, labelling all peptides in a given sample digest.



Typical MS/MS scan of reporter region

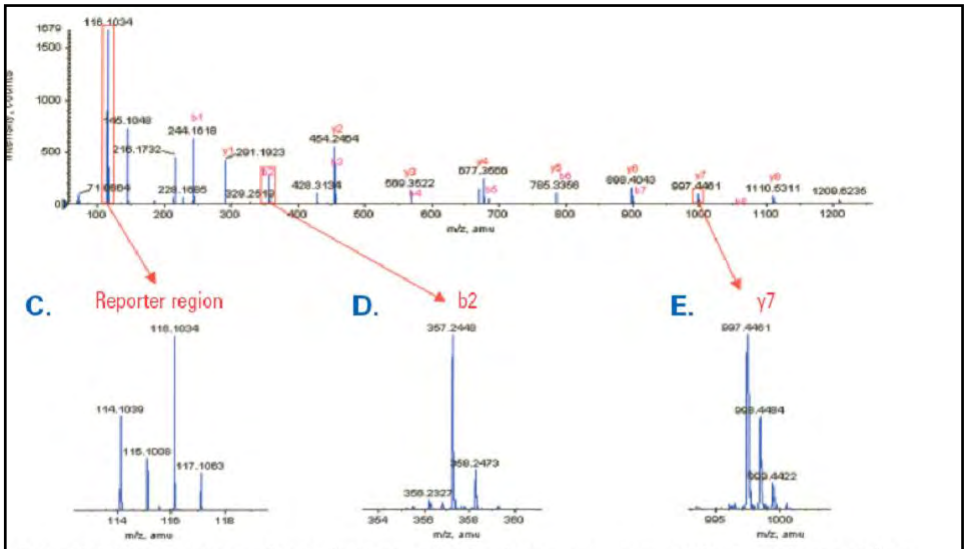
Combining multiple iTRAQ Reagent labelled digests into one sample mixture, the MS resembles the MS of an individual sample (assuming the same peptides are present).

The balance group ensures that an iTRAQ reagent-labelled peptide displays the same mass, whether labelled with iTRAQ reagent 114, 115, 116, or 117.

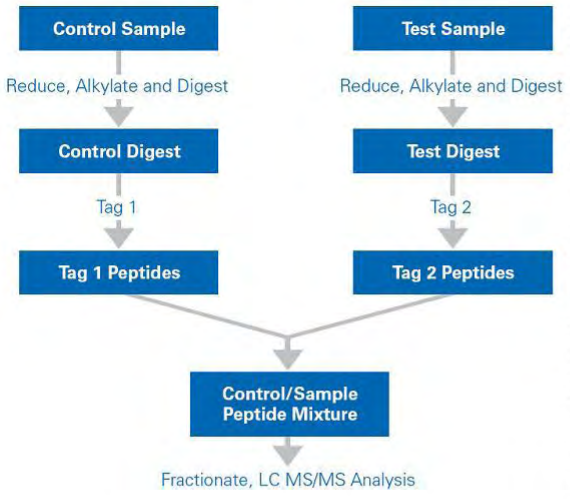
During MS/MS (along with the usual peptide fragmentation) the isobaric tag cleaves at the sites indicated:

As a result of fragmentation, there is neutral loss of the balance group, and the reporter groups are generated, displaying diagnostic ions in the low-mass region between m/z of 114-117. Because this region is free of other common ions, quantifying the peak areas of these resultant ions represents the relative amount of a given peptide in the respective sample.

These diagnostic ions are the key components in quantitative experiments.



An additive series of peptide y- and b- ions are generated and used for protein identification.
Comparison of the MS/MS spectra generated from samples labelled with any of the four different iTRAQ Reagents provide quantitative information on the differences among four different samples.



Duplex example of the iTRAQ reagent protocol.

Although up to four different samples can be analyzed in any given experimental procedure, this Figure shows an experiment using only two. Protein isolates are reduced, alkylated and digested with trypsin in an amine free buffer system, in parallel. After adjustment of the digest to 70% organic, the resulting peptides are labelled with the iTRAQ Reagents.

On completion of labeling the samples are combined. Depending on sample complexity, samples are either directly analyzed via LC/MS/MS after a one-step elution from a cation exchange column to remove reagent by-products or, in the case of complex samples, cation exchange chromatographic fractionation to reduce overall peptide complexity.

Antarctic Prion Pachyptila desolata

Slender-billed Prion