OMICS Technologies

55

Christina S. Schmitt, Philip Hewitt, Mostafa Kabiri, and Alexander Amberg

Contents

C.S. Schmitt

P. Hewitt
Merck Darmstadt KG, Institut für Toxikologie, Darmstadt, Germany

M. Kabiri
Sanofi Pharma Deutschland GmbH, A company of the Sanofi-Aventis Group DSE, Hattersheim, Germany

A. Amberg
R&D DSAR Preclinical Safety, Sanofi Deutschland GmbH, Frankfurt am Main, Germany

H.G. Vogel et al. (eds.), *Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays*,
DOI 10.1007/978-3-642-25240-2_61, © Springer-Verlag Berlin Heidelberg 2013

55.1 Introduction

Traditionally, toxicologists define the risk of a new compound to human safety using animal models supported by histopathological and biochemical approaches. However, despite decades of experience, the limitations remain:

- The extrapolation dilemma is still challenging.
- The relevance of animal data to real life continues to be a controversial issue.
- Long-term exposure in humans remains unclear.

Therefore, there is a need for the development of novel test systems that can complement or yet replace the conventional experimental approach in toxicology and thus improve the pharmaceutical drug development process and at the end the quality of new drugs.

The genomics revolution of the recent years led to development of many new and innovative technologies that can change this paradigm and address uncertainty issues in the current toxicological practice and safety assessment. The foundations of the new era build the "OMICS" triads: toxicogenomics, toxicoproteomics, and metabonomics through the identification of novel key genes, marker proteins and metabolites, or gene, protein, and metabolite profiles. These new disciplines combine toxicology with global gene, protein, and metabolite alterations in response to toxic substances and put this knowledge into a toxicological context for a better risk calculation. Global analysis of genes, proteins, and metabolites in cell or tissues has been achieved using a set of different technology platforms such as DNA and protein microarrays (chips), two-dimensional gel electrophoresis (2DGE) combined with mass spectrometer, and liquid chromatography in connection to spectral analysis. The potential of "OMICS" platforms for a better prediction, biomarker identification, and mechanistic explanations of toxicity has been demonstrated in a panel of pilot and comparative studies. Whereas the "OMICS" technologies proved superior alternative to traditional toxicological approaches for biomarker identification or mechanistic investigation, the predictive potential remains difficult and unsatisfactory. "OMICS" data are very complex in volume and content and demand the support of other sciences, for example, bioinformatics, biostatistics, and regulatory, to collect and decipher the whole information. Further variations such as strain and genetic variations, dose, and duration are challenging and demand validation. In recent years, much effort has been made to standardize study designs, experimental procedures, and data processing. Nonetheless, the replacement of conventional in vivo animal studies by "OMICS" platforms is still dreams of the future. A big step forward is the regulatory acceptance that is beginning and would be considered case-by-case. The continued development and refinement of the new methods will alleviate further regulatory appreciation.

In this chapter, Ph. Hewitt and M. Kroger describe toxicogenomics and show examples of its application in toxicological investigations. M. Kabiri deals with toxicoproteomics and outlines the established and alternative methods of global protein analysis, and provides an example of utilization. A. Amberg gives an introduction into metabonomics and presents some details of this technology.

55.2 Toxicogenomics

In toxicology, a full range of genomics technologies are now being used in efforts to uncover the cellular and biochemical mechanisms at work in response to xenobiotic/toxin exposures. The development of these new technologies represents a great opportunity to elucidate toxicological responses to pharmaceuticals, and other chemicals, at a very early stage in drug development. Toxicogenomics (or "transcriptomics") is becoming a well-accepted technology to complement traditional toxicology methods. Since molecular changes occur prior to pathological outcomes, detection of disease and organ toxicity should be possible at earlier time-points during a pathological process. In addition, these technologies are highly sensitive, so that long-term toxic effects can potentially be detected at lower doses. This has the potential to greatly impact toxicology, and to help in the risk assessment of new drug entities. Toxicogenomics represents a desire to step outside the boundaries of traditional toxicology. It is based on the measurement of thousands of genes simultaneously and has shown potential to revolutionize toxicity testing. It has been successfully used as a tool to elucidate mechanisms of toxicity as well as having the potential to predict toxicities much earlier during drug development (Schena et al. 1995; Hamadeh et al. 2002; Ganter et al. 2008).

The advanced knowledge of gene and protein expression patterns, together with modern classification algorithms, has also demonstrated practical benefits for predicting pathological events and toxic end points (Waring et al. 2001; Steiner et al. 2004). Unfortunately, these early promises are only being realized after a period of relatively expensive and deliberate test validation and generation of large reference databases, which are still essential for the future of mechanism elucidation. Without adequate study design, appropriate use of controls, and multidisciplinary development of standardized methods, acceptance has been slow.

Applications can be divided into two broad and partly overlapping classes: investigative studies and predictive toxicology. Investigative studies may help to identify new molecular targets for toxicants or provide novel and deeper insights into mechanisms of action (Man et al. 2002; Ruepp et al. 2002; Fella et al. 2005; Hewitt et al. 2005). The belief that different groups or classes of compounds will induce specific molecules or expression patterns provides the basis for predictive toxicology. Such single markers or gene/protein patterns can have a high degree of predictive power (Elcombe et al. 2002; Li et al. 2002; Petricoin et al. 2002; Ellinger-Ziegelbauer et al. 2004; Boehme et al. 2011; Hrach et al. 2011). Currently, researchers try to set up databases with expression profiles derived from known toxins. These can in the future be used to screen novel compounds in the drug discovery and preclinical evaluation processes.

The full power of toxicogenomics has yet to be realized, and there are numerous platforms available on the market. Both global expression systems (whereby all genes in a given organism are examined simultaneously) and smaller applications (hypothesis-based selection of a small number of specific genes or verification of detected genes of interest observed in microarray analysis) are widely used for many different purposes. Most people involved agree that standardization of microarray experiment procedures and of genomic signatures are keys to the broad acceptance and use of these data. Most journals now only accept papers that have used the MIAME (minimum information about a microarray experiment) guidelines. In traditional toxicology, histopathological evaluation is the gold standard to understand toxicity. Therefore, organs are fixed in paraffin and embedded in paraffin to produce tissue sections for microscopic evaluation. A huge number of formalin-fixed and paraffin-embedded (FFPE) tissues are stored in the archives of toxicology departments, providing a valuable source of molecular biological information. The enormous disadvantage is the low quality of the RNA extracted from such tissue. Thus, special technologies are needed for genomic analyses. In the meanwhile, many companies provide such platforms, either based on microarrays, qPCR, or branched DNA (bDNA) technologies. However, tissue blocks are still not commonly used for molecular profiling in the field of toxicogenomics.

This chapter will be separated into different subjects based on different levels of expression profiling: global expression arrays and multiplexed expression profiling as well as future technologies – namely next generation sequencing. All of these technologies should complement a toxicogenomics study, and their use will be dependent upon the questions being asked.

References and Further Reading

Boehme K, Dietz Y, Hewitt P, Mueller SO (2011a) Genomic profiling uncovers a molecular pattern for toxicological characterization of mutagens and promutagens in vitro. Toxicol Sci 122(1):185–197

Elcombe CR, Odum J, Foster JR, Stone S, Hasmall S, Soames AR, Kimber I, Ashby J (2002) Prediction of rodent nongenotoxic carcinogenesis: evaluation of biochemical and tissue changes in rodents following exposure to nine nongenotoxic NTP carcinogens. Environ Health Perspect 110(4):363–375

Ellinger-Ziegelbauer H, Stuart B, Wahle B, Bomann W, Ahr HJ (2004a) Characteristic expression profiles induced by genotoxic carcinogens in rat liver. Toxicol Sci 77(1):19–34

Fella K, Glückmann M, Hellmann J, Karas M, Kramer PJ, Kröger M (2005) Use of two-dimensional gel-electrophoresis in predictive toxicology: identification of potential early protein biomarkers in chemically induced hepatocarcinogenesis. Proteomics 5(7):1914–1927

Ganter B, Zidek N, Hewitt PR, Müller D, Vladimirova A (2008) Pathway analysis tools and toxicogenomics reference databases for risk assessment. Pharmacogenomics 9(1):35–54

Hamadeh HK, Bushel PR, Jayadev S, DiSorbo O, Bennett L, Li L, Tennant R, Stoll R, Barrett JC, Paules RS, Blanchard K, Afshari CA (2002) Prediction of compound signature using high density gene expression profiling. Toxicol Sci 67(2):232–240

Hewitt P, Kramer P-J, Borlak J (2005a) Toxicogenomics applied to teratogenicity studies. In: Borlak J (ed) Handbook of toxicogenomics, strategies and applications. Wiley-VCH, Weinheim, pp 435–469

Hrach J, Mueller SO, Hewitt P (2011a) Development of an in vitro liver toxicity prediction model based on longer term primary rat hepatocyte culture. Toxicol Lett 206(2):189–196

Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW (2002) Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 48(8):1296–1304

Man WJ, White IR, Bryant D, Bugelski P, Camilleri P, Cutler P, Heald G, Lord PG, Wood J, Kramer K (2002) Protein expression analysis of drug-mediated hepatotoxicity in Sprague-Dawley rat. Proteomics 2(11):1577–1585

Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA (2002) Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359(9306):572–577

Ruepp SU, Tonge RP, Shaw J, Wallis N, Prognan F (2002) Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. Toxicol Sci 65:135–150

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270(5235):467–470

Steiner G, Suter L, Boess F, Gasser R, de Vera MC, Ruepp S (2004) Discriminating different classes of toxicants by transcript profiling. Environ Health Perspect 112(12):1236–1248

Waring JF, Jolly RA, Ciurlionis R, Lurn PY, Praestgaard JT, Morfitt DC, Buratto B, Roberts C, Schadt E, Ulrich RG (2001) Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. Toxicol Appl Pharmacol 175(1):28–42

55.2.1 Total RNA Isolation

PURPOSE AND RATIONALE

For the investigation of expression changes due to xenobiotic/toxin exposure, total RNA must be extracted, whether from body fluids, tissues (including FFPE tissues), or cells. Bolton and McCarthy first published a method for RNA isolation in 1962 (Bolton and McCarthy 1962). Since then many extraction protocols have been developed and adapted to different sample types. All protocols follow the main objective to recover high yield, high quality RNA with as little contamination by DNA and protein as possible. Sample preparation has to be performed prior to any toxicogenomics technology used in investigative studies, and predictive toxicology. For all these approaches, high-quality total RNA (or mRNA) has to be extracted first.

PROCEDURE

Prior to total RNA extraction, sample lysis procedures have to be performed. Lysis conditions are very important for the success of the RNA extraction and depend strongly upon the sample used. Due to great diversity, the biological sample can be pulverized, homogenized, or otherwise disrupted to yield a mixture that contains cells, subcellular components, and other biological debris in an aqueous buffer or suspension. Here is described the protocol for the Trizol® method of RNA extraction.

Trizol® is a mono-phasic solution of phenol and guanidine isothiocyanate, maintains the integrity of the RNA, and is an improvement on the original single-step RNA isolation method described by Chomczynski and Sacchi (1987). After addition of chloroform, vigorous shaking for several minutes, and centrifugation, the RNA exclusively remains in the aqueous phase generated. RNA is recovered by precipitation with isopropyl alcohol, after incubation, and centrifugation at 12,000 *g*. The isolated RNA is then washed with 75% ethanol, and centrifuged at a lower speed. This method facilitates isolation of a variety of RNA species, both of small and large molecular size.

The resulting cleaned RNA pellet should be briefly dried (air-dry, but not to complete dryness) before redissolving in either RNase-free water, or an appropriate buffer. To remove any remaining DNA, a DNase digestion after RNA recovery is highly recommended.

EVALUATION

After sample preparation, total RNA yield can be measured by optical density. Several methods are available. Typically OD at both 260 and 280 nm gives an indication of RNA purity and quantity; thus, the ratio of OD 260/280 should be close to 2. The integrity of RNA can be checked by gel electrophoresis. A convenient platform is the Agilent 2100 Bioanalyzer which is based on capillary gel electrophoresis (Liu et al. 2003). Strong 28 S and 18 S bands or peaks should be visible to indicate high quality RNA. This will most likely not be the case for RNA extracted from FFPE tissues. An additional parameter reflecting RNA quality is the RNA integrity (RIN) introduced by Schroeder et al. (2006). The integrity is lower with many short RNA fragments, with 18 S and 28 S peaks often not being present.

CRITICAL ASSESSMENT

Since differential expression analysis means to compare the quantities of RNA species in two samples, every step during sample preparation has to be highly reproducible. In order to maximize reproducibility, complete total RNA extraction in a one step procedure is recommended. Care must always be taken when

working with RNA, to avoid contamination with RNases, which may result in RNA degradation.

MODIFICATIONS OF THE METHOD

RNA extraction using the relatively toxic Trizol® can also be substituted by numerous other technologies available. Many vendors provide ready-to-use extraction kits including column-based extraction methods. Some kits already include the DNase digestion step, either on column or in solution.

When extracting RNA from FFPE tissues an additional proteinase K digestion step is required to release RNA (Jiang et al. 1995). Due to the "extreme" cross-linking of RNA to proteins initiated by formalin, RNA cannot easily be extracted using common methods. The quality of such FFPE RNA is dependent on several parameters, including warm ischemia time, duration of fixation, embedding process, and blockage.

References and Further Reading

Bolton ET, McCarthy BJ (1962) A general method for the isolation of RNA complementary to DNA. Proc Natl Acad Sci USA 48(8):1390–1397

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162(1):156–159

Jiang YH, Davidson LA, Lupton JR, Chapkin RS (1995) A rapid RT-PCR method for detection of intact RNA in formalin-fixed paraffin-embedded tissues. Nucleic Acid Res 23(15):3071–3072

Liu CH, Ma WL, Shi R, Zhang B, Ou YQ, Zheng WL (2003) Gene expression study of saccharomyces cerevisiae with the Agilent 2100 Bioanalyser. Br J Biomed Sci 60(1):22–25

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3

55.2.2 Global Expression Profiling

The determination of gene expression changes due to toxic insult has become the area of intense research. These changes can be monitored by comparing the level of mRNA for each gene of interest in control and treated tissues/cells/fluids, etc. More recently, it has often been shown that global expression profiling can give hints to molecular changes that lead to overt toxicity (Ellinger-Ziegelbauer et al. 2011).

Global gene expression profiling can be exploited to clarify mechanism of toxicity but also to identify marker genes for the prediction of certain toxicities (Boehme et al. 2009; Boehme et al. 2011; Hrach et al. 2011). These biomarker "fingerprints" can be utilized in later studies without running further genome-wide analyses (Fig. 55.1).

55.2.2.1 Affymetrix GeneChip

PURPOSE AND RATIONALE

There are multiple platforms available that allow one to look at the gene expression of all known genes in a given organism. The aim of this chapter is not to cover all of them, but to give an overview of one such method, the Affymetrix GeneChip®. Affymetrix can be considered to be one of the market leaders in such classical microarray technologies. Correspondingly, it is a highly reproducible, robust system and is based on single-color analysis (Shi et al. 2006). Each transcript is represented by eleven 25-mer probe pairs, with both match and mismatch probes (whereby the central nucleotide is changed). By comparing whole genome expression changes, we have an objective and hypothesis-free method to gain better understanding of the relationship between toxicity and gene expression. Currently, there are several genome-wide arrays commercially available from Affymetrix, covering more than 30 different organisms.

PROCEDURE

The following paragraph describes the sample processing using the GeneChip® 3′ IVT Express Kit generating cRNA and hybridization onto GeneChip 3′ expression arrays. In a reverse transcription reaction, cDNA is synthesized from total RNA or mRNA using reverse transcriptase and a T7 Oligo (dT) primer. Double-stranded cDNA is subsequently synthesized in a reaction mix containing DNA polymerase and RNase H. The cDNA is then used as a template for in vitro transcription using biotinylated nucleotides to produce biotin-labeled amplified cRNA (User Manual, Affymetrix).

The purified material is then assessed for yield, purity, and integrity by spectrophotometric and Agilent Bioanalyzer analyses. Fragmented (35–200 bases) in vitro transcripts (cRNAs) are generated and purified before hybridizing overnight together with

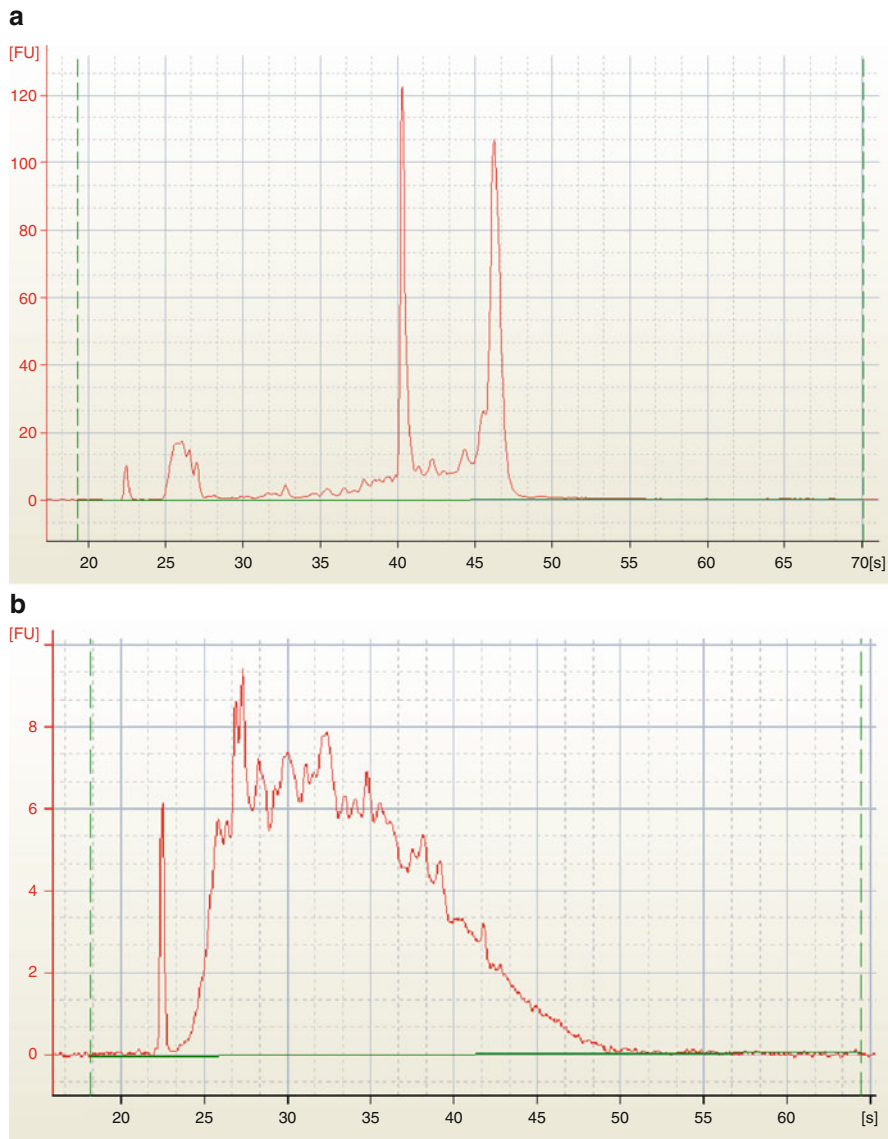

Fig. 55.1 Typical Bioanalyser spectra. Showing two distinct peaks for 18 S and 28 S RNA in high quality samples (**a**) and a trace of degraded RNA derived from FFPE tissues (**b**)

controls onto the Affymetrix GeneChip® (e.g., the Rat Expression 2.0 array contains approximately 31,000 rat-specific probe sets). The hybridized samples are stained with streptavidin-R-phycoerythrin (SAPE) and the signal is amplified using a biotinylated antibody, followed by a final staining. Washing, staining, and amplification are carried out using the manufacturer's fluidics station. The arrays are scanned using the manufacturer's fluorescent scanner (Fig. 55.2a).

EVALUATION

Normalization and scaling of the expression data across arrays can be performed based on a set of maintenance genes included on most Affymetrix arrays The raw data is firstly quality checked and transformed into expression values whereas different algorithms can be used, for example, MAS5.0 from Affymetrix or RMA. These algorithms include background correction, normalization, and summarization of the data. To identify differentially regulated genes, for example, in comparison to a control group, a threshold value of twofold is commonly applied. To aid data interpretation, it is essential that statistical analysis, false discovery rate, t-test or n-way Anova are also included. Expressionist® Refiner and Expressionist® Analyst from Genedata or GeneSpring GX

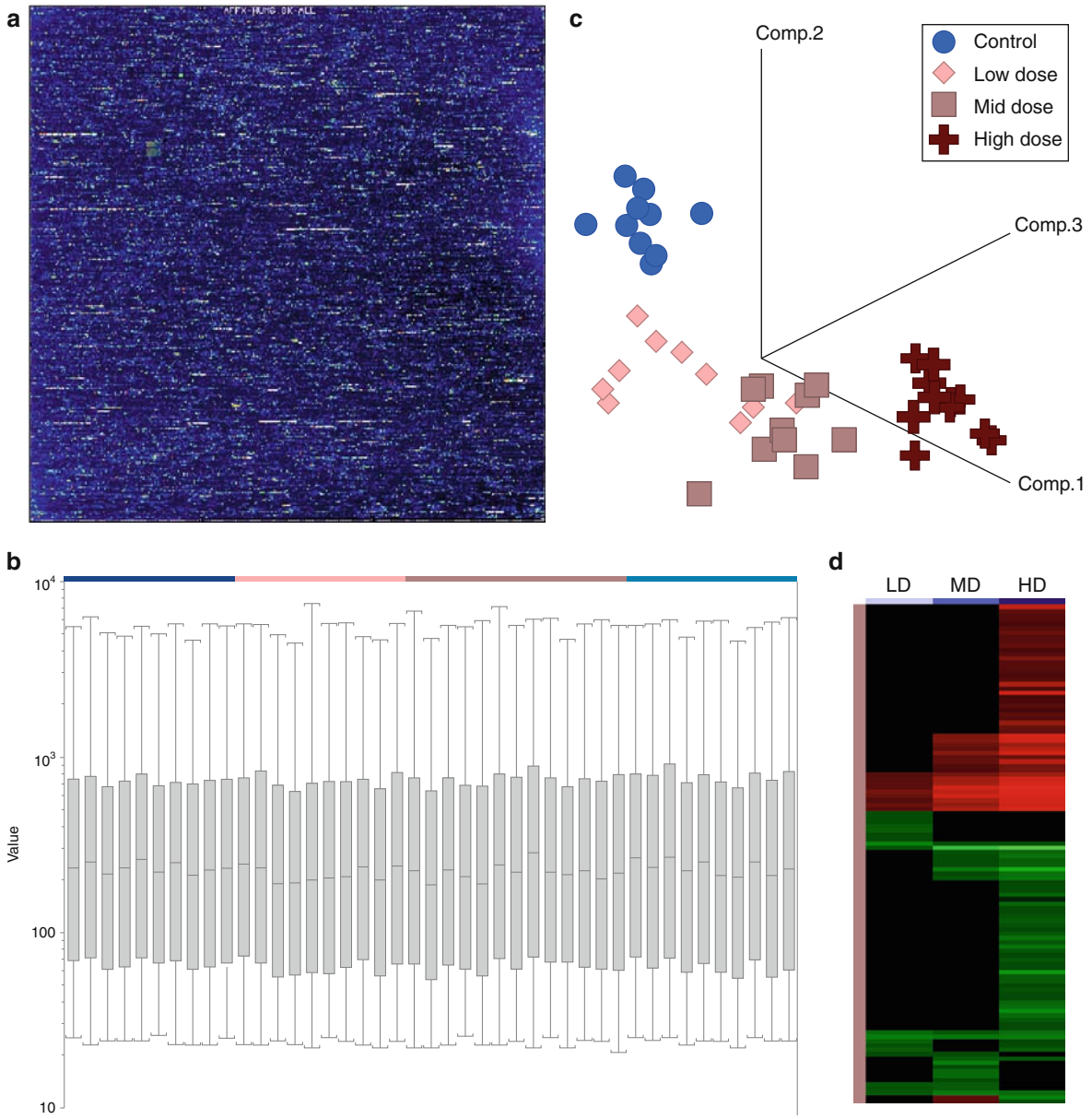

Fig. 55.2 Whole genome gene expression data of a toxicogenomic study. Computer image of a single Affymetrix GeneChip after sample hybridization (**a**). Presentation of gene expression data from rats treated with a low dose, mid dose, or high dose, respectively, of a potential drug in comparison to control animals analyzed with Expressionist® Analyst from Genedata. Non-normalized expression values of individual samples presented in separate Box Plots (**b**). Principal Component Analysis (PCA) after data normalization, showing differences between samples in their overall gene expression (**c**). The so-called heat map displays upregulated (*red*) and downregulated (*green*) genes in comparison to control animals (**d**)

from Agilent are possible software tools where such specific analysis can be performed. Dose-responses or time-effects can be evaluated by special statistical methods. An example of gene expression data from a toxicogenomic study is given in Fig. 55.2b–d.

CRITICAL ASSESSMENT

Affymetrix, as a tool, is well accepted in the scientific community and is highly reproducible (mean false change rate of triplicates $\leq 0.18\%$ and the percentage of concordant detection call is $\geq 91\%$) and sensitive

(down to 1.5 pm mRNA); therefore, chip-to-chip variation is kept to a minimum (Technical Note, Affymetrix). The major advantage of this method is that you can simultaneously monitor the expression changes of tens of thousands of individual genes. Mechanisms of toxicological response can be teased out of the data and gene expression patterns (signatures) may lead to a more predictive approach to early toxicological assessment. However, the data is cumbersome and the amount of data generated is enormous, and therefore, appropriate software tools and large databases are essential. Interpretation of such large datasets is difficult and care must be taken not to over-interpret such data. For all array technologies, a second method is recommended to confirm a small number of the gene expression changes, for example, real-time PCR (see Sect. 55.2.3).

MODIFICATIONS OF THE METHOD

The actual protocol for running Affymetrix arrays is well standardized—as recommended by the manufacturer. However, it is clear that this method is only a tool, and many researchers have reported different uses for this technology (and not only in toxicology).

There are also obviously many other companies offering similar global expression arrays. These include Agilent, Illumina, Roche NimbleGen, Applied Microarrays, to name just a few. All are based on oligonucleotides attached to an array surface where the target sequences will be captured. The most popular systems are synthesized oligonucleotides spotted onto the array (customized or standard arrays). This is in contrast to the Affymetrix array described above, where the manufacture is based on a combined chemical and photolithographic method of oligonucleotide synthesis directly on the array (Pease et al. 1994). In addition to Affymetrix, spotted cDNA arrays have been widely used for mechanistic toxicity testing (e.g., Kultima et al. 2004). Due to the availability of new genomic technologies (next-generation sequencing), the conventional microarray analysis has already lost importance, at least in other fields of research. So this new technology might also enter toxicogenomic evaluations in the near future (see Sect. 55.2.4.2).

Expression analysis using FFPE tissues is not commonly applied in toxicology but a lot is known about their use in other fields of research. Tissue blocks have been assessed for molecular toxicology processes and can successfully be used (Schmitt et al. 2009; von Landenberg et al. 2011). Due to the nature of RNA extracted from FFPE tissues, special technologies are required for successful sample processing. For example, Genisphere and NuGEN Technologies provide sample processing kits to perform expression profiling on Affymetrix and Illumina whole genome arrays. Furthermore, the DASL™ assay from Illumina was specifically designed for analyzing degraded RNA and is no available for whole genome expression analysis of FFPE tissues (Fan et al. 2004).

References and Further Reading

Affymetrix Technical Note (2002) Manufacturing quality control and validation of gene chip arrays. Part number 701309 Rev. 3

Affymetrix User Manual (2008–2010) GeneChip® 3' IVT Express Kit. P/N:702646 Rev. 8

Boehme K, Simon S, Mueller SO (2009) Gene expression profiling in Ishikawa cells: a fingerprint for estrogen active compounds. Toxicol Appl Pharmacol 236(1):85–96

Boehme K, Dietz Y, Hewitt P, Mueller SO (2011b) Genomic profiling uncovers a molecular pattern for toxicological characterization of mutagens and promutagens in vitro. Toxicol Sci 122(1):185–197

Boitier E, Amberg A, Barbié V, Blichenberg A, Brandenburg A, Gmuender H, Gruhler A, McCarthy D, Meyer K, RiefkeB RM, Schoonen W, Sieber M, Suter L, Thomas CE, Sajot N (2011) A comparative integrated transcript analysis and functional characterization of differential mechanisms for induction of liver hypertrophy in the rat. Toxicol Appl Pharmacol 252(2):85–96

Ellinger-Ziegelbauer H, Adler M, Amberg A, Brandenburg A, Callanan JJ, Connor S, Fountoulakis M, Gmuender H, Gruhler A, Hewitt P, Hodson M, Matheis KA, McCarthy D, Raschke M, Riefke B, Schmitt CS, Sieber M, Sposny A, Suter L, Sweatman B, Mally A (2010) The enhanced value of combining conventional and "omics" analyses in early assessment of drug-induced hepatobiliary injury. Toxicol Appl Pharmacol 252(2):97–111

Ellinger-Ziegelbauer H, Stuart B, Wahle B, Bomann W, Ahr HJ (2004b) Characteristic profiles induced by genotoxic carcinogens in rat liver. Toxicol Sci 77(1):19–34

Ezendam J, Staedtler F, Pennings J, Vandebriel RJ, Pieters R, Boffetta P, Harleman JH, Vos JG (2004) Toxicogenomics of subchronic hexachlorobenzene exposure in Brown Norway rats. Environ Health Perspect 112(7):782–791

Fan JB, Yeakley JM, Bibikova M, Chudin E, Wickham E, Chen J, Doucet D, Rigault P, Zhang B, Shen R, McBride C, Li HR, Fu XD, Oliphant A, Barker DL, Chee MS (2004) A versatile assay for high-throughput gene expression profiling on universal array matrices. Genome Res 14(5):878–885

Harris AJ, Shaddock JG, DelongChamp R, Dragan Y, Casciano DA (2004) Comparison of basal gene expression in cultured primary rat hepatocytes and freshly isolated rat hepatocytes. Toxicol Mech Method 14(5):257–270

Hewitt P, Kramer P-J, Borlak J (2005b) Toxicogenomics applied to teratogenicity studies. In: Borlak J (ed) Handbook of toxicogenomics, strategies and applications. Wiley-VCH, Weinheim, pp 435–469
Hook SE (2010) Promise and progress in environmental genomics: a status report on the applications of gene expression-based microarray studies in ecologically relevant fish species. J Fish Biol 77(9):1999–2022
Hrach J, Mueller SO, Hewitt P (2011b) Development of an in vitro liver toxicity prediction model based on longer term primary rat hepatocyte culture. Toxicol Lett 206(2):189–196
Jessen BA, Mullins JS, De Peyster A, Stevens GJ (2003) Assessment of hepatocytes and liver slices as in vitro test systems to predict in vivo gene expression. Toxicol Sci 75(1):208–222
Kostrubsky VE, Strom SC, Hanson J, Urda E, Rose K, Burliegh J, Zocharski P, Cai H, Sinclair JF, Sahi J (2003) Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. Toxicol Sci 76(1):220–228
Kultima K, Nystrom AM, Scholz B, Gustafson AL, Dencker L, Stigson M (2004) Valproic acid teratogenicity: a toxicogenomics approach. Environ Health Perspect 112(12):1225–1235
Martyniuk CJ, Griffitt RJ, Denslow ND (2011) Omics in aquatic toxicology: not just another microarray. Environ Toxicol Chem 30(2):263–264
Matheis KA, Com E, Gautier JC, Guerreiro N, Brandenburg A, Gmuender H, Sposny A, Hewitt P, Amberg A, Boernsen O, Riefke B, Hoffmann D, Mally A, Kalkuhl A, Suter L, Dieterle F, Staedtler F (2011) Cross-study and cross-omics comparisons of three nephrotoxic compounds reveal mechanistic insights and new candidate biomarkers. Toxicol Appl Pharmacol 252(2):112–122
Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SP (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci USA 91(11):5022–5026
Schmitt C, Walijew A, Hewitt P, Harleman JH (2009) Gene expression profiling in toxicological studies using FFPE tissues. Abstracts. Exp Toxicol Pathol 61:387–483, Abstract #P06
Von Landenberg F, Hewitt P, Mueller SO, Schmitt CS (2011) Retrospective gene expression analysis using formalin-fixed tissue blocks from toxicological studies. Toxicologist 120(2):Abtract #1590
Shi L, MAQC Consortium et al (2006a) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24(9):1151–1161
Yuan X, Jonker MJ, de Wilde J, Verhoef A, Wittink FR, van Benthem J, Bessems JG, Hakkert BC, Kuiper RV, van Steeg H, Breit TM, Luijten M (2010) Finding maximal transcriptome differences between reprotoxic and non-reprotoxic phthalate responses in rat testis. J Appl Toxicol. doi: 10.1002/jat.1601

EXAMPLES

Many researchers have used genome-wide expression profiling to elucidate toxic mechanisms and/or to find marker gene(s) for specific toxicity end points. Ellinger-Ziegelbauer et al. (2004) have used Affymetrix technology to characterize genotoxic carcinogens in rat liver. Ezendam et al. (2004) have used toxicogenomics for the elucidation of the mechanism of toxicity after sub-chromic hexachlorobenzene exposure in rats. Hewitt et al. (2005) have reported the use of the Affymetrix technology to elucidate the teratogenic mechanism of two drugs (retinoic acid and a novel pharmaceutical agent). In a larger in vivo study, it was demonstrated how toxicogenomic evaluations together with toxicoproteomics and metabonomics can contribute to the detection of liver toxicity, biliary injury, or hepatocellular hypertrophy, as well as kidney toxicity (Boitier et al. 2011; Ellinger-Ziegelbauer et al. 2011; Matheis et al. 2011). Yuan and coworkers successfully used toxicogenomic approaches to identify the reprotoxicity potential of specific phthalates in rat testicles (Yuan et al. 2010). Microarrays have even been applied in the fields of environmental biology and ecotoxicology (Hook 2010; Martyniuk et al. 2011). In addition, many in vitro studies have been reported, for example, in hepatocytes to evaluate liver toxicity or HepG2 cells for mutagenicity studies (Jessen et al. 2003; Kostrubsky et al. 2003; Harris et al. 2004; Boehme et al. 2011; Hrach et al. 2011).

55.2.3 Multiplexed Gene Expression Analysis

PURPOSE AND RATIONALE

The quantitative analysis of gene expression changes is important if we are to trust data generated by larger gene arrays. The real-time PCR technique (e.g., TaqMan® from Applied Biosystems) allows fast and very sensitive detection of even rare RNA molecules and is routinely used for analyzing a small set of distinct preselected genes. It can also be applied for the validation of array data. This high specificity is due to a complementarity between the primer set, the internal probe, and the target. It is widely believed to be the most sensitive and specific method with a wide dynamic range for mRNA quantification. The TaqMan® assay was used as gold standard in the MAQC project (Shi et al. 2006). Very small amounts of RNA are required, ensuring economic use of

precious samples, as well as the possibility of using micro-dissected tissue.

PROCEDURE

For the TaqMan® assay, purified RNA is first subjected to reverse transcription using random primers. The subsequent PCR reaction includes individual primers (both forward and reverse), a sequence-specific probe, and the polymerase. Ready-to-use primer/probe mixes can be purchased from the supplier or custom primer/probe mixes can be designed on their website (Primer Express® software). The software produces sequences that comply with requirements regarding the melting point, G/C content, length, and configuration. Furthermore, the amplicon should not exceed 150 bp. The characteristic TaqMan® probes are labeled with the fluorescent dye FAM (6-carboxy-fluorescein) and a nonfluorescent quencher together with a minor groove binder (MGB) at the 5′- and 3′-end, respectively. The MGB stabilizes the probe binding and enables higher melting temperatures. Probe and primers are provided in one mix and the TaqMan® Universal Master Mix comprises all remaining components necessary for a real-time PCR. The TaqMan® reaction exploits the 5′-nuclease activity of the Taq polymerase releasing the reporter dye from the 5′-end of the annealed TaqMan® probe during amplification. Reporter dye fluorescence is no longer transferred (Forester energy transfer) and suppressed by the quencher, resulting in an increasing fluorescent signal. The reaction is performed on the real-time PCR systems from Applied Biosystems (e.g., 7,500 Real-Time PCR System) and the accumulating reporter dye fluorescence is detected in real time. For amplification, the reaction starts with 2 min at 50°C and 10 min at 95°C followed by up to 40 cycles with 15 s at 95°C for denaturation and 1 min at 60°C for annealing/extension for each amplification cycle (Protocol, Applied Biosystems). Each sample is analyzed for the target gene of interest and at least one endogenous control (e.g., 18 S ribosomal RNA (rRNA)) for normalization. It is important to correctly choose an endogenous control which is consistently expressed in all samples independent of tissue source and treatment. A no template reaction can be run as the negative control. If a standard curve is required, different dilutions of a sample with known quantities are analyzed for each target, including the endogenous control. All samples and controls are run in triplicates (Fig. 55.3).

EVALUATION

In the resulting amplification curve, the baseline and the threshold within the exponential phase are set to determine the threshold cycle (C_T) (Protocol, Applied Biosystems). Two methods are usually used for the evaluation of real-time PCR data, namely, the standard curve method or the comparative C_T method. The standard curve method relies on the use of dilutions of cDNA reverse transcribed from a reference RNA, which will result in only a relative quantification. Other more specific standards can be used, for example, in vitro transcribed RNA, which gives an absolute quantitation; however, this method is very labor intensive and not commonly used (Martell et al. 1999). The standard curve is included in each PCR run, and therefore provides a correction control for the PCR efficiency, making inter-assay comparisons easier. The comparative C_T method uses algorithms to calculate relative expression levels, compared to a calibrator (e.g., a control sample). A detailed description of the mathematics is given by Livak and Schmittgen (2001). After calculation, the normalized expression of the target gene in the unknown sample relative to the normalized expression of the control (calibrator) sample is produced. It is important when using this method that the PCR efficiency of the target gene and the housekeeping gene is equal, then more samples can be run in one PCR run (i.e., no wells lost to the standard curve).

The housekeeping gene is one that is universally expressed, and does not change under the conditions of the assay employed. 18 S rRNA, β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), cyclophilin, mitochondrial ATP synthase 6, hypoxanthine guanine phosphoribosyl transferase 1 (HPRT), and succinate dehydrogenase complex subunit A (SDHA) have, for example, been reported (Zhong and Simons 1999; Gerard et al. 2000; Cicos et al. 2007). Assays are evaluated only when negative controls do not show any amplification products.

Statistical analyses (e.g., t-test) are performed and significant differences between treated samples compared to vehicle control are determined.

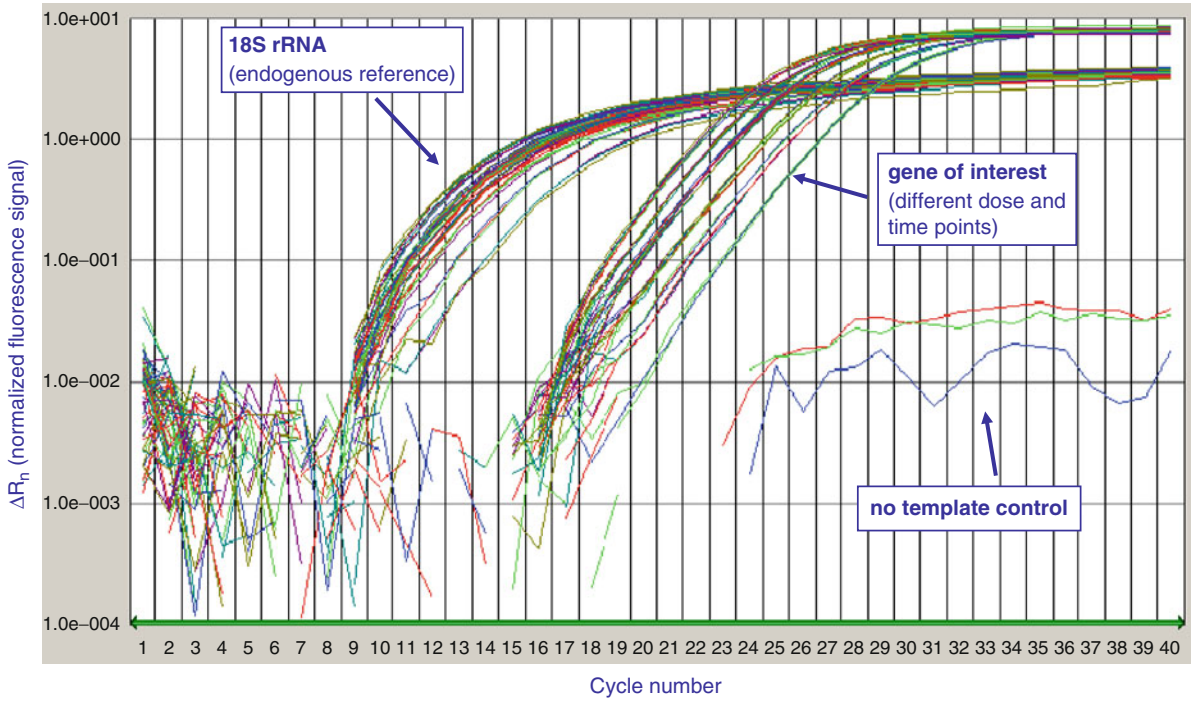

Fig. 55.3 Representative real-time PCR (TaqMan®) trace. Showing the housekeeping gene, 18 S, and the test gene of interest. Increased fluorescence intensity indicates increasing levels of cDNA and more cycles needed indicate smaller amount of starting material (RNA)

CRITICAL ASSESSMENT

Real-time PCR can be considered to be a highly sensitive, specific, and reproducible technology for quantitation of gene expression. Reliability is very high and the data generated is of the highest quality. This method would be the method of choice, when expression of a limited number of genes is required. The obvious drawback is that the number of gene expressions possible is limited. Therefore, higher-throughput gene arrays for the study of larger numbers of genes are needed.

Care must be taken when choosing housekeeping genes for normalization, as there are many citations reporting the gene expression regulation of all of these commonly used housekeepers.

Table 55.1 A selection of currently available real-time PCR systems

Company	PCR cycler
Life Technologies	7300, 7500 (Fast), 7900HT, OpenArray®, StepOne(Plus)™, ViiA™ 7 (Dx) Real-Time PCR Systems
Roche	LightCycler® 2.0, 480, 1536, Nano
Illumina	Eco® Real-Time PCR System
Eppendorf	Mastercycler® ep realplex
BioRad	MiniOpticon, MyiQ2, CFX96 Touch, CFX384 Touch
Qiagen	Rotor-Gene® Q
Cepheid	SmartCycler, GeneXpert
Agilent Technologies	Mx3000P/Mx3005P qPCR System

MODIFICATIONS OF THE METHOD

More recently intercalating double-stranded DNA-binding dyes (e.g., SYBR Green),have been introduced (Giulietti 2001) which removed the need for an expensive, specific probe to be designed. Other sophisticated tools have been developed to work in conjunction with the TaqMan® method, for example, molecular beacons, scorpions, and hybridization probes. These techniques also rely on the FRET (Fluorescence Resonance Energy Transfer) principle but the emergence of the fluorescent signal does not require the nuclease activity of the Taq polymerase. Some of the different real-time PCR providers currently available on the market are given in Table 55.1. Applied Biosystems

have introduced "microfluidics cards" or low-density gene expression arrays. These cards follow the main TaqMan® principles, but are based on a 384-well plate design. Therefore, multiple samples and genes can be monitored, quantitatively, at the same time. Maley et al. (2004) have reported the use of a multiplexed TaqMan® model for high-throughput screening applications. Instead of the nonfluorescent quencher at the 3′-end of the TaqMan® probe, a fluorescent quencher (TAMRA) is linked, especially when designing custom gene expression assays.

Other multiplexing solutions are available on the market, for example, the QuantiFast® Multiplex PCR Kit from Qiagen, which enables the analysis of up to four targets in a single tube. A flexible multiplexing PCR solution is provided by Life Technologies/Applied Biosystems. The OpenArray® technology contains several individual PCR assays on a conventional microscope slide format with 48 subarrays. The subarrays enable analysis in a mid-density manner for higher throughput applications (up to 48 samples or 224 individual assays). PCR reactions can either be TaqMan or SYBR® Green based that can be used for gene expression but also for genotyping, GWAS, and miRNA analysis.

A PCR-free system is provided by Affymetrix/Panomics. The QuantiGene® assay, based on the branched DNA technology, applies signal amplification rather than DNA amplification. Various sample types, for example, cells, tissues, FFPE tissues, blood, can be run in this assay without the need to extract pure RNA. Either a single gene or several genes can be multiplexed in one assay. After capturing the target sequence on the plate surface or on beads for single and multiplexed assays, respectively, the signal is ~400-fold increased by the use of pre-amplifiers and amplifiers which build a branch-like structure. The bead type (target) and the signal intensity are detected using the Luminex® instrument (Dunbar 2006).

References and Further Reading

Applied Biosystems Protocol (2010) TaqMan® Gene Expression Assays. P/N: 4333458 Rev. N

Campbell SJ, Finlay M, Clements JM, Wells G, Miller KM, Perry VH, Anthony DC (2004) Reduction of exitotoxicity and associated leukocyte recruitment by a broad-spectrum matrix metalloproteinase inhibitor. J Neurochem 89(6):1378–1386

Cicos S, Bukovska A, Koppel J (2007) Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 8:113

Dunbar SA (2006) Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta 363(1–2):71–82

Fox BC, Devonshire AS, Schutte ME, Foy CA, Minguez J, Przyborski S, Maltman D, Bokhari M, Marshall D (2010) Validation of reference gene stability for APAP hepatotoxicity studies in different in vitro systems and identification of novel potential toxicity biomarkers. Toxicol In Vitro 24(7):1962–1970

Gerard CJ, Andrejka LM, Macina RA (2000) Mitochondrial ATP synthase 6 as an endogenous control in the quantitative RT-PCR analysis of clinical cancer samples. Mol Diagn 5(1):39–46

Giulietti A (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25(4):386–401

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408

Maley D, Mei J, Lu H, Johnson DL, Ilysin SE (2004) Multiplexed RT-PCR for high throughput screening applications. Comb Chem High Throughput Screen 7(8):727–732

Martell M, Gomez J, Steban JI, Sauleda S, Quer J, Cabot B, Esteban R, Guardia J (1999) High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J Clin Microbiol 37(2):327–332

Oshida K, Hirakata M, Maeda A, Miyoshi T, Miyamoto Y (2011) Toxicological effect of emodin in mouse testicular gene expression profile. J Appl Toxicol. doi: 10.1002/jat.1637

Ovando BJ, Ellison CA, Vezina CM, Olson JR (2011) Toxicogenomic analysis of exposure to TCDD, PCB126 and PCB153: identification of genomic biomarkers of exposure to AhR ligands. BMC Genomics 11:583

Shi L, MAQC Consortium et al (2006b) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24(9):1151–1161

Zhang J, Peterson SM, Weber GJ, Zhu X, Zheng W, Freeman JL (2011a) Decreased axonal density and altered expression profiles of axonal guidance genes underlying lead (Pb) neurodevelopmental toxicity at early embryonic stages in the zebrafish. Neurotoxicol Teratol. doi:10.1016/j.ntt.2011.07.010

Zheng JL, Frantz G, Lewis AK, Sliwkowski M, Gao WQ (1999) Heregulin enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic shock. J Neurocytol 28(10–11):901–912

Zhong H, Simons JW (1999) Direct comparison of GAPDH, beta-actin, cyclophilin, and 28 S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 259(3):523–526

EXAMPLE

There are numerous publications where real-time PCR analysis has been used to follow the changes in expression of specific toxic relevant genes. Fox et al. (2010)

identified potential biomarkers for the identification of acetaminophen toxicity in HepG2 cells and primary rat hepatocytes by qPCR analysis. RT-qPCR analysis was applied to validate a gene marker set observed by microarray analysis describing the molecular changes in rat liver after AhR ligands exposure (Ovando et al. 2011). Testicular toxicity of emodin, a herbal medicine, was observed and analyzed by qPCR (Oshida et al. 2011). The neurotoxic effect of lead during early development was assessed by real-time PCR using zebra fish embryos (Zhang et al. 2011). Giulietti (2001) have used real-time PCR techniques to evaluate cytokine profiles in both mouse and human cells and tissues. Heregulin (a member of the neuregulin family) and its binding receptors (ErbB-2, ErbB-3, and ErbB-4) are induced by gentamicin treatment, and are therefore postulated to play an important role in hair cell regeneration following ototoxic shock (Zheng et al. 1999). Campbell et al. (2004) showed, using TaqMan®, the importance of matrix metalloproteinases in kainic acid–induced excitotoxicity in the rat brain.

55.2.4 Recent Innovations and Future Technologies

55.2.4.1 Small Noncoding RNAs in Toxicogenomics

PURPOSE AND RATIONALE

The evaluation of epigenetic factors, for example, DNA methylation, histone modifications, as well as the impact of micro RNAs (miRNAs) on mRNA regulation, has become very important in many fields of research. In the following chapter, the impact of small RNAs, with a focus on miRNA, in toxicogenomics is described. Assessing such factors has already been well established in cancer research (Munker and Calin 2011) and the importance also for toxicology has been recognized. Polymerase II promoters are often involved in the induction of toxicological effects and miRNAs are mainly transcribed by this important enzyme; thus, it has been stated that miRNAs play a crucial role in the development of certain toxicities (Taylor and Gant 2008). miRNA expression has been confirmed to be affected by extracellular signals, cellular stress, and xenobiotics (Lema and Cunningham 2010). Therefore, miRNA expression profiles could support the identification, classification, or prediction of adverse effects and serve as safety-specific biomarkers. Identifying effected miRNAs by a certain toxicant, and the posttranscriptional modification of the miRNA's target gene can be estimated which would be a crucial contribution to the clarification of a toxicity mechanism. It is also of great interest whether changes in miRNA expression affect an individual's susceptibility to xenobiotics.

miRNAs are small endogenous noncoding RNA molecules (~22 nt) with regulatory functions. The major effect of these molecules is binding to the 3′UTR region of particular mRNAs and posttranscriptionally inhibiting their translation into proteins. In the most recent release of the commonly used miRBase database (17; April 2011), 16,772 entries are recorded with 1,424 and 408 miRNA sequences for *Homo sapiens* and Rattus norvegicus, respectively (http://www.mirbase.org). In contrast to miRNAs, small interfering RNAs (siRNAs) are mainly exogenous molecules from viruses, transposons, or transgenes that achieve posttranscriptional silencing via mRNA degradation. These RNAs are used in the RNA interference (RNAi) technology to knock down genes of interest, used in gene function studies for analyzing the association of specific genes to a particular phenotype (Hannon 2002). Specifically, in toxicology, it can be studies whether the genes of interest correspond to certain toxicity.

PROCEDURE

miRNA expression can be analyzed with different technologies, including northern blotting, qPCR, microarrays, and next-generation sequencing. Since most technologies are described in other parts of this chapter, they will not be described in detail here.

Small RNAs are not extracted with common RNA extraction methods. Therefore, it has to be ensured that the extraction kit used does not lose the small RNA molecules. Products that provide small RNA enrichment (< 200 nt) in total RNA or separated small RNA extractions are available on the market. For example, the mirVana™ Kit from Ambion, Life Technologies, or the miRNeasy Mini Kit from Qiagen. Furthermore, phenol-based RNA extraction methods usually yield total RNA including small RNAs. The quality check for miRNAs can be performed using the Agilent Small RNA Assay on the 2100 Bioanalyzer.

For qPCR analysis, a special reverse transcription of miRNA is necessary. Regular oligo d(T) or random priming is not suitable due to the short nature of miRNA molecules which have no poly-A tail. Applied Biosystems describes a reverse transcription procedure where a specific stem-loop RT primer is annealed to the 3′ end of the mature miRNA (Protocol, Applied Biosystems). The primer is extended by reverse transcriptase producing a longer cDNA molecule containing the primer sequence and the first strand cDNA complementary to the mature miRNA sequence. The TaqMan® MicroRNA assay is based on the TaqMan® assay already described in Sect. 55.2.3. Here, a forward primer, as well as the TaqMan® probe, is designed specifically for the small RNA sequence, whereas the reverse primer is complementary to the RT primer sequence. Analysis of siRNA can also be performed by the same assay procedure using the TaqMan® siRNA Assay from Applied Biosystems.

EVALUATION

Quantitation of miRNA expression in the TaqMan® assay is performed based on the C_T values, similar to the evaluation described in Sect. 55.2.3. Comparable to transcriptomics data, statistically relevant differentially expressed miRNAs should be observed. Several miRNA databases are available online to obtain more information on the miRNAs of interest, for example, http://www.mirbase.org/, http://mirnamap.mbc.nctu.edu.tw/, or http://www.microRNA.org. It is often of great interest to additionally know the mRNA targets of the relevant miRNAs. Therefore, the MicroCosm Targets website is a valuable source of information (www.ebi.ac.uk/enright-srv/microcosm/).

CRITICAL ASSESSMENT

In molecular toxicology, it is often not sufficient to have information of the transcriptome. Proteins are the actual active molecules and mRNA expression alone does not mean that a protein is expressed. Due to the complexity of the proteome and the adjacent technologies, protein expression profiles are often not available. Having mRNA and the adjacent regulatory miRNA expression patterns would be a step toward protein translation which could help to postulate/predict the proteomic status of an organ/cell. An issue in interpretation of miRNA data is that many mRNAs are eligible to be targeted by one miRNA. Therefore, miRNA expression is suggested to be analyzed in conjunction with gene expression. This results in the need for additional experiments on different expression platforms. Due to the short length of miRNA primer, binding is often relatively instable and only one miRNA sequence-specific primer can be included. Others have also reported high false-positive and false-negative rates in miRNA microarray experiments (Choudhuri 2010).

MODIFICATIONS OF THE METHOD

Other qPCR technologies require a polyadenylation reaction before the reverse transcription with a universal primer. This cDNA consists of a miRNA complementary sequence at the 3′ end and a universal 5′ end. The qPCR is subsequently run with a miRNA-specific and a universal primer. The NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen) or miScript PCR System (Qiagen) are examples applying this polyadenylation reaction. Other providers use locked nucleic acid (LNA™) oligonucleotides as primers. LNA™ are chemically modified nucleic acids providing more stable binding to miRNA and therefore enabling higher melting temperatures (Exiqon).

References and Further Reading

Applied Biosystems Protocol (2011) TaqMan® Small RNA Assays. P/N: 4364031 Rev. E

Choudhuri S (2010) Small noncoding RNAs: biogenesis, function, and emerging significance in toxicology. J Biochem Mol Toxicol 24(3):195–216

Hannon GJ (2002) RNA interference. Nature 418(6894):244–251

Klaassen CD, Lu H, Cui JY (2011) Epigenetic regulation of drug processing genes. Toxicol Mech Methods 21(4):312–324

Lema C, Cunningham MJ (2010) MicroRNAs and their implications in toxicological research. Toxicol Lett 198(2):100–105

Munker R, Calin GA (2011) MicroRNA profiling in cancer. Clin Sci (Lond) 121(4):141–158

Nakajima M, Yokoi T (2011) MicroRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. Pharmacol Ther 131(3):330–337

Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpyleva SI, Kovalchuk O (2010) Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. Int J Cancer 127(8):1785–1794

Taylor EL, Gant TW (2008) Emerging fundamental roles for non-coding RNA species in toxicology. Toxicology 246(1):34–39

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10(1):57–63

EXAMPLES

Several Phase I enzymes and transporters, including Cyp2E1 and MDR1, were found to be posttranscriptionally regulated by miRNAs (Nakajima and Yokoi 2011; Klaassen et al. 2011). miRNAs also regulate many transcription factors involved in xenobiotic toxicity. Thus, miRNA expression is involved in the translation of PPARα, P53, PXR, and NRF2, to name only a few. A miRNA targeting MRP1 (and MRP1 itself) was found to be a key factor in the establishment of resistance to the cytostatic Cisplatin (Pogribny et al. 2010). Lema and Cunningham reviewed how several toxicants effect miRNA expression and their targets leading to a distinct adverse effect, for example, hepatocyte proliferation induced by PPARα agonists, Tamoxifen-induced hepatocarcinogenesis or acetaminophen-induced hepatotoxicity (Lema and Cunningham 2010). Choudhuri reported that neurotoxicity, developmental toxicity, hepatotoxicity, and carcinogenesis are all affected by miRNA expression (Choudhuri 2010). Furthermore, Wang and coworkers have suggested several miRNA biomarkers for drug-induced liver injury (Wang et al. 2009).

55.2.4.2 Next-Generation Sequencing

PURPOSE AND RATIONALE

The emergence of next-generation sequencing (NGS) technologies has revolutionized the field of genomic research. Steadily decreasing cost for analysis of a genome has enabled their expansion. This is leading the displacement of traditional microarray technologies from the market. NGS platforms are able to perform massively parallel sequencing, have a higher dynamic range, and are more sensitive compared to microarrays. So far these technologies have not yet been extensively applied in the field of toxicology, but this is sure to change in future. NGS is a further development from the shotgun sequencing technology which was already an improvement of the Sanger sequencing. Numerous genomic analyses are possible, including DNA and transcriptome sequencing for genotyping and copy number variation, linkage analysis, gene regulation, and epigenetic evaluations (e.g., ChIP-Seq, methylation, small RNA discovery and analysis, histone modification) (Mardis 2007).

PROCEDURE

The most widely used NGS system is the Genome Analyzer from Illumina. It is easiest to handle with superior data quality and accuracy (Zhang et al. 2011). The Illumina NGS systems are based on the TruSeq™ technologies (Data Sheet, Illumina). For library preparation, DNA is fragmented, a blunt end generated, and an adenosine is added to the 3′end. In case of transcriptome analysis, mRNA is reverse transcribed using random primers to generate double-stranded cDNA. Adapter oligos containing universal primer sites with a 5′ overhang are ligated to both ends and fragments are purified after size selection. The fragments are hybridized with their adapter onto a flow cell and bridges built for amplification. The generated cluster can now be sequenced by synthesis. Here all four bases with different labels are added simultaneously and the hybridized base is identified by fluorescence signal (Technology Spotlight, Illumina).

EVALUATION

After read-generation "base-calling" is performed. With this information, sequences can be aligned to a reference genome or assembly can be carried out for de novo sequences.

Proper alignment or assembly is critical and many open source bioinformatic tools are available online, for example, ELAND, MAQ, and BLAST. More information about these software tools are described in the review from Zhang and coworkers (Zhang et al. 2011). High professional information technology infrastructure is not available in every lab; thus, vendors of NGS technologies also provide so-called cloud computing end-user software for data processing (e.g., the CASAVA package from Illumina). Uniquely mapped reads are then used for further analyses. To observe differentially expressed genes, for example, all reads of a specific gene transcript are counted and compared between samples. Furthermore, normalization, transformation, and statistical analyses are carried out in a similar way to microarray data analysis.

CRITICAL ASSESSMENT

NGS technologies have the potential to be of great added value to genomic research. An enormous amount of data can be obtained in a relatively short period of time from limited samples. Additionally, these technologies have the potential to pave the way toward personalized medicine. In the case of toxicogenomics, by sequencing individual genomes, interindividual differences could be potentially

Table 55.2 A selection of currently available next-generation sequencing systems

Company	Template preparation	Sequence by	System	Read length	Max output
Illumina	Bridge amplification	Synthesis	HighSeq 2000	2x100b	600 Gb
			Genome Analyzer IIx	2x150b	95 Gb
			MySeq	2x150b	~1 Gb
Roche	Emulsion PCR	Pyro-sequencing	GS-FLX+	<1,000b	700 Mb
			GS Junior	400b	~35 Mb
Applied Biosystems	Emulsion PCR	Ligation	SOLiD 4	50b	100 Gb
			5,500xl Genetic Analyzer	75b	10–15 Gb
			5,500 Genetic Analyzer	75b	7–9 Gb

detected after exposure to a toxicant/new drug entity. However, many issues exist concerning the use of NGS. Especially the handling of the huge amount of data generated is extremely challenging, specifically concerning data transfer, storage, analysis, and interpretation (Metzker 2010). Due to the short read length, reads often cannot uniquely be aligned and therefore cannot be included in the analyses, repetitive sequences alignment is especially difficult. Improvement of software tools is thus essential to be able to properly adopt this technology. These technologies still need to be validated for data quality, reliability, reproducibility, and biological relevance. In 2008, a consortium has been founded coordinated by the FDA to deal with these challenges, namely, the MAQC-III project, called Sequencing Quality Control (SEQC) (www.fda.gov/MicroArrayQC).

Furthermore, sequencing is still relatively expensive—when compared to the costs of whole genome microarrays. This is an especially sensitive issue for toxicogenomics where usually large numbers of animals are need to be analyzed/sequenced; therefore, costs would be too high to use sequencing in routine studies. To reduce costs, it can be considered to sequence only preselected regions rather than analyzing the whole genome. As the technologies are continually and rapidly being improved, there is hope that in the near future they will become affordable soon.

MODIFICATIONS OF THE METHOD

Three major NGS technologies are currently available on the market. One is the technology from Illumina described above, with different sequencers available (HighSeq, HiScanSQ, Genome AnalyzerIIx and the MySeq) and the two others are from Roche and Applied Biosystems. The major differences of all technologies are in template preparation or in sequencing. Templates can be prepared by emulsion PCR or the amplification is performed on a solid phase. The 454 Sequencing Systems (Roche) are pyrosequencer and performs emulsion PCR for template preparation. Sequencing by ligation is required on the SOLiD platform (Applied Biosystems). Here the template is amplified on a solid phase. In addition, all platforms differ in the obtained number and length of reads (Table 55.2). All sequencing technologies are described and compared by Voelkerding et al. (2009) and Metzker (2010).

References and Further Reading

Ilumina Data Sheet (2011) TruSeq™ RNA and DNA Sample Preparation Kits v2. Pub.No. 970-2009-039

Illumina Technology Spotlight (2010) Illumina Sequencing Technology. Pub.No. 770-2007-002

Mardis ER (2010) The impact of next-generation sequencing technology on genetics. Trends Genet 24(3):133–141

Metzker ML (2010) Sequencing technologies – the next generation. Nat Rev Genet 11(1):31–46

Su Z, Li Z, Chen T, Li QZ, Fang H, Ding D, Ge W, Ning B, Hong H, Perkins RG, Tong W, Shi L (2011) Comparing next-generation sequencing and microarray technologies in a toxicological study of the effects of Aristolochic acid on rat kidneys. Chem Res Toxicol. doi: 10.1021/tx200103b

Voelkerding KV, Dames SA, Durtschi JD (2009) Next-generation sequencing: from basic research to diagnostics. Clin Chem 55(4):641–658

Woollard PM, Mehta NA, Vamathevan JJ, Van Horn S, Bonde BK, Dow DJ (2011) The application of next-generation sequencing technologies to drug discovery and development. Drug Discov Today 16(11–12):512–519

Zhang J, Chiodini R, Badr A, Zhang G (2011b) The impact of next-generation sequencing on genomics. J Genet Genomics 38(3):95–109

EXAMPLES

The first toxicogenomics study applying NGS was described by Su and coworker in 2011. They compared

the RNA-Seq data to data from an Affymetrix microarray analysis, which was based on a real-life toxicological study where rats were treated with aristolochic acid. Differentially expressed genes compared to control animal were assessed. NGS was observed to be more sensitive in identifying a great number of deregulated genes. Although they found only 40–50% common genes, gene expression patterns obtained from both technologies were similar, as was the biological outcome. For example, typical nephrotoxic and carcinogenicity GO processes were observed with both technologies (Su et al. 2011). NGS has been applied in several phases of drug discovery and development process, and has been extensively reviewed by Woolland et al. (2011). NGS can support target identification, but can also be used in drug resistance studies, biomarker discovery, and RNA-protein identification studies. For personalized medicine, the identification of interindividual variations is important for therapy which might improve drug efficacy and safety.

55.3 Application of Toxicoproteomics in Profiling Drug Effects

PURPOSE AND RATIONALE

Traditionally, toxicologists define the preliminary risk of a new compound to human safety using animal models supported by histopathological and biochemical approaches. However, despite decades of experience, the extrapolation dilemma and the relevance of animal data to real-life, long-term exposure in humans remained unclear. The genomics revolution of the recent years led to development of many new and innovative technologies that can change this paradigm and address uncertainty issues in the current toxicological practice and safety assessment through the identification of novel key genes, marker proteins, or protein profiles. Thus, these technologies provide a superior alternative to traditional rodent and canine bioassays to identify and accurately assess the safety of chemicals and drug candidates for human safety.

Toxicogenomics, the use of DNA microarray for comprehensive RNA expression analysis, has recently caused a great deal of interest (Pennie et al. 2000; Nuwaysir et al. 1999). This technology has been used to monitor changes in gene expression in response to drug treatment. However, analysis of the information produced by toxicogenomics has proven to be unsatisfactory (Anderson and Seilhamer 1997; Mann 1999; Srinivas et al. 2001). Fundamental studies have illustrated the usefulness and potential of the toxicoproteomics, the proteomic approach, to complement RNA microarray data. Proteomic technology helps identify corresponding changes in the level of protein, which is critical because the protein is the basic component of a cell. Additionally, toxicoproteomics helps resolve issues involving differential protein modifications. These are critical for the function of many proteins, in that they may lead to changes in the activity of gene products. Primarily, the manifestation of protein modifications is the reason for undesired, compound-related effects. Toxicoproteomics helps to determine such changes, and to gain insight into the mode of action of drugs (Kumagai et al. 2006; Gao et al. 2009). Furthermore, toxicoproteomics technologies can also be applied in identification, characterization, and evaluation of new prognostic and diagnostic biomarkers (Merrick 2006; Bjørnstad et al. 2006; Provan et al. 2006; Glückmann et al. 2007).

Toxicoproteomics studies, as they have been conducted so far, focused mainly on investigation of toxic effects of known toxicants and reference chemicals. These studies were of paramount importance to show the potential of proteomics technologies and their application in toxicological research. In recent publications, however, researchers report investigations with new chemical entities within pharmaceutical and chemical industry, and scientific institutions. These studies herald the next period of practicing toxicoproteomics with more focus on issue resolution or biomarker identification. To achieve this goal, the knowledge from proteomics approach needs to be combined with the information from classical disciplines such as toxicology and pathology.

PROCEDURE

55.3.1 Available Technology Platforms

The most common implementation of proteomic analysis involves protein separation 2-D gel electrophoresis (2DGE), quantification of proteins with analytical methods for their identification in mass

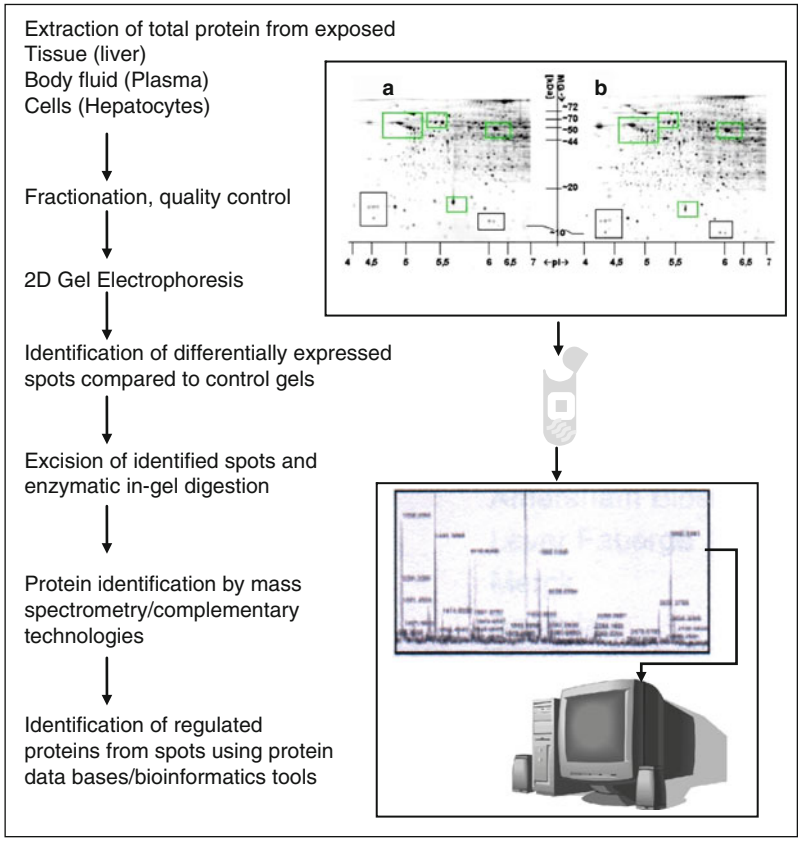

Fig. 55.4 A general flow chart of sequential steps for conducting standard proteomics studies

spectrometer (MS), and at the very least data integration and analysis using bioinformatic tools. Figure 55.4 represents the sequential steps for conducting standard proteomics studies.

55.3.1.1 Protein Extraction and Fractionation

The first step in the protein identification and characterization process by different proteomics technologies is the extraction of proteins from cell cultures, body fluids, or tissue samples. As total protein extracts harbor many abundant proteins such as immunoglobulins or albumin, the removal of abundant proteins could be considered for the purpose of increasing number of detectable and identifiable proteins (Merrick 2006).

The first step in the preparation of protein extracts is to mix samples (cell or tissue pieces) with a lysis buffer, for example, 20 mM Tris-HCL, pH 7.4, 1% CelLytic-M (Sigma). To avoid the enzymatic degradation of proteins by proteases, protease inhibitors are added. When organ samples are used for protein extraction, as soon as these constituents are added, tissue are homogenized using a Polytron type homogenizer. During the homogenization process, the tube is kept submersed in a water ice bath to maintain the sample at 4°C. After homogenization, samples are centrifuged to separate the solubilized proteins from cell debris and insoluble membrane components. The supernatant is aspirated and can be divided into aliquots. At the end, the total protein concentration can be determined using a BCA protein assay with BSA as the standard. Depending on the purpose and experimental design, a protein fractionation can be followed, or the protein extracts are analyzed without further processing. If the protein extracts are not used immediately, they can be frozen at < -70°C until tested.

55.3.1.2 2DGE

Initially, proteins in a sample are separated according to their isoelectric point in a pH gradient. Next, the proteins are separated according to size on a SDS-polyacrylamide gel. A dye marker such as coomassie blue, silver, or fluorescent dyes then detects the resolved proteins. In order to analyze differentially expressed protein spots in an experimental set of gels,

a computerized detection and matching system is required. Finally, MS identifies selected protein spots. The 2-D gel electrophoresis allows separation of around 3,000 proteins. Even though the 2DGE is an effective method for the separation and quantitation of proteins from different sources, it has some limitations. Working with 2DGE technique takes time and demands intensive skilled labor, and hence is not practical for high throughput. Other limitations like lack of reproducibility narrow the application of 2DGE. Liquid chromatography is an alternative and straightforward method with the advantage of direct connection to mass fingerprinting technologies (Link et al. 1999).

55.3.1.3 MS

Mass fingerprinting of excised and trypsin-digested gel spots is the method of choice to identify proteins. The masses of the tryptic fragments in a sample are accurately and quickly measured using a matrix assisted laser-desorption/ionization time-of-flight (MALDI-TOF) instrument. In this technique, purified or partially purified proteins are mixed with a crystal-forming matrix, placed on an inert metal target, and subjected to a pulsed laser beam to produce the phase ions that traverse a field-free flight tube. They are then separated according to their mass-dependent velocities. The mass peak list obtained is searched by means of in silico digest of sequence databases for comparison and identification of proteins.

EVALUATION

55.3.2 Performed Studies to Figure Out the Mechanism of Organ Toxicity by Proteome Analysis

The proteomic investigation has been applied to a series of compounds to examine the response of in vitro and in vivo models after exposure to toxicants. The main focus of these studies has been to understand the mechanism of their toxicity. In an attempt to develop a rodent liver proteomic toxicity database, Anderson and colleagues characterized the effect of a range of xenobiotics on protein expression in the liver (Anderson et al. 1996a). Using this database, it was possible to detect, classify, and characterize a broad range of hepatotoxins. Toxicoproteomics was the key tool used to gain new insights into the molecular mechanism involved in cyclosporine A (CsA) nephrotoxicity. The initial study reported by Steiner et al. (1996) investigated changes in the kidney protein pattern of CsA treated rats in order to determine the nephrotoxic mechanism of this drug. Using this proteomic approach, the investigators discovered an association between decreased calcium binding protein, calbindin-D 28 kDa, and CsA-mediated medullar nephrotoxicity (Aicher et al. 1998). Since these early studies, the potential of toxicoproteomics to identify protein changes associated with nephrotoxicity has

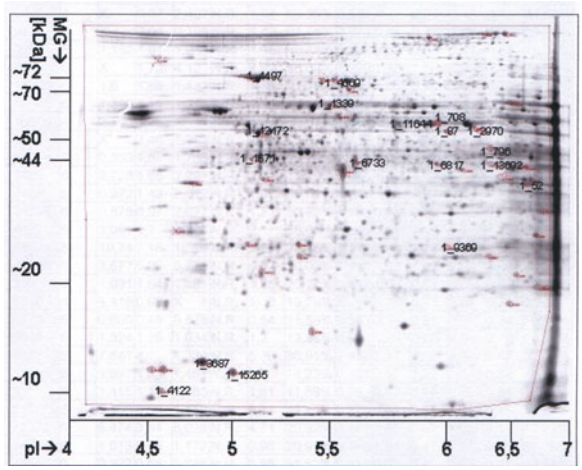

Fig. 55.5 Protein pattern of treated cells compared to control obtained after separation of 350 µg protein sample. Cells were exposed to 0.5 mM fenofibrate for 24 h. Spot IDs marks differentially regulated proteins

Table. 55.3 Identified proteins from rat primary hepatocytes involved in energy metabolism and electron transfer

Protein identification[a]	Molecular mass (kDa)[b]	pI[b]	Regulation
ATPase chain B, liver	51.35	4.95	Down
ATP synthase δ-chain	15.79	4.74	Down
NADH ubiquinone oxidoreductase	26.52	6	High
malate dehydrogenase	36.48	6.16	Down
NAD-specific isocitrate dehydrogenase	39.6	6.47	High
Enoyl-CoA hydratase	31.5	8.4	High
Aldehyde dehydrogenase	48.27	6.06	High
Guanidino acetate N-methyl transferase	26.4	5.7	High

[a]Peptide masses were identified in the positive ion reflector mode (MALDI-TOF) and protein identification was performed using the program MS-Fit
[b]Theoretical calculated pI and molecular masses

been shown in further investigative animal and clinical studies (Witzmann and Li 2004; Janech et al. 2007; Mischak et al. 2009; Klawitter et al. 2010). Data on H1 receptor antagonist, pyrilamine, and the non-genotoxic carcinogenic analogue, methapyrilene, showed differing proteomic profiles despite a similar chemical structure. Widespread changes in hepatic protein composition were observed for methapyrilene but not for pyrilamine (Cunningham et al. 1995). The proteomic approach further assisted numerous mechanistic investigations followed by explanation of regulatory changes implemented at the protein level. This included testing of pharmaceuticals for carcinogenic potential, hepatocellular hypertrophy, and peroxisome proliferation (Arce et al. 1998; Anderson et al. 1996b). The proteome profiling in these studies was partially used for lead prioritization, emphasizing the potential role for toxicoproteomics in lead candidate selection.

To investigate the significance of the 2-DE technology in determining changes in protein expression, Kabiri et al. (unpublished data) exposed rat primary hepatocytes to the peroxisome proliferator fenofibrate. Several important aspects of this study are discussed here. To investigate the proteome profile, we isolated and cultured primary hepatocytes in the presence of various concentrations of fenofibrate. To reduce the complexity, protein extracts were narrowed by prefractionation procedure and cytosolic fraction was collected for further analysis. After 2-DE separation, protein spots with an alteration in their abundance were excised and subjected to the MS. Figure 55.5 shows the 2-D pattern obtained after separation of 350 μg protein sample from treated cells as an example. When primary cells were treated with 0.5 mM fenofibrate over 24 h, a total of 30 protein spots were strongly affected. Some of these are summarized in Table 55.3. Many of the identified proteins are involved in cell proliferation, protein metabolism, and energy. In addition, proteins associated with defense reactions to cellular stress are expressed at high levels in response to fenofibrate exposure. These results are consistent with mRNA abundance as indicated in microarray experiments (data not shown). However, we detected expressed changes in protein, for which no changes at RNA level were measured. We have investigated the comparative effects of additional compounds on the rat primary hepatocytes and the results show the potential of toxicoproteomics to serve as a complementary technology to microarray-based approach.

CRITICAL ASSESSMENT OF THE METHOD

55.3.3 Alternative Proteomic Technologies and Options

Although 2DGE is unchallenged in its ability to resolve thousand of proteins, it has several limiting factors. Firstly, it is labor intensive, requires large quantities of proteins, and may not be suitable to serve as an effected diagnostic tool. Secondly, the application of 2DGE is limited because it fails to detect proteins at the extremes of separation either by size or by isoelectric point, and because it is insufficiently sensitive for low-abundant proteins (Moseley 2001). Therefore, additional innovative methods are needed to measure broad protein abundances and activity.

55.3.3.1 SELDI-TOF/Protein Biochips

SELDI-TOF is beginning to offer an alternative to 2DGE. Surface-enhanced laser-desorption/ionization (SELDI) is an affinity-based mass spectrometric method in which proteins of interest are selectively adsorbed to a chemically modified surface on a biochip (Weinbergera et al. 2002, Ciphergen Protein Chip® Arrays).

This system has already been used to identify markers of prostate cancer and changes in renal cell carcinoma. It also has been applied to the discovery of new toxicity markers (Grizzle et al. 2004; Jr et al. 1999; Paweletz et al. 2001; Vlahou et al. 2001). Taking advantage of the recent development in SELDI and the protein chip technology, it will be possible to simultaneously analyze protein profiles of body fluids such as serum and urine samples very rapidly. The SELDI mass spectrometry in conjunction with bioinformatics tools could greatly facilitate the discovery of new and improved toxicologic biomarkers (Collins et al. 2010).

Protein biochips (gel-based microarrays) represent a further development of proteomics technologies with the potential for screening of protein subsets. In this technology, proteins (mostly antibodies) are immobilized and trapped in gel-based matrices and thus are made accessible for target proteins. The combination with dye marker like fluorescence and chemiluminescence and detection of their signal intensities allows detection of protein modifications or deregulations. The gel-based protein chips can also be

combined with MALDI-TOF MS to achieve a specific analysis of protein subclasses (Rubina et al. 2003; Rubina et al. 2008).

55.3.3.2 ICAT

A recent and exciting development by Aebersold and colleagues is the isotope-codes affinity tag (ICAT) method, which can be used to label proteins before separation (Gygi et al. 1999). By using two different isotopes for labeling, it is possible to perform a binary comparison in a single step. After labeling, test and control samples are pooled and digested with proteases to produce peptide fragments. ICAT-labeled peptides are separated and analyzed by tandem MS. Due to the fact that the ICAT method is designed to combine labeling, separation, and the analysis of peptides into a single automated procedure, it is possible to scan several thousand peptide pairs a day.

55.3.4 Phosphoproteomics and Detection of Posttranslational Modifications

Proteomics is complicated by the fact that the absolute quantification does not always reflect the molecular function of proteins, because protein activities are highly regulated posttranslationally. Posttranslational modifications modulate the function of proteins and thus directly impact their capacity to participate in cellular regulatory events (Cravatt and Sorensen 2000). Due to posttranslational modifications, the numbers of proteins in human are estimated to be at least three times the amount of genes. Therefore, the elucidation of protein posttranslational modifications is the most important justification for biochemical and structural relationships. Hence, these modifications need to be evaluated.

However, establishing a proteomics platform initially requires implementation and combination of a series of systems to allow a flexible and reliable approach for analysis and identification of differences observed on 2-D gels. Proteomics in this sense is more interdisciplinary, combining aspects of biology, chemistry, toxicology and pharmacology, bioinformatic and information sciences. Use of bioinformatics is essential for analyzing the massive amount of data generated by proteomics.

The throughput of proteomics is currently much lower than that of RNA microarrays, largely due to the requirement of MS analysis, or similar technologies. However, the microarray-based approaches of protein detection may overcome this limitation.

While the combination of 2DGE with protein analytic techniques has been established for toxicoproteomics, the integration of bioinformatic and appropriate software is yet to be implemented.

For toxicoproteomics, the ideal proteomics platform would be one that is:

- Sensitive enough to detect high- and low-abundance proteins
- Easily implemented and performed quickly
- Able to detect modifications and alternative splice forms in addition to abundance
- Able to deliver sophisticated data for protein-protein networking

There are many obstacles to overcome in regard to current limitations of toxicoproteomic technologies. Thus, in the near future, proteomics will play an important role in the research toxicology.

MODIFICATIONS OF THE METHOD

55.3.5 Subcellular Proteomics

One-step characterization of a eukaryotic cell proteome is difficult if not impossible to achieve. There is a growing trend in eukaryotes proteomics toward characterization of subcellular and organellar structures. The reason for this shift from global proteomics to subcellular proteomics is the complexity of eukaryotic cells and subcellular organelles. Therefore, the proteomic analysis of subcellular organelles will be an important aspect of toxicoproteomics (Lee et al. 2010; Gatto et al. 2010).

References and Further Reading

Aicher L, Wahl D, Arce A, Grenet O, Steiner S (1998) New insights into cyclosporine A nephrotoxicity by proteome analysis. Electrophoresis 19(11):1998–2003

Anderson L, Seilhamer L (1997) A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 18:533–537

Anderson LN, Esquer-Blasco R, Richardson F, Foxworthy P, Eacho P (1996a) The effect of peroxisome proliferators on protein abundances in mouse liver. Toxicol Appl Pharmacol 137:75–89

Anderson NL, Taylor J, Hofmann JP, Esquer-Blasco R, Swift S, Anderson NG (1996b) Simultaneus measurement of hundreds of liver proteins: application in assessment of liver function. Toxicol Pathol 24(1):72–76

Arce A, Aicher L, Wahl D, Anderson NL, Meheus L, Raymackers J, Cordier A, Steiner S (1998) Changes in the liver protein pattern of female wistar rats treated with the hypoglycaemic agent SDZ PGU 693. Life Sci 63(25):2243–2250

Bjørnstad A, Larsen BK, Skadsheim A, Jones MB, Andersen OK (2006) The potential of ecotoxicoproteomics in environmental monitoring: biomarker profiling in mussel plasma using ProteinChip array technology. J Toxicol Environ Health A 69:77–96

Collins BC, Sposny A, McCarthy D, Brandenburg A, Woodbury R, Pennington SR, Gautier J-C, Hewitt Ph, Gallagher WM (2010) Use of SELDI MS to discover and identify potential biomarkers of toxicity in InnoMed PredTox: a multi-site, multi-compound study. Proteomics 10:1592–1608

Cravatt BF, Sorensen EJ (2000) Chemical strategy for the global analysis of protein function. Curr Opin Chem Biol 4:663–668

Cunningham ML, Pippin LL, Anderson NL, Wenk ML (1995) The hepatocarcinogen methapyrilene but not the analogue pyrilamine induces sustained hepatocellular replication and protein interactions in F344 rats in a 13 week feed study. Toxicol Appl Pharmacol 131:216–223

Gao Y, Holland RD, Yu L-R (2009) Quantitative proteomics for drug toxicity. Brief Funct Genomic Proteomic 8(2):158–166

Gatto L, Vizca´no AJ, Hermjakob H, Huber W, Lilley KS (2010) Organelle proteomics experimental designs and analysis. Proteomics 10:3957–3969

Glückmann M, Fella K, Waidelich D, Merkel D, Kruft V, Kramer PJ, Walter Y, Hellmann J, Karas M, Kröger M (2007) Prevalidation of potential protein biomarkers in toxicology using iTRAQ™ reagent technology. Proteomics 7:1564–1574

Grizzle WE, Adam BL, Bigbee WL, Conrads TP, Carroll C, Feng Z, Izbicka E, Jendoubi M, Johnsey D, Kagan J, Leach RJ, McCarthy DB, Semmes OJ, Srivastava S, Srivastava S, Thompson IM, Thornquist MD, Verma M, Zhang Z, Zou Z (2004) Serum protein expression profiling for cancer detection: validation of a SELDI-based approach for prostate cancer. Dis Markers 19(4–5):185–195

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999

Janech MG, Raymond JR, Arthur JM (2007) Proteomics in renal research. Am J Physiol Renal Physiol 292(2):F501–F512

Jr GW, Cazares LH, Leung SM, Nasim S, Adam BL, Yip TT, Schellhammer PF, Gong L, Vlahou A (1999) Proteinchip(R) surface enhanced laser desorption/ionization (SELDI) mass spectrometry: a novel protein biochip technology for detection of prostate cancer biomarkers in complex protein mixtures. Prostate Cancer Prostatic Dis 2(5/6):264–276

Klawitter J, Klawitter J, Kushner E, Jonscher K, Bendrick-Peart J, Leibfritz D, Christians U, Schmitz V (2010) Association of immunosuppressant-induced protein changes in the rat kidney with changes in urine metabolite patterns: a proteometabonomic study. J Proteome Res 9(2):865–875

Kumagai K, Ando Y, Kiyosawa N, Ito K, Kawai R, Yamoto T, Manabe S, Teranishi M (2006) Toxicoproteomic investigation of the molecular mechanisms of cycloheximide-induced hepatocellular apoptosis in rat liver. Toxicology 228:299–309

Lee YH, Tan HT, Chung MCM (2010) Subcellular fractionation methods and strategies for proteomics. Proteomics 10:3935–3956

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR (1999) Direct analysis of protein complexes using mass spectrometry. Nat Biotechnol 17:676–682

Mann M (1999) Quantitqative proteomics? Nat Biotechnol 17:954–955

Merrick BA (2006) Toxicoproteomics in liver injury and inflammation. Ann N Y Acad Sci 1076:707–717

Merrick BA (2008) The plasma proteome, adductome and idiosyncratic toxicity in toxicoproteomics research. Brief Funct Genomic Proteomic 7(1):35–49

Mischak H, Spandiari P, Sadrieh N, Hanig J (2009) Profiling of rat urinary proteomic patterns associated with drug-induced nephrotoxicity using CE coupled with MS as a potential model for detection of drug-induced adverse effects. Proteomics Clin Appl 3(9):1062–1071

Moseley MA (2001) Current trends in differential expression proteomics: isotopically coded tags. Trends Biotechnol 19: S40–S48

Nuwaysir EF, Bittner M, Trent J, Barrett JC, Afshari CA (1999) Microarrays and toxicology: the advent of toxicogenomics. Mol Carcinog 24:53–159

Paweletz CP, Trock B, Pennanen M, Tsangaris T, Magnant C, Liotta LA, Petricoin EF 3rd (2001) Proteomic patterns of nipple aspirate fluids obtained by SELDI-TOF: potential for new biomarkers to aid in the diagnosis of breast cancer. Dis Markers 17:301–307

Pennie WD, Tugwood JD, Oliver GJA, Kimber I (2000) The principles and practice of toxicogenomics. Toxicol Sci 54:277–283

Provan F, Bjørnstad A, Pampanin DM, Lyng E, Fontanillas R, Andersen OK, Koppe W, Bamber S (2006) Mass spectrometric profiling–a diagnostic tool in fish? Mar Environ Res 62(Suppl):S105–S108

Rubina AY, Dementieva EI, Stomakhin AA, Darii EL et al (2003) Hydrogel-based protein microchips: manufacturing, properties, and applications. Biotechniques 34:1008–1022

Rubina AY, Kolchinsky A, Makarov AA, Zasedatelev AS (2008) Why 3-D? Gel-based microarrays in proteomics. Proteomics 8:817–831

Srinivas PR, Srivastava S, Hanash S, Wright GL Jr (2001) Proteomics in early detection of cancer. Clin Chem 47:1901–1911

Steiner S, Aicher L, Raymacher J, Meheus L, Eyquer-Blasco R, Anderson NL, Cordier A (1996) Cyclosporine A decreases the protein level of the calcium-binding protein calbindin-D 28 kDa in rat kidney. Biochem Pharmacol 51(3):253–258

Vlahou A, Schellhammer PF, Mendrinos S, Patel K, Kondylis FI, Gong L, Nasim S, Wright GL Jr (2001) Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. Am J Pathol 158:1491–1502

Weinbergera SR, Boschettib E, Santambienb P, Brenacb V (2002) Surface-enhanced laser desorption–ionization retentate chromatography mass spectrometry (SELDI–RC–MS): a new

method for rapid development of process chromatography conditions. J Chromatogr B 782:307–316
Witzmann FA, Li J (2004) Proteomics and nephrotoxicity. Contrib Nephrol 141:104–123

55.4 Metabonomics

Metabonomics, or "metabolomics" or "metabolic profiling" as it is often mentioned in the literature, is closely related to the other "-omics" technologies toxicogenomics and toxicoproteomics, linking genotype to phenotype. This is also reflected by the most cited definition of metabonomics that was published in 1999 as "the quantitative measurement of the dynamic multiparametric response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al. 1999). Therefore, metabonomics means the investigation of endogenous metabolites in biofluids or tissues and the changes on this system caused by different factors such as drug treatment, environmental influences, nutrition, lifestyle, genetic effects, or diseases. Metabonomics has become increasingly popular in drug development, molecular medicine, and other biotechnology fields, since it profiles directly the phenotype and changes therefore in contrast to other "-omics" technologies (Dieterle et al. 2011). It is also often described as the analysis of the "metabolic profile" or "metabolome," which is defined as all small, nonprotein metabolites with molecular weight not more than 1,500–2,000 Da, estimated up to several thousand different molecules. Therefore, analytical technologies for metabonomics are faced with the challenge to analyze all these chemically diverse high and low molecular weight molecules in a wide variety of different concentrations simultaneously. There are various technologies capable to analyze these metabonomics samples, including nuclear magnetic resonance Spectroscopy (NMR) and mass spectrometry coupled with liquid chromatography (LC-MS) or gas chromatography (GC-MS), which will be described later in this chapter. Metabonomics investigations can be performed by the principal approaches of global or targeted metabonomics analysis. In global metabonomics analysis, all the changes on the metabolic profile, measured by the different analytical technologies, are analyzed by statistical methods followed by an identification of the changed analytical signal with the help of databases with known endogenous metabolites. In targeted metabonomics analysis, the changes of a predefined subset of known endogenous metabolites of interest are quantified in the analyzed samples for which no statistical analysis and good databases are needed which is often crucial in metabonomics analysis.

References and Further Reading

Dieterle F, Riefke B, Schlotterbeck G, Ross A, Senn H, Amberg A (2011b) NMR and MS methods for metabonomics. Methods Mol Biol 691:385–415
Lindon JC, Holmes E, Nicholson JK (2003) So what's the deal with metabonomics? Anal Chem 75:384A–391A
Lindon JC, Holmes E, Nicholson JK (2004) Metabonomics and its role in drug development and disease diagnosis. Expert Rev Mol Diagn 4:189–199
Nicholson JK, Lindon JC, Holmes E (1999) "Metabonomics": understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29:1181–1189
Nicholson JK, Wilson ID (2003) Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov 2:668–676
Shockcor JP, Holmes E (2002a) Metabonomic applications in toxicity screening and disease diagnosis. Curr Top Med Chem 2:35–51
Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TWM, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane AN, Lindon JC, Marriott P, Nicholls AW, Reily MD, Thaden JJ, Viant MR (2007) Proposed minimum reporting standards for chemical analysis. Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 3:211–221

55.4.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

PURPOSE AND RATIONALE

The principle of nuclear magnetic resonance (NMR) analysis is described in many publications (Ernst et al. 1990; Goldman 1991). For the acquisition of a NMR spectrum, a liquid sample is placed in a static magnetic field. After irradiation with high-frequency pulses (pulse-sequences), the response of the NMR sample is detected by an induced current. The highest field strength available today in NMR spectrometers is 21 T corresponding to 900 MHz ^{1}H (proton) frequency. Most applications in metabolic profiling are using 600 MHz (14.1 T) instruments.

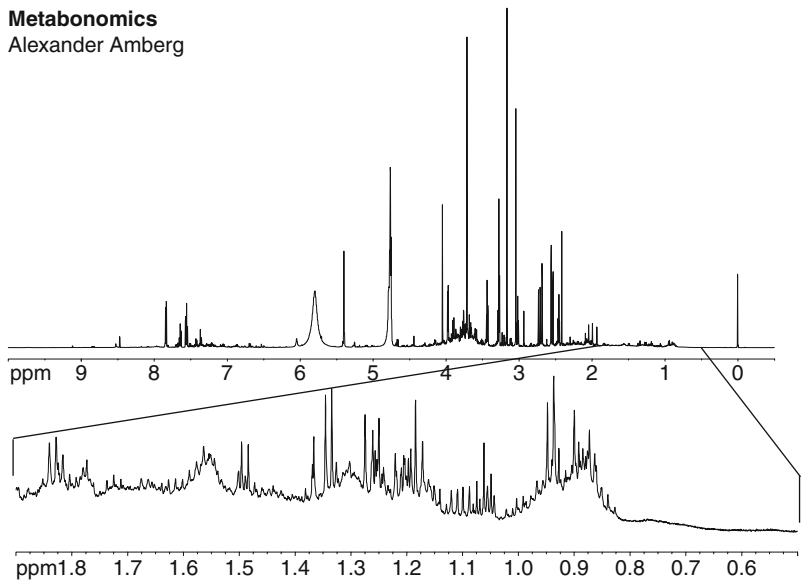

Fig. 55.6 Typical 600 MHz ^{1}H-NMR spectrum of rat urine after dilution with 10% D_2O buffer

The amplitude response of a NMR spectrometer is perfectly linear to the concentration of the sample, which allows easy quantification of compound concentrations for metabolic profiling in the µM to mM range. All steps involved in the acquisition and processing of NMR data, including preparation and exchange of samples, can be performed fully automated for hundreds of samples without the need for manual interaction (Dieterle et al. 2011).

PROCEDURE

With NMR it is possible to analyze all different kinds of liquid samples, including the noninvasively biofluids urine and blood (plasma or serum) but also many other biofluids like seminal fluid, amniotic fluids, cerebrospinal fluid, synovial fluid, etc. Organs, tissue, or cells can be analyzed after extraction of the endogenous metabolites using standard extraction methods (Lindon et al. 2006).

NMR analysis of biofluids needs no sophisticated sample preparation, details for the different biofluids are described by Lindon et al. 2000. In most cases, adding H_2O/D_2O (90:10) or buffer to account for pH variation or to reduce viscosity is sufficient as sample preparation before the NMR measurement (Keun et al. 2002). This means that one potential source of variance due to sample extraction procedures is absent (Dieterle et al. 2011).

The most used analytical technology in NMR metabonomics is ^{1}H proton spectroscopy of biofluids. For the analysis of these samples, water suppression techniques have to be applied, since endogenous metabolites can be present in low µM concentrations in an aqueous environment. Different water suppression techniques are available and described by Prince (1999). Additionally, blood serum/plasma samples or tissue extracts have high concentrations of macromolecules like proteins and lipoproteins, this means the NMR signals of these macromolecules have to be removed in the spectra by employing diffusion editing methods to avoid overlapping with NMR signals from the other endogenous metabolites (Dieterle et al. 2011).

After recording of the NMR raw data, the NMR signals (FID) are processed by application of the Fourier transformation (FT) followed by a phase correction and baseline correction to obtain the final NMR spectrum. A typical example of a 600 MHz ^{1}H spectrum of rat urine is shown in Fig. 55.6.

EVALUATION

The next step applied to the NMR spectra is the exclusion of spectral regions without interest in metabonomics. This includes the exclusion of the water region between 4.6 and 5.0 ppm, as this region does not contain any information due to the water suppression. In urine samples also, the urea is excluded because the amplitude of the strong urea signal is falsified due to proton exchange with water.

Small changes in the chemical shift of NMR signals can be caused by variations of the pH, salt

concentrations, overall dilution, etc. To reduce this effect for data analysis and interpretation, different mathematical methods can be applied. Normally the equidistant binning method is used for this, which is the integration of the signals in small spectral regions called "bins" or "buckets" of 0.04 ppm, for example (Dieterle et al. 2011).

To better identify the changes of a NMR signal in samples with varying concentration like urine, normalization methods of the quantified concentration have to be performed. Mostly, creatinine normalization is used for which each peak is divided by the creatinine signals at 3.05 and 4.05 ppm. The assumption behind this method is that the excretion of creatinine into urine is constant over time. Additionally, integral normalization is used very often for which each peak is divided by the total integral of all peaks. Alternatively also quotient normalization can be used for which each peak is divided by the corresponding peak of a reference spectrum (Dieterle et al. 2011).

The last and most important, but also often very time intensive step then is the assignment of the changed NMR signals of interest. This can be performed by reference spectra, many reference spectra are also available in different public and commercial databases (Dieterle et al. 2011).

CRITICAL ASSESSMENT OF THE METHOD

The inter-laboratory comparability of NMR data was tested for a set of samples shipped to different laboratories. Data were acquired with NMR spectrometers operated at different field strengths. Compared to any other analytical technology, NMR shows an impressive analytical reproducibility and repeatability reflecting itself in a coefficient of variation of 2% for a study invoking a large set of spectra (Goldman 1991). Thus, the observed variances in NMR spectra of a biological study are highly dominated by biological effects (Dieterle et al. 2011). This is also supported by other authors who see the main advantages of NMR metabonomics in the nonselectivity, lack of sample bias, and cross-laboratory/cross-platform reproducibility and in the good reference databases that are available for NMR. Some of the limitations are the lower sensitivity compared to mass spectrometry and the issue with the resolving of many different metabolites in the same region of a NMR spectrum (Keun et al. 2002; Robertson 2005; Lindon et al. 2006).

MODIFICATION OF THE METHOD

With the use of two-dimensional NMR techniques, more structural information can be extracted from the NMR spectrum, which can be used especially for the elucidation of an unknown structure of a newly found biomarker in a biological sample. But the use of 2D NMR in metabonomics is limited to small sample arrays as the measuring time is up to several hours per sample (Dieterle et al. 2011).

Intact tissue samples can directly be analyzed only by magic angle spinning (MAS) NMR without sample extraction. For this a small sample of intact tissue is placed into the spectrometer and is analyzed directly. But this technology requires specialized equipment and expertise for the conduction and is therefore used less often like the other technologies (Robertson 2005).

References and Further Reading

Bollard ME, Stanley EG, Lindon JC, Nicholson JK, Holmes E (2007) NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. NMR Biomed 18:143–162

Connor SC, Wu W, Sweatman BC, Manini J, Haselden JN, Crowther DJ, Waterfield CJ (2004) Effects of feeding and body weight loss on the 1H-NMR-based urine metabolic profiles of male Wistar Han rats: implications for biomarker discovery. Biomarkers 9:156–179

Dieterle F, Riefke B, Schlotterbeck G, Ross A, Senn H, Amberg A (2011c) NMR and MS methods for metabonomics. Methods Mol Biol 691:385–415

Ernst RR, Bodenhausen G, Wokaun A (1990) Principles of nuclear magnetic resonance in one and two dimensions. Oxford University Press, Oxford

Goldman M (1991) Quantum description of high-resolution NMR in liquids. Oxford University Press, Oxford

Keun HC, Ebbels TM, Antti H, Bollard ME, Beckonert O, Schlotterbeck G, Senn H, Niederhauser U, Holmes E, Lindon JC, Nicholson JK (2002) Analytical reproducibility in (1)H NMR-based metabonomic urinalysis. Chem Res Toxicol 15:1380–1386

Lenz EM, Wilson ID (2007) Analytical strategies in metabonomics. J Proteome Res 6:443–458

Lindon JC, Nicholson JK, Holmes E, Everett JR (2000) Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. Concept Magn Reson 12:289–320

Lindon JC, Holmes E, Nicholson JK (2006b) Metabonomics techniques and applications to pharmaceutical research & development. Pharm Res 23:1075–1088

Nicholson JK, Connelly J, Lindon JC, Holmes E (2002) Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 1:153–161

Prince WS (1999) Water signal suppression in NMR spectroscopy. Annu Rep NMR Spectrosc 38:289–354
Robertson DG (2005b) Metabonomics in toxicology: a review. Toxicol Sci 85:809–822
Shockcor JP, Holmes E (2002b) Metabonomic applications in toxicity screening and disease diagnosis. Curr Top Med Chem 2:35–51

55.4.2 Liquid Chromatography: Mass Spectrometry (LC-MS)

PURPOSE AND RATIONALE

Other metabonomics technologies which are capable to analyze a complex mixture of molecules simultaneously include the separation of the different compounds by chromatographic methods, like liquid chromatography (LC) in combination with mass spectrometry (MS) used as detector. In LC analysis, liquid samples flow with a mobile phase at high pressure through a column that is packed with a stationary phase of different particles. In most metabonomics applications, a reversed-phase LC is used. The separation here is based on adsorption of the compounds on a nonpolar stationary phase followed by elution of the compounds as a result of the hydrophobic interaction with the mobile phase by using a gradient from a polar to nonpolar solvent. As a consequence compounds are eluted over time from the column based on their polarity with different retention times. These analyses are known as HPLC (high performance liquid chromatography) or UPLC (ultra performance liquid chromatography), which are using higher pressures that results in better separations. The opposite principle using a polar stationary phase and a gradient from a nonpolar to polar solvent is called normal phase LC. These approaches are known as HILIC (hydrophilic interaction liquid chromatography).

After LC separation, the compounds are analyzed and quantified by mass spectrometry (MS). For this the solvent that is coming from the column is vaporized, afterward the remaining compounds are ionized, and the charged molecules are accelerated and separated in electromagnetic fields according to their mass-to-charge (m/z) ratio. In most metabonomics investigations, electrospray ionization mass spectrometry (ESI-MS) is used as ionization method in negative mode (ESI−) or positive mode (ESI+).

PROCEDURE

The samples which can be used for metabonomics LC-MS analysis are the same already described for NMR analysis. The analysis of the noninvasively biofluid urine can be challenging due to the high salt content, the complex composition, and the varying dilution. To overcome these problems, urine samples can be desalted before LC-MS analysis by solid phase extraction (Wagner et al. 2007) or column switching procedures (Waybright et al. 2006).

For the analysis of blood plasma or serum, it is of key importance to selectively remove proteins before analysis without affecting the low molecular weight metabolome. This is mandatory to reduce signal suppression of low-abundance compounds and to avoid protein precipitation under reversed-phase (RP) liquid chromatography conditions. Several procedures for deproteinization exist, such as extraction of low molecular weight compound by organic solvents, acids, or denaturation of proteins by heat (Want et al. 2006; Boernsen et al. 2005; Trygg et al. 2005). With regard to reproducibility, number of metabolic features detected, and robustness, extraction by methanol followed by evaporation and resuspension in the mobile LC phase proved to be the best method (Dieterle et al. 2011).

The most common method for metabonomics analysis is a reversed-phase LC (e.g., LC column packed with C_{18} particles) by using a water/acetonitrile (both with 0.1% formic acid) gradient as mobile phase (e.g., starting from 5% to 90% acetonitrile). As mass analyzers, mainly quadrupole- and time-of-flight (TOF)-based analyzers and hybrid forms of them are used (Dunn and Ellis 2005; Ackermann et al. 2006). Quadrupoles are robust, flexible, have a high linear dynamic range, but are limited in full-scan data acquisition due to long duty cycles. TOF instruments on the other hand have fast scanning capabilities, wide mass range, and high resolution. Quadrupole TOF (Q-TOF) hybrid instruments combine the stability and robustness of the quadrupole analyzer with TOF features and allow for MS-MS experiments (Dieterle et al. 2011).

Nontargeted metabolite profiling approaches require a sensitive full-scan mode and exact masses. Therefore, Q-TOF instruments or linear ion trap FT-MS instruments are advantageous. In contrast, for targeted analysis of selected metabolites, triple quadrupole instruments and Q TRAP instruments with their capability for multiple reaction monitoring are frequently used (Dieterle et al. 2011).

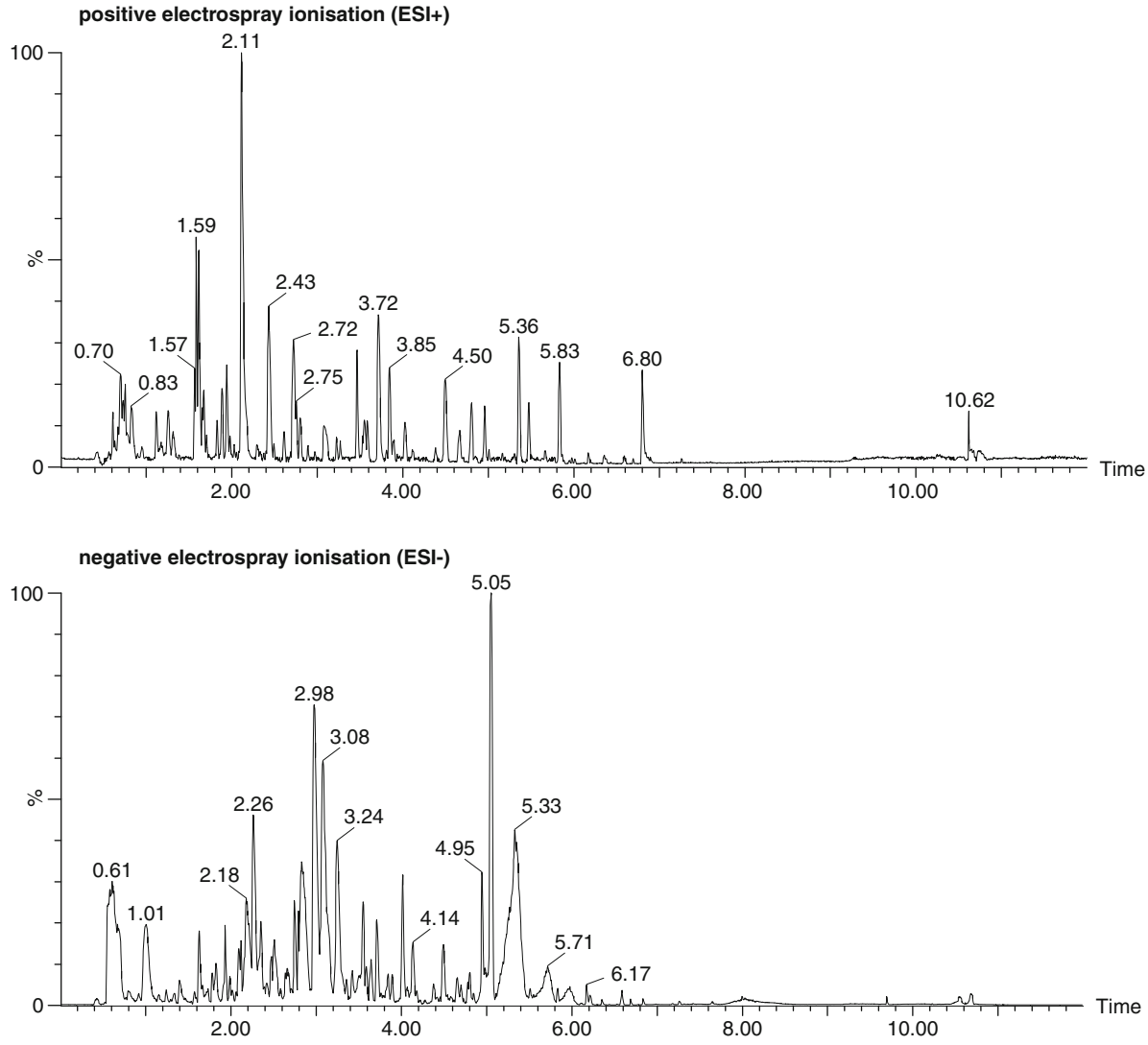

Fig. 55.7 Typical UPLC-TOF-MS chromatogram of rat urine with positive electrospray ionization (ESI+) and negative electrospray ionization (ESI−) after dilution of urine with water (1:3)

A typical example of an UPLC-TOF-MS chromatogram of rat urine is shown in Fig. 55.7.

EVALUATION

Full-scan LC-MS chromatograms contain many data (full mass spectra at the different retention times) from all the analyzed compounds in complex samples like urine, serum, or plasma. Therefore, an extraction of the relevant information (most expressed as m/z to retention time pairs) and de-noising from full-scan metabolic profiles is an important step in processing and statistical analysis of such LC-MS data. But a prerequisite for statistical analysis is to apply data alignment procedures and to reduce variance between samples that is not attributed to true differences. The major sources of such variances are nonlinear shifts in retention time, peak overlap, and m/z shifts. In general, three preprocessing strategies are used for LC-MS data sets. First, the spectra are aligned along the chromatographic and spectral axis. Then the dimensionality is reduced by binning or bucketing procedures. With the last step, significant peaks are automatically detected and quantified. Several commercial and open source routines for automatic

alignment, de-noising, deconvolution, and extraction of peak have been published and are reviewed by Katajamaa et al. (2007).

For samples with varying concentrations like urine, analogue normalization methods which are already described for NMR metabonomics analysis can be used.

Also in LC-MS metabonomics analysis, the last and most important step is the assignment of the changed LC-MS signals (m/z to retention time pairs) of interest with reference LC-MS spectra/chromatograms, which are also available in different public and commercial databases (Weckwerth and Morgenthal 2005; Dieterle et al. 2011).

CRITICAL ASSESSMENT OF THE METHOD

The main advantage of LC-MS metabonomics analysis is the better sensitivity with lower detection limits compared to NMR. This is very important in metabonomics analysis especially if novel biomarkers want to be identified. Other advantages are better resolutions by chromatographic separation of complex samples mainly for analysis of higher molecular compounds. As already described, some limitations of LC-MS metabonomics analysis are the potential for sample bias and the lower cross-laboratory/cross-platform reproducibility compared to NMR (Robertson 2005). But in summary, NMR and LC-MS approaches are highly complementary and use of both is often necessary for molecular characterization (Lindon et al. 2006).

MODIFICATION OF THE METHOD

HILIC (hydrophilic interaction liquid chromatography) as an alternative approach to reversed-phase LC was recently applied in metabonomics studies as a complementary tool to study polar metabolites (Idborg et al. 2005). However, until now, HILIC has not reached the level of reliability, stability, and reproducibility of HPLC or UPLC methods (Dieterle et al. 2011).

Also alternative mass spectrometer like ion traps, Fourier transform and Orbitrap instruments can be used in metabonomics analysis. Benefits of ion trap instruments are their capability to perform progressive fragmentation steps (MS^n), compact size, and fast full scanning but with low resolution. Linear ion traps quadrupole hybrid instruments (Q TRAP or QqLIT) combine the MS^n capabilities of ion trap instruments with the neutral loss and precursor ion scan capabilities of triple quadrupole instruments. Therefore, the shortcomings of both approaches are overcome.

References and Further Reading

Ackermann BL, Hale JE, Duffin KL (2006) The role of mass spectrometry in biomarker discovery and measurement. Curr Drug Metab 7:525–539

Boernsen KO, Gatzek S, Imbert G (2005) Controlled protein precipitation in combination with chip-based nanospray infusion mass spectrometry. an approach for metabolomics profiling of plasma. Anal Chem 77:7255–7264

Dieterle F, Riefke B, Schlotterbeck G, Ross A, Senn H, Amberg A (2011a) NMR and MS methods for metabonomics. Methods Mol Biol 691:385–415

Dunn W, Ellis DI (2005) Metabolomics: current analytical platforms and methodologies. Trends Anal Chem 4:285–294

Idborg H, Zamani L, Edlund PO, Schuppe-Koistinen I, Jacobsson SP (2005) Metabolic fingerprinting of rat urine by LC/MS Part 1. Analysis by hydrophilic interaction liquid chromatography–electrospray ionization mass spectrometry. J Chromatogr B 828:9–13

Katajamaa M, Oresic M (2007) Data processing for mass spectrometry-based metabolomics. J Chromatogr A 1158:318–328

Lindon JC, Holmes E, Nicholson JK (2006a) Metabonomics techniques and applications to pharmaceutical research & development. Pharm Res 23:1075–1088

Robertson DG (2005a) Metabonomics in toxicology: a review. Toxicol Sci 85:809–822

Trygg J, Gullberg J, Johansson AI, Jonsson P, Antti H, Marklund SL, Moritz T (2005) Extraction and GC/MS analysis of the human blood plasma metabolome. Anal Chem 77:8086–8094

Wagner S, Scholz K, Sieber M, Kellert M, Voelkel W (2007) Tools in metabolomics: an integrated validation approach for LC-MS metabolic profiling of mercapturic acid in human urine. Anal Chem 79:2918–2926

Want EJ, O'Maille G, Smith CA, Brandon TR, Uritboonthai W, Qin C, Trauger SA, Siuzdak G (2006) Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass spectrometry. Anal Chem 78:743–752

Want EJ, Coen M, Masson P, Keun HC, Pearce JT, Reily MD, Robertson DG, Rohde CM, Holmes E, Lindon JC, Plumb RS, Nicholson JK (2010) Ultra performance liquid chromatography-mass spectrometry profiling of bile acid metabolites in biofluids: application to experimental toxicology studies. Anal Chem 82:5282–5289

Waybright TJ, Van QN, Muschik GM, Conrads TP, Veenstra TD, Issaq HJ (2006) LC-MS in metabonomics: optimization of experimental conditions for the analysis of metabolites in human urine. J Liq Chromatogr Relat Technol 29:2475–2497

Weckwerth W, Morgenthal K (2005a) Metabolomics: from pattern recognition to biological interpretation. Drug Discov Today 10:1551–1558

Wilson ID, Plumb R, Granger J, Major H, Williams R, Lenz EM (2005) HPLC-MS-based methods for the study of metabolomics. J Chromatogr B 817:67–76

55.4.3 Gas Chromatography: Mass Spectrometry (GC-MS)

PURPOSE AND RATIONALE

An alternative chromatographic method which can be applied for metabonomics analysis is the use of gas chromatography (GC) in combination with mass spectrometry (MS) as detector. In GC analysis, gaseous compounds from the samples are analyzed or compounds which are vaporized before analysis. The compounds to be analyzed flow with an inert carrier gas, such as helium or nitrogen, as mobile phase through a column. The stationary phase in the column is a microscopic layer of a liquid film of a polymer coated on the surface of the column, normally a nonpolar but sometimes also a more polar stationary phase. The separation of a complex mixture is based on the interaction of the compounds from the sample between the liquid stationary phase and the gaseous mobile phase by using a temperature gradient of the gas and column which is located in an oven. As a consequence compounds are eluted over time from the column with different retention times which is based primarily on their boiling point or vapor pressure but also on the interaction with the stationary phase.

After GC separation, the compounds are analyzed and quantified by mass spectrometry (MS). This is very similar to the previously described LC-MS analysis, including ionization of the compounds and acceleration and separation in vacuum of the charged molecules in electromagnetic fields according to their mass-to-charge (m/z) ratio. As the ionization method in GC-MS metabonomics investigations, typically electron ionization (EI) is used or chemical ionization (CI) as a softer alternative method with the help of a reagent gas like methane.

PROCEDURE

The samples which can be used for metabonomics GC-MS analysis are the same already described for NMR and LC-MS analysis. But since these are primarily very polar samples and the prerequisite for GC-MS is the analysis of volatile compounds, sample preparations methods have to be performed before analysis. This includes normally an extraction procedure that maximizes the number and amounts of the endogenous metabolites combined with a derivatization that converts polar compounds (e.g., sugars, amino acids, organic acids, etc.) into volatile compounds (Dieterle et al. 2011). Most extraction procedures are based on methods from Bligh and Dyer's (Bligh and Dyer 1959; Peña-Alvarez et al. 2004) with little variations and optimization combined with 2-stage derivatization methods (Gullberg et al. 2004; Schröder et al. 2003). In the first step, a methoxymation converts aldehyde and keto groups into oximes using hydroxylamines or alkoxyamines to reduce the number of tautomeric forms (due to the limited rotation along the C = N bond). The second step of silylation then derivatizes polar functional groups (e.g., −OH, −SH, −NH) into trimethylsilyl groups (TMS ethers, TMS sulfides, TMS amines) resulting in more volatile compounds (Dieterle et al. 2011).

After this sample preparation, GC analysis is normally performed with a nonpolar column (e.g., DB5-MS with 0.18 μm film column) by using a temperature gradient typically from 70°C to 320°C with an increase of 15°C/min and a flow of 1 ml/min of the carrier gas helium. The mass analyzers in GC-MS are analogous to the previously described LC-MS mass analyzers, this means mainly quadrupole- and time-of-flight (TOF)-based analyzers are used.

As ionization methods in GC-MS, an EI or CI can be used depending on the results that are favored. With EI ionization, the molecules break down into different fragments that give some structural information of the molecules. On the other hand, CI ionization is a less energetic process and often results in less fragmentations and the formation of the molecular ion species to access the mass of the molecules (Dieterle et al. 2011).

A typical example of a GC-TOF-MS chromatogram of rat urine is shown in Fig. 55.8.

EVALUATION

Full-scan GC-MS chromatograms have the same format and are in principle the same like LC-MS chromatograms, they differ only in the chromatographic method that was used. Therefore, the same processing, normalization, and assignment methods previously described for the evaluation of LC-MS chromatograms are valid and are used for GC-MS chromatograms.

For GC-MS, reference spectra/chromatograms are also available in different public and commercial databases (Dieterle et al. 2011).

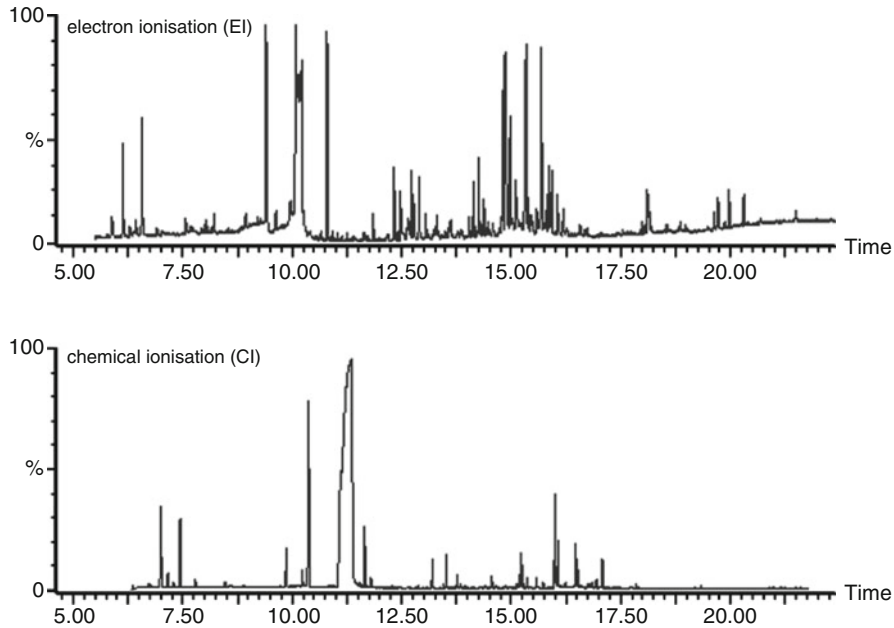

Fig. 55.8 Typical GC-TOF-MS chromatogram of rat urine with electron ionization (EI) and chemical ionization (CI) after extraction and silylation derivatization of the urine

CRITICAL ASSESSMENT OF THE METHOD

All advantages and limitations which were already described for the LC-MS analysis are also valid for metabonomics analysis with GC-MS compared to NMR. Further advantages are that GC-MS analyses show no problems with ion suppression of co-eluting compounds as observed in LC-MS analysis. Also, the assignment of the identity of peaks via a database of mass spectra is straightforward, due to the extensive and reproducible fragmentation patterns obtained in full-scan mode. Further limitations of GC-MS analyses exist for thermally labile compounds at the temperatures required for their separation or for compounds that are not volatile at all. Also since most endogenous metabolites in metabonomics analysis contain polar functional groups and are therefore less volatile, additional sample preparation steps of derivatization prior to GC-MS analysis are needed in most analysis to extend the application range of GC-based methods (Koek et al. 2011). But in summary, also GC-MS approaches are highly complementary to NMR and LC-MS approaches and the use of all the different technologies is often necessary for molecular characterization (Lindon et al. 2006).

MODIFICATION OF THE METHOD

Besides normal GC-MS also two-dimensional GC×GC-MS techniques can be used to increase the resolution of peaks in complex mixtures (Shellie et al. 2005; Van Mispelaar et al. 2003). Many different columns are available for GC analysis with different stationary phases, common GC columns are packed, for example, with different polysiloxane, polyethylene glycol polymers, resulting in columns with different polarities and other different characteristics.

Modifications in the mass analyzers which were already described for LC-MS analysis are also valid for metabonomics analysis with GC-MS (Weckwerth and Morgenthal 2005; Dieterle et al. 2011).

References and Further Reading

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Gullberg J, Jonsson P, Nordström A, Sjöström M, Moritz T (2004) Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomics studies with gas chromatography/mass spectrometry. Anal Biochem 331:283–295

Koek MM, Jellema RH, van der Greef J, Tas AC, Hankemeier T (2011) Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives. Metabolomics 7:307–328

Peña-Alvarez A, Díaz L, Medina A, Labastida C, Capella S, Vera LE (2004) Characterization of three Agave species by gas chromatography and solid-phase microextraction-gas chromatography-mass. J Chromatogr A 1027:131–136

Schröder NW, Schombel U, Heine H, Göbel UB, Zähringer U, Schumann RR (2003) Acylated cholesteryl galactoside as a novel immunogenic motif in Borrelia burgdorferi sensu stricto. J Biol Chem 278:33645–33653

Shellie RA, Welthagen W, Zrostliková J, Spranger J, Ristow M, Fiehn O, Zimmermann R (2005) Statistical methods for comparing comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry results: metabolomic analysis of mouse tissue extracts. J Chromatogr A 1086:83–90

Van Mispelaar VG, Tas AC, Smilde AK, Schoenmakers PJ, van Asten AC (2003) Quantitative analysis of target components by comprehensive two-dimensional gas chromatography. J Chromatogr A 1019:15–29J Chromatogr. A 1019: 15-29

Weckwerth W, Morgenthal K (2005b) Metabolomics: from pattern recognition to biological interpretation. Drug Discov Today 10:1551–1558

References and Further Reading

Ackermann BL, Hale JE, Duffin KL (2006) The role of mass spectrometry in biomarker discovery and measurement. Curr Drug Metab 7:525–539

Affymetrix Technical Note (2002) Manufacturing quality control and validation of gene chip arrays. Part number 701309 Rev. 3

Affymetrix User Manual (2008–2010) GeneChip® 3' IVT Express Kit. P/N:702646 Rev. 8

Aicher L, Wahl D, Arce A, Grenet O, Steiner S (1998) New insights into cyclosporine A nephrotoxicity by proteome analysis. Electrophoresis 19(11):1998–2003

Anderson L, Seilhamer L (1997) A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 18:533–537

Anderson LN, Esquer-Blasco R, Richardson F, Foxworthy P, Eacho P (1996a) The effect of peroxisome proliferators on protein abundances in mouse liver. Toxicol Appl Pharmacol 137:75–89

Anderson NL, Taylor J, Hofmann JP, Esquer-Blasco R, Swift S, Anderson NG (1996b) Simultaneus measurement of hundreds of liver proteins: application in assessment of liver function. Toxicol Pathol 24(1):72–76

Applied Biosystems Protocol (2010) TaqMan® Gene Expression Assays. P/N: 4333458 Rev. N

Applied Biosystems Protocol (2011) TaqMan® Small RNA Assays. P/N: 4364031 Rev. E

Arce A, Aicher L, Wahl D, Anderson NL, Meheus L, Raymackers J, Cordier A, Steiner S (1998) Changes in the liver protein pattern of female wistar rats treated with the hypoglycaemic agent SDZ PGU 693. Life Sci 63(25):2243–2250

Bjørnstad A, Larsen BK, Skadsheim A, Jones MB, Andersen OK (2006) The potential of ecotoxicoproteomics in environmental monitoring: biomarker profiling in mussel plasma using ProteinChip array technology. J Toxicol Environ Health A 69:77–96

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Boehme K, Simon S, Mueller SO (2009) Gene expression profiling in Ishikawa cells: a fingerprint for estrogen active compounds. Toxicol Appl Pharmacol 236(1):85–96

Boehme K, Dietz Y, Hewitt P, Mueller SO (2011) Genomic profiling uncovers a molecular pattern for toxicological characterization of mutagens and promutagens in vitro. Toxicol Sci 122(1):185–197

Boernsen KO, Gatzek S, Imbert G (2005) Controlled protein precipitation in combination with chip-based nanospray infusion mass spectrometry. an approach for metabolomics profiling of plasma. Anal Chem 77:7255–7264

Boitier E, Amberg A, Barbié V, Blichenberg A, Brandenburg A, Gmuender H, Gruhler A, McCarthy D, Meyer K, RiefkeB RM, Schoonen W, Sieber M, Suter L, Thomas CE, Sajot N (2011) A comparative integrated transcript analysis and functional characterization of differential mechanisms for induction of liver hypertrophy in the rat. Toxicol Appl Pharmacol 252(2):85–96

Bollard ME, Stanley EG, Lindon JC, Nicholson JK, Holmes E (2007) NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. NMR Biomed 18:143–162

Bolton ET, McCarthy BJ (1962) A general method for the isolation of RNA complementary to DNA. Proc Natl Acad Sci USA 48(8):1390–1397

Campbell SJ, Finlay M, Clements JM, Wells G, Miller KM, Perry VH, Anthony DC (2004) Reduction of exitotoxicity and associated leukocyte recruitment by a broad-spectrum matrix metalloproteinase inhibitor. J Neurochem 89(6): 1378–1386

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162(1):156–159

Choudhuri S (2010) Small noncoding RNAs: biogenesis, function, and emerging significance in toxicology. J Biochem Mol Toxicol 24(3):195–216

Cicos S, Bukovska A, Koppel J (2007) Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 8:113

Collins BC, Sposny A, McCarthy D, Brandenburg A, Woodbury R, Pennington SR, Gautier J-C, Hewitt Ph, Gallagher WM (2010) Use of SELDI MS to discover and identify potential biomarkers of toxicity in InnoMed PredTox: a multi-site, multi-compound study. Proteomics 10:1592–1608

Connor SC, Wu W, Sweatman BC, Manini J, Haselden JN, Crowther DJ, Waterfield CJ (2004) Effects of feeding and body weight loss on the 1H-NMR-based urine metabolic profiles of male Wistar Han rats: implications for biomarker discovery. Biomarkers 9:156–179

Cravatt BF, Sorensen EJ (2000) Chemical strategy for the global analysis of protein function. Curr Opin Chem Biol 4:663–668

Cunningham ML, Pippin LL, Anderson NL, Wenk ML (1995) The hepatocarcinogen methapyrilene but not the analogue pyrilamine induces sustained hepatocellular replication and protein interactions in F344 rats in a 13 week feed study. Toxicol Appl Pharmacol 131:216–223

Dieterle F, Riefke B, Schlotterbeck G, Ross A, Senn H, Amberg A (2011) NMR and MS methods for metabonomics. Methods Mol Biol 691:385–415

Dunbar SA (2006) Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta 363(1–2):71–82

Dunn W, Ellis DI (2005) Metabolomics: current analytical platforms and methodologies. Trends Anal Chem 4:285–294

Elcombe CR, Odum J, Foster JR, Stone S, Hasmall S, Soames AR, Kimber I, Ashby J (2002) Prediction of rodent nongenotoxic carcinogenesis: evaluation of biochemical and tissue changes in rodents following exposure to nine nongenotoxic NTP carcinogens. Environ Health Perspect 110(4):363–375

Ellinger-Ziegelbauer H, Stuart B, Wahle B, Bomann W, Ahr HJ (2004) Characteristic expression profiles induced by genotoxic carcinogens in rat liver. Toxicol Sci 77(1):19–34

Ellinger-Ziegelbauer H, Adler M, Amberg A, Brandenburg A, Callanan JJ, Connor S, Fountoulakis M, Gmuender H, Gruhler A, Hewitt P, Hodson M, Matheis KA, McCarthy D, Raschke M, Riefke B, Schmitt CS, Sieber M, Sposny A, Suter L, Sweatman B, Mally A (2010) The enhanced value of combining conventional and "omics" analyses in early assessment of drug-induced hepatobiliary injury. Toxicol Appl Pharmacol 252(2):97–111

Ernst RR, Bodenhausen G, Wokaun A (1990) Principles of nuclear magnetic resonance in one and two dimensions. Oxford University Press, Oxford

Ezendam J, Staedtler F, Pennings J, Vandebriel RJ, Pieters R, Boffetta P, Harleman JH, Vos JG (2004) Toxicogenomics of subchronic hexachlorobenzene exposure in Brown Norway rats. Environ Health Perspect 112(7):782–791

Fan JB, Yeakley JM, Bibikova M, Chudin E, Wickham E, Chen J, Doucet D, Rigault P, Zhang B, Shen R, McBride C, Li HR, Fu XD, Oliphant A, Barker DL, Chee MS (2004) A versatile assay for high-throughput gene expression profiling on universal array matrices. Genome Res 14(5):878–885

Fella K, Glückmann M, Hellmann J, Karas M, Kramer PJ, Kröger M (2005) Use of two-dimensional gel-electrophoresis in predictive toxicology: identification of potential early protein biomarkers in chemically induced hepatocarcinogenesis. Proteomics 5(7):1914–1927

Fox BC, Devonshire AS, Schutte ME, Foy CA, Minguez J, Przyborski S, Maltman D, Bokhari M, Marshall D (2010) Validation of reference gene stability for APAP hepatotoxicity studies in different in vitro systems and identification of novel potential toxicity biomarkers. Toxicol In Vitro 24(7):1962–1970

Ganter B, Zidek N, Hewitt PR, Müller D, Vladimirova A (2008) Pathway analysis tools and toxicogenomics reference databases for risk assessment. Pharmacogenomics 9(1):35–54

Gao Y, Holland RD, Yu L-R (2009) Quantitative proteomics for drug toxicity. Brief Funct Genomic Proteomic 8(2):158–166

Gatto L, Vizca´no AJ, Hermjakob H, Huber W, Lilley KS (2010) Organelle proteomics experimental designs and analysis. Proteomics 10:3957–3969

Gerard CJ, Andrejka LM, Macina RA (2000) Mitochondrial ATP synthase 6 as an endogenous control in the quantitative RT-PCR analysis of clinical cancer samples. Mol Diagn 5(1):39–46

Giulietti A (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25(4):386–401

Glückmann M, Fella K, Waidelich D, Merkel D, Kruft V, Kramer PJ, Walter Y, Hellmann J, Karas M, Kröger M (2007) Prevalidation of potential protein biomarkers in toxicology using iTRAQ™ reagent technology. Proteomics 7:1564–1574

Goldman M (1991) Quantum description of high-resolution NMR in liquids. Oxford University Press, Oxford

Grizzle WE, Adam BL, Bigbee WL, Conrads TP, Carroll C, Feng Z, Izbicka E, Jendoubi M, Johnsey D, Kagan J, Leach RJ, McCarthy DB, Semmes OJ, Srivastava S, Srivastava S, Thompson IM, Thornquist MD, Verma M, Zhang Z, Zou Z (2004) Serum protein expression profiling for cancer detection: validation of a SELDI-based approach for prostate cancer. Dis Markers 19(4–5):185–195

Gullberg J, Jonsson P, Nordström A, Sjöström M, Moritz T (2004) Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of Arabidopsis thaliana samples in metabolomics studies with gas chromatography/mass spectrometry. Anal Biochem 331:283–295

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999

Hamadeh HK, Bushel PR, Jayadev S, DiSorbo O, Bennett L, Li L, Tennant R, Stoll R, Barrett JC, Paules RS, Blanchard K, Afshari CA (2002) Prediction of compound signature using high density gene expression profiling. Toxicol Sci 67(2):232–240

Hannon GJ (2002) RNA interference. Nature 418(6894): 244–251

Harris AJ, Shaddock JG, DelongChamp R, Dragan Y, Casciano DA (2004) Comparison of basal gene expression in cultured primary rat hepatocytes and freshly isolated rat hepatocytes. Toxicol Mech Method 14(5):257–270

Hewitt P, Kramer P-J, Borlak J (2005) Toxicogenomics applied to teratogenicity studies. In: Borlak J (ed) Handbook of toxicogenomics, strategies and applications. Wiley-VCH, Weinheim, pp 435–469

Hook SE (2010) Promise and progress in environmental genomics: a status report on the applications of gene expression-based microarray studies in ecologically relevant fish species. J Fish Biol 77(9):1999–2022

Hrach J, Mueller SO, Hewitt P (2011) Development of an in vitro liver toxicity prediction model based on longer term primary rat hepatocyte culture. Toxicol Lett 206(2):189–196

Idborg H, Zamani L, Edlund PO, Schuppe-Koistinen I, Jacobsson SP (2005) Metabolic fingerprinting of rat urine by LC/MS Part 1. Analysis by hydrophilic interaction liquid chromatography–electrospray ionization mass spectrometry. J Chromatogr B 828:9–13

Illumina Technology Spotlight (2010) Illumina Sequencing Technology. Pub.No. 770-2007-002

Ilumina Data Sheet (2011) TruSeq™ RNA and DNA Sample Preparation Kits v2. Pub.No. 970-2009-039

Janech MG, Raymond JR, Arthur JM (2007) Proteomics in renal research. Am J Physiol Renal Physiol 292(2):F501–F512

Jessen BA, Mullins JS, De Peyster A, Stevens GJ (2003) Assessment of hepatocytes and liver slices as in vitro test systems to predict in vivo gene expression. Toxicol Sci 75(1):208–222

Jiang YH, Davidson LA, Lupton JR, Chapkin RS (1995) A rapid RT-PCR method for detection of intact RNA in formalin-

fixed paraffin-embedded tissues. Nucleic Acid Res 23(15):3071–3072

Jr GW, Cazares LH, Leung SM, Nasim S, Adam BL, Yip TT, Schellhammer PF, Gong L, Vlahou A (1999) Proteinchip(R) surface enhanced laser desorption/ionization (SELDI) mass spectrometry: a novel protein biochip technology for detection of prostate cancer biomarkers in complex protein mixtures. Prostate Cancer Prostatic Dis 2(5/6):264–276

Katajamaa M, Oresic M (2007) Data processing for mass spectrometry-based metabolomics. J Chromatogr A 1158:318–328

Keun HC, Ebbels TM, Antti H, Bollard ME, Beckonert O, Schlotterbeck G, Senn H, Niederhauser U, Holmes E, Lindon JC, Nicholson JK (2002) Analytical reproducibility in (1)H NMR-based metabonomic urinalysis. Chem Res Toxicol 15:1380–1386

Klaassen CD, Lu H, Cui JY (2011) Epigenetic regulation of drug processing genes. Toxicol Mech Methods 21(4):312–324

Klawitter J, Klawitter J, Kushner E, Jonscher K, Bendrick-Peart J, Leibfritz D, Christians U, Schmitz V (2010) Association of immunosuppressant-induced protein changes in the rat kidney with changes in urine metabolite patterns: a proteometabonomic study. J Proteome Res 9(2):865–875

Koek MM, Jellema RH, van der Greef J, Tas AC, Hankemeier T (2011) Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives. Metabolomics 7:307–328

Kostrubsky VE, Strom SC, Hanson J, Urda E, Rose K, Burliegh J, Zocharski P, Cai H, Sinclair JF, Sahi J (2003) Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. Toxicol Sci 76(1):220–228

Kultima K, Nystrom AM, Scholz B, Gustafson AL, Dencker L, Stigson M (2004) Valproic acid teratogenicity: a toxicogenomics approach. Environ Health Perspect 112(12):1225–1235

Kumagai K, Ando Y, Kiyosawa N, Ito K, Kawai R, Yamoto T, Manabe S, Teranishi M (2006) Toxicoproteomic investigation of the molecular mechanisms of cycloheximide-induced hepatocellular apoptosis in rat liver. Toxicology 228:299–309

Lee YH, Tan HT, Chung MCM (2010) Subcellular fractionation methods and strategies for proteomics. Proteomics 10:3935–3956

Lema C, Cunningham MJ (2010) MicroRNAs and their implications in toxicological research. Toxicol Lett 198(2):100–105

Lenz EM, Wilson ID (2007) Analytical strategies in metabonomics. J Proteome Res 6:443–458

Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW (2002) Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 48(8):1296–1304

Lindon JC, Nicholson JK, Holmes E, Everett JR (2000) Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. Concept Magn Reson 12:289–320

Lindon JC, Holmes E, Nicholson JK (2003) So what's the deal with metabonomics? Anal Chem 75:384A–391A

Lindon JC, Holmes E, Nicholson JK (2004) Metabonomics and its role in drug development and disease diagnosis. Expert Rev Mol Diagn 4:189–199

Lindon JC, Holmes E, Nicholson JK (2006) Metabonomics techniques and applications to pharmaceutical research & development. Pharm Res 23:1075–1088

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR (1999) Direct analysis of protein complexes using mass spectrometry. Nat Biotechnol 17:676–682

Liu CH, Ma WL, Shi R, Zhang B, Ou YQ, Zheng WL (2003) Gene expression study of saccharomyces cerevisiae with the Agilent 2100 Bioanalyser. Br J Biomed Sci 60(1):22–25

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408

Maley D, Mei J, Lu H, Johnson DL, Ilysin SE (2004) Multiplexed RT-PCR for high throughput screening applications. Comb Chem High Throughput Screen 7(8):727–732

Man WJ, White IR, Bryant D, Bugelski P, Camilleri P, Cutler P, Heald G, Lord PG, Wood J, Kramer K (2002) Protein expression analysis of drug-mediated hepatotoxicity in Sprague-Dawley rat. Proteomics 2(11):1577–1585

Mann M (1999) Quantitqative proteomics? Nat Biotechnol 17:954–955

Mardis ER (2010) The impact of next-generation sequencing technology on genetics. Trends Genet 24(3):133–141

Martell M, Gomez J, Steban JI, Sauleda S, Quer J, Cabot B, Esteban R, Guardia J (1999) High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J Clin Microbiol 37(2):327–332

Martyniuk CJ, Griffitt RJ, Denslow ND (2011) Omics in aquatic toxicology: not just another microarray. Environ Toxicol Chem 30(2):263–264

Matheis KA, Com E, Gautier JC, Guerreiro N, Brandenburg A, Gmuender H, Sposny A, Hewitt P, Amberg A, Boernsen O, Riefke B, Hoffmann D, Mally A, Kalkuhl A, Suter L, Dieterle F, Staedtler F (2011) Cross-study and cross-omics comparisons of three nephrotoxic compounds reveal mechanistic insights and new candidate biomarkers. Toxicol Appl Pharmacol 252(2):112–122

Merrick BA (2006) Toxicoproteomics in liver injury and inflammation. Ann N Y Acad Sci 1076:707–717

Merrick BA (2008) The plasma proteome, adductome and idiosyncratic toxicity in toxicoproteomics research. Brief Funct Genomic Proteomic 7(1):35–49

Metzker ML (2010) Sequencing technologies – the next generation. Nat Rev Genet 11(1):31–46

Mischak H, Spandiari P, Sadrieh N, Hanig J (2009) Profiling of rat urinary proteomic patterns associated with drug-induced nephrotoxicity using CE coupled with MS as a potential model for detection of drug-induced adverse effects. Proteomics Clin Appl 3(9):1062–1071

Moseley MA (2001) Current trends in differential expression proteomics: isotopically coded tags. Trends Biotechnol 19: S40–S48

Munker R, Calin GA (2011) MicroRNA profiling in cancer. Clin Sci (Lond) 121(4):141–158

Nakajima M, Yokoi T (2011) MicroRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. Pharmacol Ther 131(3):330–337

Nicholson JK, Wilson ID (2003) Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov 2:668–676

Nicholson JK, Lindon JC, Holmes E (1999) "Metabonomics": understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29:1181–1189

Nicholson JK, Connelly J, Lindon JC, Holmes E (2002) Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 1:153–161

Nuwaysir EF, Bittner M, Trent J, Barrett JC, Afshari CA (1999) Microarrays and toxicology: the advent of toxicogenomics. Mol Carcinog 24:53–159

Oshida K, Hirakata M, Maeda A, Miyoshi T, Miyamoto Y (2011) Toxicological effect of emodin in mouse testicular gene expression profile. J Appl Toxicol. doi:10.1002/jat.1637

Ovando BJ, Ellison CA, Vezina CM, Olson JR (2011) Toxicogenomic analysis of exposure to TCDD, PCB126 and PCB153: identification of genomic biomarkers of exposure to AhR ligands. BMC Genomics 11:583

Paweletz CP, Trock B, Pennanen M, Tsangaris T, Magnant C, Liotta LA, Petricoin EF 3rd (2001) Proteomic patterns of nipple aspirate fluids obtained by SELDI-TOF: potential for new biomarkers to aid in the diagnosis of breast cancer. Dis Markers 17:301–307

Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SP (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci USA 91(11):5022–5026

Peña-Alvarez A, Díaz L, Medina A, Labastida C, Capella S, Vera LE (2004) Characterization of three Agave species by gas chromatography and solid-phase microextraction-gas chromatography-mass. J Chromatogr A 1027:131–136

Pennie WD, Tugwood JD, Oliver GJA, Kimber I (2000) The principles and practice of toxicogenomics. Toxicol Sci 54:277–283

Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA (2002) Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359(9306):572–577

Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpyleva SI, Kovalchuk O (2010) Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. Int J Cancer 127(8):1785–1794

Prince WS (1999) Water signal suppression in NMR spectroscopy. Annu Rep NMR Spectrosc 38:289–354

Provan F, Bjørnstad A, Pampanin DM, Lyng E, Fontanillas R, Andersen OK, Koppe W, Bamber S (2006) Mass spectrometric profiling–a diagnostic tool in fish? Mar Environ Res 62(Suppl):S105–S108

Robertson DG (2005) Metabonomics in toxicology: a review. Toxicol Sci 85:809–822

Rubina AY, Dementieva EI, Stomakhin AA, Darii EL et al (2003) Hydrogel-based protein microchips: manufacturing, properties, and applications. Biotechniques 34:1008–1022

Rubina AY, Kolchinsky A, Makarov AA, Zasedatelev AS (2008) Why 3-D? Gel-based microarrays in proteomics. Proteomics 8:817–831

Ruepp SU, Tonge RP, Shaw J, Wallis N, Prognan F (2002) Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. Toxicol Sci 65:135–150

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270(5235):467–470

Schmitt C, Walijew A, Hewitt P, Harleman JH (2009) Gene expression profiling in toxicological studies using FFPE tissues. Abstracts. Exp Toxicol Pathol 61:387–483, Abstract #P06

Schröder NW, Schombel U, Heine H, Göbel UB, Zähringer U, Schumann RR (2003) Acylated cholesteryl galactoside as a novel immunogenic motif in Borrelia burgdorferi sensu stricto. J Biol Chem 278:33645–33653

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3

Shellie RA, Welthagen W, Zrostliková J, Spranger J, Ristow M, Fiehn O, Zimmermann R (2005) Statistical methods for comparing comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry results: metabolomic analysis of mouse tissue extracts. J Chromatogr A 1086:83–90

Shi L, MAQC Consortium et al (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24(9):1151–1161

Shockcor JP, Holmes E (2002) Metabonomic applications in toxicity screening and disease diagnosis. Curr Top Med Chem 2:35–51

Srinivas PR, Srivastava S, Hanash S, Wright GL Jr (2001) Proteomics in early detection of cancer. Clin Chem 47:1901–1911

Steiner S, Aicher L, Raymacher J, Meheus L, Eyquer-Blasco R, Anderson NL, Cordier A (1996) Cyclosporine A decreases the protein level of the calcium-binding protein calbindin-D 28 kDa in rat kidney. Biochem Pharmacol 51(3):253–258

Steiner G, Suter L, Boess F, Gasser R, de Vera MC, Ruepp S (2004) Discriminating different classes of toxicants by transcript profiling. Environ Health Perspect 112(12):1236–1248

Su Z, Li Z, Chen T, Li QZ, Fang H, Ding D, Ge W, Ning B, Hong H, Perkins RG, Tong W, Shi L (2011) Comparing next-generation sequencing and microarray technologies in a toxicological study of the effects of Aristolochic acid on rat kidneys. Chem Res Toxicol. doi:10.1021/tx200103b

Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TWM, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane AN, Lindon JC, Marriott P, Nicholls AW, Reily MD, Thaden JJ, Viant MR (2007) Proposed minimum reporting standards for chemical analysis. Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 3:211–221

Taylor EL, Gant TW (2008) Emerging fundamental roles for non-coding RNA species in toxicology. Toxicology 246(1):34–39

Trygg J, Gullberg J, Johansson AI, Jonsson P, Antti H, Marklund SL, Moritz T (2005) Extraction and GC/MS analysis of the human blood plasma metabolome. Anal Chem 77:8086–8094

Van Mispelaar VG, Tas AC, Smilde AK, Schoenmakers PJ, van Asten AC (2003) Quantitative analysis of target components by comprehensive two-dimensional gas chromatography. J Chromatogr A 1019:15–29

Vlahou A, Schellhammer PF, Mendrinos S, Patel K, Kondylis FI, Gong L, Nasim S, Wright GL Jr

(2001) Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. Am J Pathol 158:1491–1502

Voelkerding KV, Dames SA, Durtschi JD (2009) Next-generation sequencing: from basic research to diagnostics. Clin Chem 55(4):641–658

Von Landenberg F, Hewitt P, Mueller SO, Schmitt CS (2011) Retrospective gene expression analysis using formalin-fixed tissue blocks from toxicological studies. Toxicologist 120(2):Abtract #1590

Wagner S, Scholz K, Sieber M, Kellert M, Voelkel W (2007) Tools in metabolomics: an integrated validation approach for LC-MS metabolic profiling of mercapturic acid in human urine. Anal Chem 79:2918–2926

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10(1):57–63

Want EJ, O'Maille G, Smith CA, Brandon TR, Uritboonthai W, Qin C, Trauger SA, Siuzdak G (2006) Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass spectrometry. Anal Chem 78:743–752

Want EJ, Coen M, Masson P, Keun HC, Pearce JT, Reily MD, Robertson DG, Rohde CM, Holmes E, Lindon JC, Plumb RS, Nicholson JK (2010) Ultra performance liquid chromatography-mass spectrometry profiling of bile acid metabolites in biofluids: application to experimental toxicology studies. Anal Chem 82:5282–5289

Waring JF, Jolly RA, Ciurlionis R, Lurn PY, Praestgaard JT, Morfitt DC, Buratto B, Roberts C, Schadt E, Ulrich RG (2001) Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. Toxicol Appl Pharmacol 175(1):28–42

Waybright TJ, Van QN, Muschik GM, Conrads TP, Veenstra TD, Issaq HJ (2006) LC-MS in metabonomics: optimization of experimental conditions for the analysis of metabolites in human urine. J Liq Chromatogr Relat Technol 29:2475–2497

Weckwerth W, Morgenthal K (2005) Metabolomics: from pattern recognition to biological interpretation. Drug Discov Today 10:1551–1558

Weinbergera SR, Boschettib E, Santambienb P, Brenacb V (2002) Surface-enhanced laser desorption–ionization retentate chromatography mass spectrometry (SELDI–RC–MS): a new method for rapid development of process chromatography conditions. J Chromatogr B 782:307–316

Wilson ID, Plumb R, Granger J, Major H, Williams R, Lenz EM (2005) HPLC-MS-based methods for the study of metabolomics. J Chromatogr B 817:67–76

Witzmann FA, Li J (2004) Proteomics and nephrotoxicity. Contrib Nephrol 141:104–123

Woollard PM, Mehta NA, Vamathevan JJ, Van Horn S, Bonde BK, Dow DJ (2011) The application of next-generation sequencing technologies to drug discovery and development. Drug Discov Today 16(11–12):512–519

Yuan X, Jonker MJ, de Wilde J, Verhoef A, Wittink FR, van Benthem J, Bessems JG, Hakkert BC, Kuiper RV, van Steeg H, Breit TM, Luijten M (2010) Finding maximal transcriptome differences between reprotoxic and non-reprotoxic phthalate responses in rat testis. J Appl Toxicol. doi:10.1002/jat.1601

Zhang J, Peterson SM, Weber GJ, Zhu X, Zheng W, Freeman JL (2011a) Decreased axonal density and altered expression profiles of axonal guidance genes underlying lead (Pb) neurodevelopmental toxicity at early embryonic stages in the zebrafish. Neurotoxicol Teratol. doi:10.1016/j.ntt.2011.07.010

Zhang J, Chiodini R, Badr A, Zhang G (2011b) The impact of next-generation sequencing on genomics. J Genet Genomics 38(3):95–109

Zheng JL, Frantz G, Lewis AK, Sliwkowski M, Gao WQ (1999) Heregulin enhances regenerative proliferation in postnatal rat utricular sensory epithelium after ototoxic shock. J Neurocytol 28(10–11):901–912

Zhong H, Simons JW (1999) Direct comparison of GAPDH, beta-actin, cyclophilin, and 28 S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 259(3):523–526