

iTRAQ, a New Prospect of Toxicogenomics

Tanojit Sur

Department of Genetics, Calcutta University, Kolkata, West Bengal, India

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ABSTRACT Toxicogenomics is a high throughput molecular profiling technologies, it is the study of the structure and function of the genome as it responds to adverse xenobiotic exposure. Toxicoproteomics is a very young branch of toxicogenomics and it contains several advanced techniques which open a new way of toxicology research. iTRAQ or Isobaric Tag for Relative and Absolute Quantitation is an quantitative and advantageous method of toxicogenomics. iTRAQ contains a set of eight isobaric reagents namely iTRAQ 114- iTRAQ 121 and this reagents contains three group such as reporter group, balance group and peptide group. iTRAQ analysis rapidly used in different types of cancer therapy and also measured global protein content from malignant and non-malignant tissues. In this technique 8 labels helps for multiplexing experiments and post-translational modifications can also be analyzed. iTRAQ contains very few disadvantages but the major goal of iTRAQ is to identify new biomarkers and signatures of toxicity for classifying toxicants for health risk and for observing toxicity.

INTRODUCTION

Toxicogenomics combines toxicology with genomics or other high throughput molecular profiling technologies such as transcriptomics, proteomics and metabolomics. In pharmaceutical research toxicogenomics is defined as the study of the structure and function of the genome as it responds to adverse xenobiotic exposure. It is the toxicological subdiscipline of pharmacogenomics, which is broadly defined as the study of inter-individual variations in whole-genome or candidate gene single-nucleotide polymorphism(SNP) maps, haplotype markers, and alterations in gene expression that might correlate with drug responses (Lesko et al. 2003; Lesko and Woodcock 2004) Toxicogenomics is a very young branch of toxicology but it also contain some branches, toxicoproteomics is one of them which has been boosted by quantitative and qualitative proteomic technologies. Toxicoproteomics studies applying advanced methodologies must be carried out to pave the way for commencing a new phase in toxicology research. Toxicoproteomics contains several techniques such as ICAT or Isotope-Coded Affinity Tag, SILAC or Stable Isotope Labeling by Amino Acids in Cell Culture, iTRAQ or Isobaric Tag for Relative and Absolute Quantitation. Here

Address for correspondence:

Tanojit Sur
4B, Nrityagopal Ghosal Road,
Ariadaha, Shantineer,
Flat No. - 2A
Kolkata 700 057, West Bengal, India
Telephone: +918961268900, +919163391471
E-mail: surtanojit.gen.cu@gmail.com

we discuss about the method iTRAQ, which is a major breakthrough in quantitative proteomics as well as toxicogenomics. It is a peptide labeling method and the development of isobaric tags takes a completely different approach than older methods (Ross et al. 2004). At this time iTRAQ is the most widely used chemical labeling tags in proteomics research and by investigators of other biomedical fields, who seek novel protein identification and quantitation. The method is based on the covalent labeling of the N-terminus and side chain amines of peptides from protein digestions with tags of varying mass. There are currently two mainly used reagents: 4-plex and 8-plex, which can be used to label all peptides from different samples/treatments. These samples are then pooled and usually fractionated by nano liquid chromatography and analyzed by tandem mass spectrometry (MS/MS). A database search is then performed using the fragmentation data to identify the labeled peptides and hence the corresponding proteins. The fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they originated. There are four tags available for iTRAQ tagging, which makes it possible to perform up to four multiplex analysis simultaneously. Protein quantitation can then be achieved by comparing the MS intensity of the peptides derived from the two samples. iTRAQ is a recently developed protein quantitation technique that utilizes four isobaric amine specific tags. In single MS mode the differentially labelled versions of a peptide are indistinguishable. However, in tandem MS mode (in

which peptides are isolated and fragmented) each tag generates a unique reporter ion. Protein quantitation is then achieved by comparing the intensities of the four reporter ions in the MS/MS spectra (Shadforth et al. 2005). The principal advantage of iTRAQ over ICAT, SILAC and metabolic labelling is that four samples can be analyzed simultaneously, thereby reducing the amount of mass spectrometry time needed for analysis. iTRAQ has several uses in toxicoproteomics such as invention of new biomarkers and toxicity signature, discovery of early markers in drug toxicity, target organ analysis like heart, kidney, liver etc, biofluids analysed such as serum, plasma, urine, cerebrospinal fluid. There are four tags available for iTRAQ tagging, which makes it possible to perform up to four multiplex analyses simultaneously. For example, in the study of tumor progression, one can simultaneously compare normal, cancer as well as pre-cancerous tissue using iTRAQ tagging. To ensure complete labeling, it is necessary to measure the protein concentration before labelling. Intact protein can be labelled by using iTRAQ (Wiese et al. 2007). In cancer research, iTRAQ has been used to study breast cancer (Overall and Dean 2006) as well as endometrial carcinoma (De Souza et al. 2005) iTRAQ also has been widely used for studying signalling pathway. iTRAQ reagents consist of reporter, balancer and target groups. The target group is N-hydroxysuccinamide, which reacts specifically with the α -amino group of lysine and N-terminal of peptides. The reporter group can contain up to 8 differently tagged sites, allowing for detection of mass differences of 1 to 8 Da.

iTRAQ REAGENTS

iTRAQ reagents are provided as a set of eight, isobaric reagents (same mass) reagents: iTRAQ reagent 114, iTRAQ reagent 115, iTRAQ reagent 116, iTRAQ reagent 117, iTRAQ reagent 118, iTRAQ reagent 119, iTRAQ reagent 120 and iTRAQ reagent 121. The use of these reagents allows multiplexing of up to eight different samples in a single LC/MS/MS experiment (Gan et al. 2007). iTRAQ reagents are used to analyze normal, diseased and drug-treated states in the same experimental time course study, run duplicate or triplicate analyses of the same sample in one experiment. iTRAQ reagents are

non-polymeric consisting of a reporter group, a balance group and a peptide reactive group. iTRAQ reagents are amine specific and yield labelled peptides which are identical in mass and hence 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. iTRAQ reagents consist of a reporter group, a balance group and a peptide reactive group (Ernoul et al. 2008) (Fig. 1). The peptide reactive group covalently links an iTRAQ reagent isobaric tag with each lysine side chain and N-terminus group of a peptide, labeling all peptides in a given sample digest (Chong et al. 2006). The balance group ensures that an iTRAQ reagent-labeled peptide displays the same mass, whether labelled with iTRAQ reagent 114, 115, 116 or 117. The reporter group gives strong signature ions in MS/MS, maintains charge state and ionization efficiency of peptide. This reporter group is based on N-methylpiperazine. Due to the isobaric mass design of the iTRAQ reagents, differentially-labelled peptides appear as a single peak in MS scans, reducing the probability of peak overlapping. When iTRAQ-tagged peptides are subjected to MS/MS analysis, the mass balancing carbonyl moiety is released as a neutral fragment, liberating the isotope-encoded reporter ions which provides relative quantitative information on proteins (Aggarwal et al. 2006; Zieske 2006).

iTRAQ WORKFLOW

In the iTRAQ reagents labeling protocol, at first block the cysteine, digest and label each sample in a single tube. The single tube process eliminates potential sample loss in individual samples that may cause inaccuracies in quantitation (Ross et al. 2004). For very complex samples, such as a profiling shotgun approach on whole cell lysates, a 2D separation is an absolute requirement (at the least). Anyone attempting to use iTRAQ with a 1D separation on such samples is wasting their time and/or not realizing what they are missing. The isobaric tags for relative and absolute quantification (iTRAQ) method or iTRAQ-TAILS enables the quantitation of multiple samples simultaneously. This method has the ability to simultaneously analyze from 4-8 samples in multiplex

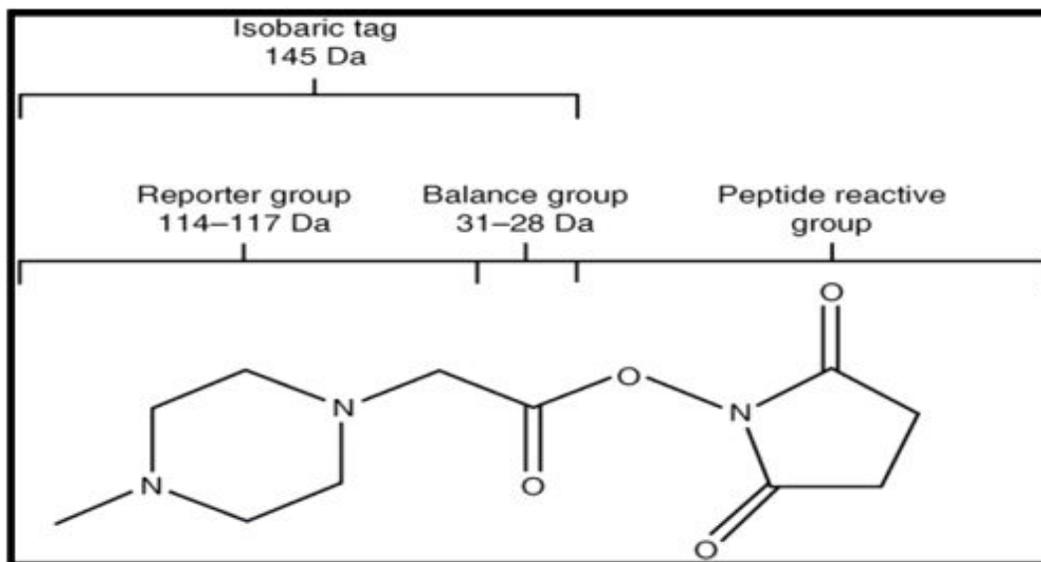


Fig. 1. Structure Of i-TRAQ reagent

experiments using four- and eight-plex iTRAQ reagents. This method provides high accuracy identification and quantification of samples and allows for ore reproducible analysis of sample replicates (Kleifeld et al. 2011). 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. The labeled samples are pooled together. SCX chromatography is generally used for the fractionation of iTRAQ-labeled peptides before LC-MS/MS analysis. However, SCX suffers from clustering of similarly charged peptides and the need to desalt fractions (Hao et al. 2011). The separation and identification of proteomics is a challenging job due to their complex structures and closely related physico-chemical behaviors. Most effectively used kinds of chromatography are liquid chromatography–mass spectrometry (LC–MS) (Neverova and Van Eyk, 2005; Hortin et al. 2006, nano-reversed phase liquid chromatography (nano-RPLC) (Wang et al. 2005). The labeled samples are then prior to further finer separation and purification using reverse phase chromatography. Reversed phase high performance liquid chromatography is the most popular mode of chromatography due to its wide range of applications because of the availabilities of various mobile and stationary phases. Multidimensional liquid chromatography

coupled with tandem-mass spectrometry has wide range of applications. To use the LC-MS/MS combination in proteome analysis, a form of complexity reduction is needed in order to detect and analyze as many components as possible in the sample (Patterson and Aebersold 2003). This is achieved, for instance, by combining two orthogonal peptide separation methods, such as cation exchange and capillary reversed phase chromatography, with MS/MS. This combination of multidimensional chromatography and tandem mass spectrometry became known as MudPit in proteomics, which has been applied to identify up to tens of thousands of proteins from highly complex protein mixtures (Link et al.1999; Wolters et al. 2001; Davis et al. 2001; Washburn et al. 2001; Lipton et al. 2002; Smith et al. 2002; Usaite et al. 2008). The use of multidimensional enrichment and separation techniques in proteomic analysis has greatly enhanced protein coverage and dynamics, allowing many previously undetected low abundance proteins to be identified (Roe and Griffin 2006). The purified labeled peptide fragments are then analyzed by MS/MS. MS/MS is nowadays well established as a method for protein identification (Hernandez et al. 2006). The different masses 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 (Lu et al. 2004). Upon fragmentation on the MS/MS level, detached reporter ions create signals in the low mass range (m/z 113-119 and m/z 121) and peptide backbones remain unmodified and generate fragments that are identical in m/z for all samples. Fragmented signature ions provide quantitative information about the peptides from different conditions upon integration of the peak areas (Hansen et al. 2003). Fragmentation of peptides can be achieved by post-source decay (PSD) during MALDI (Spengler et al. 1992a, b), or collision-induced dissociation (CID) in a collision cell (Shevchenko et al. 1996). In addition to the peptide mass the tandem mass spectrum contains information on the peptide mass and structural information originating from the peptide sequence. Both PSD and CID result dominantly in the cleavage of the peptide bond along the peptide backbone and generate fragment ion ladders either from the N terminus or the C terminus (Roepstorff and Fohlman 1984).

APPLICATION OF iTRAQ

A multiple affinity removal system was made use of to carry out immune depletion of the serum samples from normal controls as well as ovarian cancer patients. This helped in removing the high abundance proteins, leaving behind only the medium and low abundance proteins for iTRAQ analysis (Unwin 2005). iTRAQ analysis rapidly used in different types of cancer therapy and also measured global protein content from malignant and non-malignant tissues (Feldman et al. 2004; Nemunaitis et al. 2007; Petricoin et al. 2004). iTRAQ technique is now widely used in the discovery of blood transfusing biomarkers (Aeberseld et al. 2005; Hale et al. 2003). As a new method of quantitative proteomics, the technique of iTRAQ allows for the quantitative analysis of four samples simultaneously and displays its advantages of high-flux, food reproducibility, and high sensitivity; it also provides a potential technological platform for studying the mechanisms of the development and progression of prostate cancer (Sun et al. 2010). iTRAQ coupled with offline 2DLC-MS/MS to analyze a rare specimen of the poorly understood, potentially blinding ophthalmic condition Macular Telangiectasia type 2 (MacTel type 2). The technique using an internal standard consisting of pooled

samples for each iTRAQ experiment to allow for multiple comparisons between different regions of the retina and different tissue donors (Len et al. 2012). iTRAQ method is used for functional quantitation of mitochondrial protein phosphorylation (Boja 2009). The proteomic approach of isobaric tags for relative and absolute quantification (iTRAQ), followed by LC-MS/MS, is a successful treatment of gastric cancer. Isobaric tagging for relative and absolute quantitation (iTRAQ). These methods are becoming more widely used in ecotoxicology studies to identify and characterize protein bioindicators of adverse effect. In teleost fish, iTRAQ has been used successfully in different fish species (e.g. fathead minnow, goldfish, largemouth bass) and tissues (e.g. hypothalamus and liver) to quantify relative protein abundance. Of interest for ecotoxicology is that many proteins commonly utilized as bioindicators of toxicity or stress are quantifiable using iTRAQ on a larger scale, providing a global baseline of biological effect from which to assess changes in the proteome (Martinyuk et al. 2012). iTRAQ with (^{18}O) stable isotope labeling (iTRAQ plus (^{18}O)) was established to identify N-glycosylation site, quantify the glycopeptides and non-glycosylated peptides, and obtain N-glycosylation site ratio on the target glycoprotein (Zhang et al. 2012). iTRAQ technique recently used for the identification of diseased gene and protein of interest in Colorectal cancer (Haab 2005). iTRAQ, which stands for isobaric tagging for relative and absolute quantification, is a method used to determine differential protein expression. One of the applications of this method is the identification of proteins that are up or down regulated in virulent organisms. The outer membrane proteins of various serotypes of *Haemophilus parasuis*, a swine respiratory pathogen, and *Ornithobacterium rhinotracheale*, an avian respiratory pathogen, were subjected to iTRAQ analysis in order to determine proteins that could serve as potential vaccine and diagnostic candidates (Mandy 2009). iTRAQ reagents are used for analysis of Embryonal Carcinoma cell Line, which are stem cells of tetracarzinomas-tumors that develop in the gonads of both humans and mice. These tumor cells are essentially indistinguishable, at least in terms of differentiative and neoplastic potentials, from the embryonal stem (ES) cells of early (peri-implantation) mammalian embryo (Lin et al. 2010). iTRAQ

combined with LC-ESI-QTOF-MS quantitative proteomics is a powerful tool for discovery of biomarkers of breast cancer (Gui et al. 2011). Discovery of early-diagnosis biomarkers by iTRAQ analysis is the key to improve the early-diagnosis and prognosis of human lung squamous carcinoma (hLSC) (Zeng et al. 2012). iTRAQ is a useful tool in predicting cellular function and fate by determining the proteomic component. iTRAQ allows identification and quantification of proteins between multiple samples, to determine the expression of membrane-bound proteins in two previously characterized human NK cell populations. One population was derived from umbilical cord blood (UCB) stem cells (CD34+38-Lin-) and the other from expanded CD3-depleted adult peripheral blood. iTRAQ was employed for multiplex peptide labeling of proteins from fractionated membranes followed by two-dimensional high-performance liquid chromatography (2D-HPLC), and tandem mass spectrometry was used to identify protein signatures (Lund et al. 2007). iTRAQ also used to understand Endometriosis, a painful reproductive disease afflicting about up to 20% of women. It is one of the most frequent benign gynaecological diseases (Domon and Aebersold 2006). iTRAQ technique was employed for Proteomic profiling of human respiratory epithelia and revealed biomarkers of exposure and harm by tobacco smoke components (Sexton et al. 2011).

ADVANTAGES OF iTRAQ

1. iTRAQ labelled peptides that allow for identification and relative quantitation of the proteins in one experiment (Unwin et al. 2005)
2. All tryptic peptides are labeled resulting in increased confidence and higher quality data
3. In this technique up to 8 labels can be used for multiplexing experiments
4. Improved MS/MS fragmentation results in more confident peptide or protein identifications
5. Post translational modifications, such as phosphorylation, can be analyzed (Gafken and Lampe 2006).
6. The principal advantage of iTRAQ over ICAT, SILAC and metabolic labelling is that four samples can be analyzed simul-

taneously, thereby reducing the amount of mass spectrometry time needed for analysis. iTRAQ has several uses in toxicoproteomics such as invention of new biomarkers and toxicity signature, discovery of early markers in drug toxicity, target organ analysis like heart, kidney, liver etc, biofluids analysed such as serum, plasma, urine, cerebro spinal fluid (Tannu and Hemby 2006).

7. iTRAQ is a time consuming and sample intensive technique for biomarker discovery applications.
8. Another aspect that makes iTRAQ unique from other methods is that quantitation occurs in the MS/MS scan, i.e. when the peptide is sequenced, relative quantitative comparisons are obtained unambiguously for each peptide sequenced (Rudella et al. 2006).
9. From a data analysis point of view, iTRAQ is the easiest to handle in principle. Simple workflow labels peptides allowing rapid progression to LC/MS/MS analysis and easy data interpretation with software for relative and absolute quantitation (Collett et al. 2005).
10. Increase confidence in identification and quantitation by tagging multiple peptides per protein to gain more statistically significant information.
11. Perform absolute quantitation across numerous sample states, for the synchronous uniform comparison of normal, diseased and/or drug treated states.
12. iTRAQ is a highly sensitive approach. It helps to identify proteins across extreme pI and MW, it detects a great number of fragment peptides per protein and low abundance proteins are more often discerned.
13. Another major advantage of using iTRAQ is that it will allow for identification of any type of protein, including high molecular weight proteins, acidic proteins, and basic proteins, all of which are problematic when using alternative methods such as 2D gel electrophoresis.

DISADVANTAGES OF iTRAQ

The key disadvantages of iTRAQ include:-

1. More mass spectrometry time is required because of the increased number of peptides;

2. Samples must be prepared according to very strict guidelines (Aggarwal et al. 2006).
3. General disadvantage of iTRAQ technique, is that they only provide relative quantification. So we can only compare relative abundances. Therefore, researchers can compare two different samples, but they only get a relative value for the concentration of an individual protein (Wu et al. 2006).
4. An inherent drawback of the reported iTRAQ technology is due to the enzymatic digestion of proteins prior to labelling, which artificially increases sample complexity.

CONCLUSION

Toxicogenomics has matured as a field and is now producing exciting new insights into a range of questions in systemic biology. The true value of a genomic or proteomic study is in its ability to provide unique information about cellular responses. A major goal of iTRAQ is to identify new biomarkers and signatures of toxicity for classifying toxicants for health risk and for observing toxicity. It aims to better understand both, the consequences of acute exposure to toxicants as well as long-term development of diseases. The determination of individual proteins or groups of proteins associated with the exposure to toxic substances could be reflective of a common mechanism of toxicity. The iTRAQ reagents are versatile in their ability to provide quantitative information from experiments involving affinity pull-downs, time course analysis, membrane protein studies, discovery and validation analysis for biomarker elucidation, and absolute quantitation of target proteins of interest. SO finally we said that iTRAQ is a very straightforward technique because the labeling chemistry works very well, and labeling efficiency is very important for how well the quantification works. iTRAQ has advantages over the previous technologies, and it is a very good method for quantitative proteomics as well as toxicogenomics. iTRAQ is also more sensitive than previous methods for protein quantification because a lot of the protein changes that we were not able to see before and this high sensitivity is needed to be able to see changes in low-abundance proteins. Despite some of its weaknesses, iTRAQ is a powerful tool for pro-

teomics research. Continual improvements in its usability and technical specifications should make iTRAQ a must have for anyone doing proteomics.

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