

# Gene Expression Profiles of Nonylphenol as Representative EDCs in Normal Human Kidney HK-2 Cells

Seung Jun Kim<sup>1,2</sup>, Moon-Ju Oh<sup>2,3</sup>, Hye-Won Park<sup>2</sup>, Jun-Sub Kim<sup>2</sup>, Saswati Paul<sup>1</sup>, Jung-Mi Ha<sup>2</sup>, Youn-Jung Kim<sup>4</sup>, Jae-Chun Ryu<sup>4</sup>, Chul-Woo Lee<sup>5</sup>, Hyun-Mi Kim<sup>5</sup>, Kyung-Hee Choi<sup>5</sup> & Seung Yong Hwang<sup>1,2</sup>

<sup>1</sup>Department of Biochemistry, Hanyang University, Sangrok-gu, Ansan, Gyeonggi-do, Korea

<sup>2</sup>GenoCheck Co. Ltd., Sangrok-gu, Ansan, Gyeonggi-do, Korea

<sup>3</sup>Department of Bio-Nanotechnology, Hanyang University, Sangrok-gu, Ansan, Gyeonggi-do, Korea

<sup>4</sup>Cellular and Molecular Toxicology Laboratory, Korea Institute of Science and Technology, Cheongryang, Seoul, Korea

<sup>5</sup>Environmental Exposure Assessment Division, National Institute of Environmental research, Seo-gu, Incheon, Korea

Correspondence and requests for materials should be addressed to S.Y. Hwang (syhwang@hanyang.ac.kr)

Accepted 21 April 2008

## Abstract

Nonylphenol (NP) is an organic compound of the wider family of alkylphenols and is a product of industrial synthesis formed during the alkylation process of phenols, particularly in the synthesis of polyethoxylate detergents. NP is an endocrine disruptor. Endocrine-disrupting chemicals (EDCs) are exogenous compounds that have the potential to hamper with hormonal regulations and the normal endocrine system and consequently cause health effects. In numerous chemical substances, the action mechanism of an endocrine disruptor is not clearly understood. In the present study, *in vitro* gene expression profiles were analyzed in nonylphenol-treated HK-2 cells using an Agilent Human 4 × 44 K whole genome array including 41,000 transcripts. Gene expression profiles were analyzed 3 and 48 hrs after exposure to NP with 2 different doses. We analyzed the gene expression profiles in order to understand the biological effects at level of gene functions and their time-dependent effects. A total of 1,727 genes were identified as being either over or down-expressed over 2-fold changes ( $P$ -value < 0.05) in NP treated HK-2 cells. The functional classification of differentially expressed genes showed that cell death related genes were regulated by NP in HK-2 cells. 79 genes were time-dependent and differentially expressed,

while 259 genes were concentration-dependent, all of which were selected using an ANOVA method. These data may support the understanding of the toxicity of nonylphenol in normal human kidney cells.

**Keywords:** EDCs, Nonylphenol, Microarray, Toxicogenomics, HK-2

## Introduction

Endocrine-disrupting chemicals (EDCs) come from a variety of man-made sources, such as plastics, pharmaceuticals, ordinary household chemicals, and industrial chemicals. They adjust the hormonal functions of various species by acting as sex hormones, which prevent normal hormonal binding and a breakdown of natural hormones. EDCs became the focus of both public and scientific interest when defects in sexual behavior and reproductive ability of wild-life animals were recognized as corresponding to EDCs steroid-like or anti-steroid androgenic properties<sup>1</sup>. NP (4-nonylphenol) is a representative environmental EDC with weak estrogenic activity<sup>2</sup>. NP is used as an additive or surfactant in the manufacturing of plastics, and it is a degradation product of nonylphenol polyethoxylates, which are widely used. NP has been shown to have estrogenic activity in *in vitro* and *in vivo* assays<sup>3</sup>. Although weak androgenic NP activity was identified by Sohoni and Sumpter (1998)<sup>4</sup>, a more recent research using a yeast two-hybrid system revealed the anti-androgenic effects of NP<sup>5</sup>. Ferguson *et al.* (2000)<sup>6</sup> demonstrated the toxicity of NP to mothers and offspring, but found no alterations in open-field activity and running wheel activity in offspring after prenatal NP exposure. Nonylphenols (NP) are toxic degradation products of alkylphenol ethoxylates (APEs). Research has identified NP as the most critical metabolite of APEs because of its resistance to bio-degradation, its ability to bio-accumulate, and its toxicity<sup>7,8</sup>.

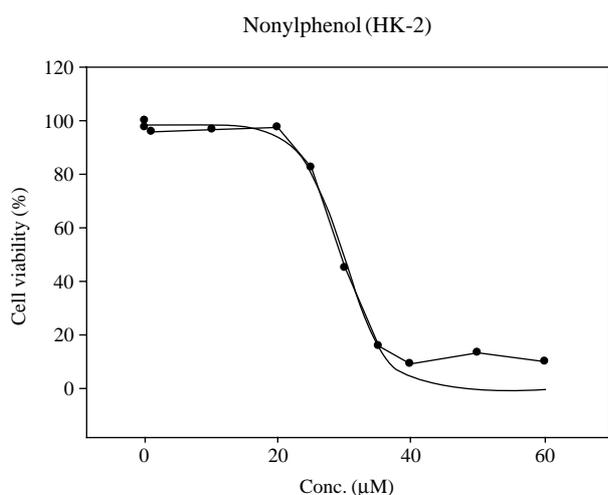
Toxicogenomics is a type of analysis by which the activity of a particular toxin or chemical substance on a living sample can be identified based upon a profiling of its known effects on genetic material. Toxicogenomics evolved from the desire to characterize how genomes respond to environmental stressors or toxicants by combining genome-wide mRNA expression

profiling with global protein expression patterns that are interpreted by the use of bioinformatics in order to understand the role of gene-environment interactions in disease and dysfunctions. It is leading the next revolution toward a better understanding of molecular pathology<sup>9,10</sup>. Nuwaysir *et al.* popularized the term 'toxicogenomics' to describe the use of microarrays to measure the responses of toxicologically-relevant genes and to identify selective sensitive biomarkers of toxicity<sup>11</sup>. In this study, we describe the global gene expression profiles in HK-2 cells in order to understand the molecular responses of nonylphenol. Using an Agilent Human oligonucleotide array, the gene expression profile at different dose and time points has been analyzed after treating with nonylphenol. Differentially expressed genes were analyzed using GO functional classification and a KEGG pathway<sup>12</sup>.

## Results

### Cytotoxicity Test and Cell Proliferation Test

Cell viability after exposure to Nonylphenol was analyzed using an MTT assay. The change in absorbance, which is reflective of the change in cell death, is expressed as the % of control. The IC<sub>20</sub> of cell proliferation was defined as the concentration that causes a 20% reduction in the cell viability versus the solvent treated control. The IC<sub>20</sub> values were directly determined from the linear dose-response curves. The IC<sub>20</sub> values of nonylphenol are calculated as 25



**Figure 1.** MTT assay. Cell viability after exposure to Nonylphenol was analyzed by MTT assay. The change in absorbance, which is reflective of the change in cell death, is expressed as the % of control. 20% inhibition concentration (IC<sub>20</sub>) values of nonylphenol are calculated as 25 µM.

µM, as shown in Figure 1.

### Gene Expression Data Analysis

Gene expression profiling by nonylphenol-treatment was analyzed using an Agilent human 4 × 44 K whole genome array. Expression data were analyzed using a GeneSpring GX 7.3.1. Local background values were subtracted from the signal means, and a small constant was added to all differences in order to allow for log transformation of the background-corrected signals. Following a log transformation, LOWESS normalization<sup>13</sup> was applied to remove intensity-dependent dye bias from each slide. The resulting values were adjusted so that the median normalized signal for each gene would be constant across all slides and dye combinations.

Total gene expression profiles were analyzed using hierarchical clustering, as shown in Figure 2<sup>14</sup>. A heat map of clustering shows the similarity between gene expressions by nonylphenol. Red indicates an up-regulation, green is a down-regulation and black is no change. High-dose samples showed that the gene expression patterns were very similar.

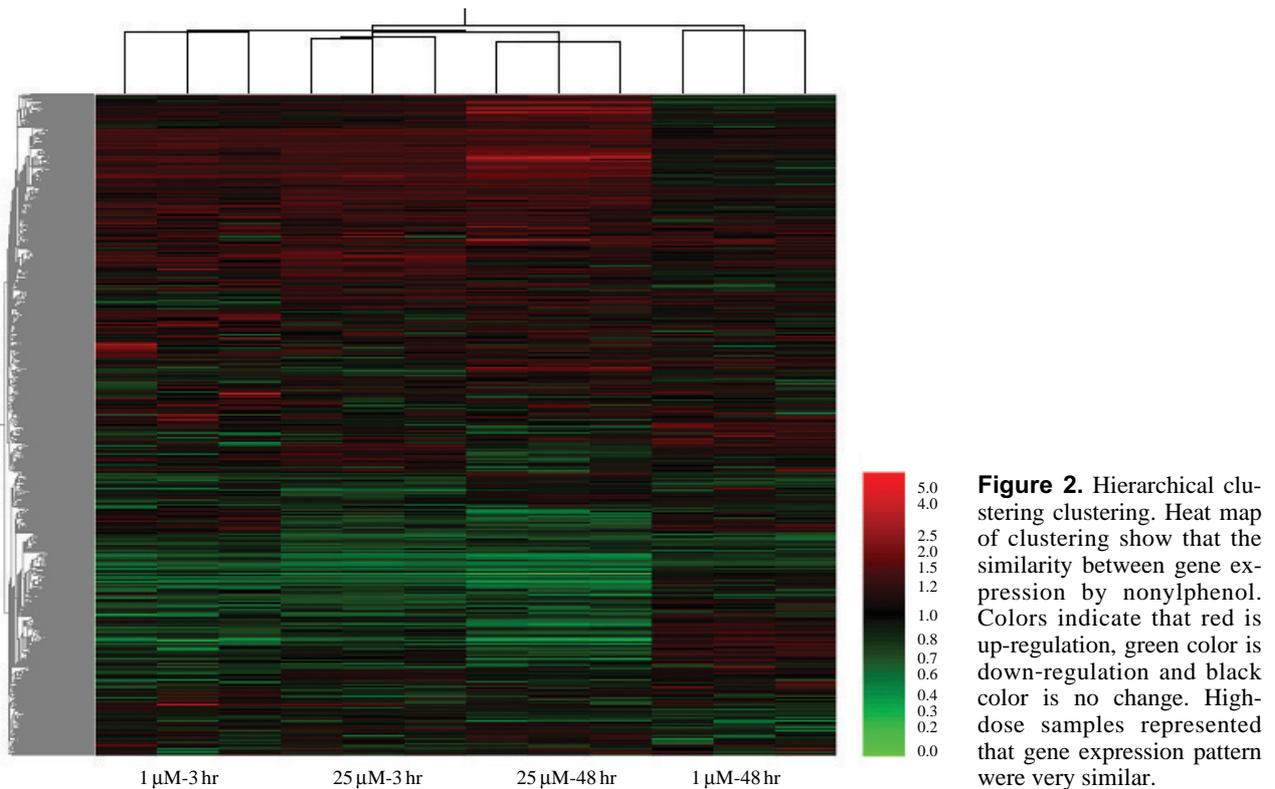
Differentially expressed genes in each point were selected using a volcano plot method<sup>15</sup>. Volcano plots are constructed using fold-change values and p-values. Volcano plots allow you to visualize the relationship between fold-change (magnitude of change) and statistical significance (which takes both the magnitude of change and variability into consideration). In each point, 406, 86, 272 and 1,205 genes were differentially expressed at 1 µM-3 hr, 1 µM-48 hr, 25 µM-3 hr and 25 µM-48 hr. A total of 1,727 genes were differentially expressed above at least one point, as shown in Table 1. These genes were analyzed to functional classification, then 79 genes were selected by time-parameter 1-way ANOVA. These genes mean to difference of acute and late responses. Also, 259 genes were selected by dose-parameter one way ANOVA and were analyzed into functional classification.

### Functional Analysis

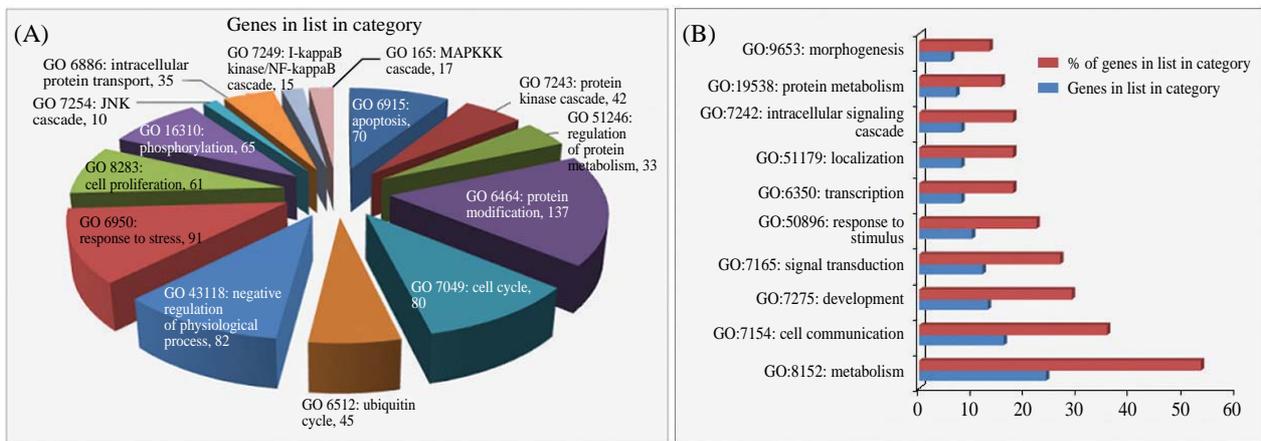
To examine the functional classification of differentially expressed genes in nonylphenol-treated HK-2 cells, 1,727 genes were analyzed using Gene Ontol-

**Table 1.** Differentially expressed genes

Sample	Up	Down	Total
1 µM-3 hr	175	231	406
1 µM-48 hr	62	24	86
25 µM-3 hr	90	182	272
25 µM-48 hr	499	706	1205



**Figure 2.** Hierarchical clustering. Heat map of clustering show that the similarity between gene expression by nonylphenol. Colors indicate that red is up-regulation, green color is down-regulation and black color is no change. High-dose samples represented that gene expression pattern were very similar.



**Figure 3.** Functional classification. (A) Ontology of 1,727 genes that differentially expressed above at least one point. (B) Function of 79 genes that were selected by time-parameter 1-way ANOVA.

ogy databases. The results of classification are shown in Figure 3. Apoptosis (GO:6915, p-value:1.1e-4) related genes including BCL10, F2R, GADD45A, IL4, VEGFA gene and protein kinase cascade (GO:7243, p-value:2.9e-4) related genes including STAT1, EGF, GJA1, ADORA2B, BIRC2 gene were differentially expressed by nonylphenol-treatment as shown in Figure 3 and Table 2. An SOP method, Kim *et al.* (2007),

was used for a related pathway analysis. MAPK signaling pathway, TGF-beta signaling pathway, JNK cascade, NFkB signaling pathway and cell apoptosis pathway related genes were differentially expressed. Also, to examine the functional classification of time-parametric differentially expressed genes, 79 genes were analyzed using GeneSpring software. In the biological process category, genes involved in metabo-

**Table 2.** Function category of differentially expressed 1,727 genes by exposure of nonylphenol.

Category	Genes in list in category	% of genes in list in category	p-value
GO:6915: apoptosis	70	8.216	1.1e-4
GO:7243: protein kinase cascade	42	4.93	2.9e-4
GO:51246: regulation of protein metabolism	33	3.873	3.62e-4
GO:6464: protein modification	137	16.08	4.65e-4
GO:7049: cell cycle	80	9.39	4.69e-4
GO:6512: ubiquitin cycle	45	5.282	0.00155
GO:43118: negative regulation of physiological process	82	9.624	0.00347
GO:6950: response to stress	91	10.68	0.00524
GO:8283: cell proliferation	61	7.16	0.00558
GO:16310: phosphorylation	65	7.629	0.00571
GO:7254: JNK cascade	10	1.174	0.01
GO:6886: intracellular protein transport	35	4.108	0.0127
GO:7249: I-kappaB kinase/NF-kappaB cascade	15	1.761	0.021
GO:165: MAPKKK cascade	17	1.995	0.0231

lism (GO:8152), cell communication (GO:7154), development (GO:7275), signal transduction (GO:7165), response to stimulus (GO:50896) are highly observed in the 79 selected time-parametric genes, as shown in Figure 3-B. Genes involved in the cellular physiological process (GO:50875), intracellular transport (GO:46907), protein metabolism (GO:19538), and cell cycle (GO:7049) are highly observed in 259 dose-dependent genes. (data not shown)

## Discussion

Toxicogenomics approaches have been carried out in the field of toxicology in order to investigate the molecular mechanism and assess toxicity. In several studies using -omics technologies, the toxicological mechanism were revealed and potentially biomarker were candidate. In this study, we examined the profiles of gene expression in response to nonylphenol in normal human kidney cells. Microarray data analysis showed variations of gene expression by nonylphe-

nol-treated HK-2 cells at two-dose and two-time points.

ESR, an estrogen receptor gene that is encoded to binding proteins with nonylphenol as an antagonist, showed no changed in expression. AR, an androgen receptor genes is up-regulated by nonylphenol. The PDZK1 (PDZ domain containing 1) gene, which is a representative of estrogen-responsiveness genes, showed an over-expression<sup>16,17</sup>. These data suggest there is no problem experimentations.

In 1,727 differentially expressed genes, several components of cell proliferation were also induced after exposure to nonylphenol. These are sixty-one genes including IL4, IL8, CSF2, IGF2, ERBB4, SMAD4, CD5, EDD1. Apoptosis genes including VEGFA, NR4A1, DDT3, TNFRSF8 and ANGPTL4 were over expressed. MAPK signaling pathway genes including FGFR, EGF, DUSP, MAPK3 and PPP-3R were over expressed by nonylphenol-treatment. Expression of cell proliferation, apoptosis related genes and MAPK signaling pathway related genes were regulated by nonylphenol; that is, nonylphenol has shown kidney cytotoxicity through the MAPK signaling pathway. Several components of the metabolism are included 79 genes that were selected to examine difference of response between acute and late. These are twenty-four genes including AGT, GARS, ADIPOQ, E2F1, PPARG. ADIPOQ, adiponectin, C1Q and collagen domain containing gene encoded protein circulates in the plasma, which is involved with metabolic and a key hormonal processes. ADIPOQ gene have been identified as up-regulated with the exposure of nonylphenol<sup>18</sup>. Nonylphenol regulated genes were published the in the Comparative Toxicogenomics Database (CTD). The CTD elucidates molecular mechanisms by which environmental chemicals affect human disease. (<http://ctd.mdibl.org/>)

Using the microarray tools, we examined the difference of gene expression in nonylphenol treated human kidney cells. The insights provided by these expression profiles, may help in understanding the molecular effects in kidney response induced by nonylphenol.

## Materials and Methods

### Chemical

Nonylphenol (NP) was purchased from Sigma-Aldrich and were dissolved in ethanol.

### Cell Culture

The immortalized proximal tubule epithelial cell

line from a normal adult human kidney (HK-2) was a gift from Prof. Soto (Tufts University School of Medicine, Boston, MA, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen Life Technologies) supplemented with 5% fetal bovine serum (Invitrogen Life Technologies), DMEM with 10% fetal bovine serum and an RPMI 1640 medium (Invitrogen Life Technologies) with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Subculturing was conducted every 3 or 4 days so as not to exceed  $1 \times 10^6$  cells/mL<sup>19</sup>.

### Cytotoxicity Test

An MTT assay was performed for the detection of cell viability. 24 well plate was used for the cytotoxicity assay. MCF-7, HK-2 and LNCaP were seeded at a seeding density of  $2 \times 10^4$  cells/mL,  $5 \times 10^4$  cells/mL and  $5 \times 10^4$  cells/mL each. Cells were exposed to various concentrations of nonylphenol in culture medium at 37°C for a 48 h exposure time. MTT (4 mg/mL in PBS) solution was added to each well and incubated for 3 h. DMSO solution was added to each tube and transfer in 96 well plate. The OD of the purple formazan product was measured at a wavelength of 540 nm. The IC<sub>20</sub> of cell proliferation was defined as the concentration that causes a 20% reduction in cell viability versus the solvent treated control. The IC<sub>20</sub> values were directly determined from the linear dose-response curves.

### Preparation of Fluorescent DNA Probe and Hybridization

Total RNA was extracted from a drug-treated rat prefrontal cortex using a TRI REAGENT (MRC, OH) according to the manufacturer's instructions. The quantity of each RNA concentration was quantified using NanoDrop and the RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., CA). Each total RNA sample (30 µg) was labeled with Cyanine (CY3) or Cyanine (Cy5) conjugated dCTP (Amersham, Piscataway, NJ) by a reverse transcription reaction using reverse transcriptase, SuperScrip II (Invitrogen, Carlsbad, California). The labeled cDNA mixture was then concentrated using an ethanol precipitation method. The concentrated Cy3 and Cy5 labeled cDNAs were resuspended in 30 µL of hybridization solution (GenoCheck, Korea). After two labeled cDNAs were mixed, placed on Agilent Human 4 × 44 K whole genome array (G4112F, Agilent Technologies, Inc., CA) and covered by a MAUI FL chamber (Biomicro systems, Inc. UT). The slides were hybridized for 12 hr at 62°C MAUI system (Biomicro systems, Inc. UT). The hybridized slides were washed in 2 X SSC, 0.1% SDS for 2 min, 1 X

SSC for 3 min, and then 0.2 X SSC for 2 min at room temperature. The slides were centrifuged at 3,000 rpm for 20 sec to dry.

### Microarray Data Analysis

Hybridized slides were scanned with an Axon Instruments GenePix 4000B scanner (Axon, CA) and the scanned images were analyzed with the software program GenePix Pro 5.1 (Axon, CA), GeneSpring GX 7.3.1 (Agilent Technologies, Inc., CA) and Microsoft Excel. Spots that were judged as substandard by visual examination of each slide were flagged and excluded from further analysis. Spots that had dust artifacts or spatial defects were manually flagged and excluded. To filter out the unreliable data, spots with signal-to-noise (signal-background-background SD) ratios below 10 were not included in the data. Data were normalized by Global, lowess, print-tip and scaled normalization for data reliability. Fold change filters included the requirement that the genes be present in at least 200% of the controls for up-regulated genes and lower than 50% of the controls for down-regulated genes. Data were clustered groups of genes that behave similarly across a drug treated experiments using GeneSpring GX 7.3.1 (Agilent Technologies, Inc., CA). We used an algorithm, based on the Pearson correlation, to separate genes of similar patterns. Differentially expressed genes were analyzed by GO functional classification and KEGG pathway<sup>11</sup>.

### Acknowledgements

This subject was supported by the National Institute of Environmental Research as part of "Toxicogenomics research for assessment of endocrine-disruption".

### References

1. Colborn, T., vom Saal, F.S. & Soto, A.M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101**, 378-384 (1993).
2. White, R., Jobling, S., Hoare, S.A., Sumpter, J.P. & Parker, M.G. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135**, 175-182 (1994).
3. Laws, S.C., Carey, S.A., Ferrell, J.M., Bodman, G.J. & Cooper, R.L. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol Sci.* **54**, 154-167 (2000).
4. Sohoni, P. & Sumpter, J.P. Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**, 327-339 (1998).

5. Lee, H.J., Chattopadhyay, S., Gong, E.Y., Ahn, R.S. & Lee, K. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. *Toxicol Sci.* **75**, 40-46 (2000).
6. Ferguson, S.A., Flynn, K.M., Delclos, K.B. & Newbold, R.R. Maternal and offspring toxicity but few sexually dimorphic behavioral alterations result from nonylphenol exposure. *Neurotoxicol. Teratol.* **22**, 583-591 (2000).
7. Tyler, C.R., Jobling, S. & Sumpter, J.P. Endocrine disruption in wildlife: A critical review of the evidence. *Crit. Rev. Toxicol.* **28**, 319-361 (1998).
8. Ahel, M., Giger, W. & Koch, M. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment I. Occurrence and transformation in sewage treatment. *Water Res.* **23**, 1131-1142 (1994).
9. Ekins, S. (Ed.). Computational toxicology-Risk assessment for pharmaceutical and environmental chemicals. *Wiley-interscience*, 2007.
10. Kim, J.H. *et al.* Possible implications of in vitro assay system for toxicity evaluation using DNA microarray. *Biochip J.* **1**, 57-64 (2007).
11. Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C. & Afshari, C.A. Microarrays and toxicology: The advent of toxicogenomics. *Mol. Carcinog.* **24**, 153-159 (1999).
12. Kim, J.S. *et al.* SOP (Search of Omics Pathway): A Web-based tool for visualization of KEGG pathway diagrams of omics data. *Mol. Cell. Toxicol.* **3**, 208-213 (2007).
13. Yang, Y.H. *et al.* Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation, *Nucleic Acids Res.* **30**, e15 (2002).
14. Golub, T.R. *et al.* Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* **286**, 531-537 (1999).
15. Chen, J.J., Wang, S.-J., Tsai, C.-A. & Lin, C.-J. Selection of differentially expressed genes in microarray data analysis. *The Pharmacogenomics J.* **7**, 212-220 (2007).
16. Inoue, A. *et al.* Comparative profiling of the gene expression for estrogen responsiveness in cultured human cell lines. *Toxicol. Vitro* **21**, 741-752 (2007).
17. Ando, S. *et al.* Breast cancer: from estrogen to androgen receptor. *Mol. Cell. Endocrinol.* **193**, 121-128 (2002).
18. Hong, E.J., Park, S.H., Choi, K.C., Leung, P.C. & Jeung, E.B. Identification of estrogen-regulated genes by microarray analysis of the uterus of immature rats exposed to endocrine disrupting chemicals. *Reprod. Biol. Endocrinol.* **4**, 49 (2006).
19. Romiti, N., Tramonti, G. & Chieli, E. Influence of different chemicals on MDR-1 P-glycoprotein expression and activity in the HK-2 proximal tubular cell line. *Toxicol. Appl. Pharmacol.* **183**, 83-91 (2002).