

A conceptual and practical overview of cDNA microarray technology: implications for basic and clinical sciences

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Abstract

cDNA microarray is an innovative technology that facilitates the analysis of the expression of thousands of genes simultaneously. The utilization of this methodology, which is rapidly evolving, requires a combination of expertise from the biological, mathematical and statistical sciences. In this review, we attempt to provide an overview of the principles of cDNA microarray technology, the practical concerns of the analytical processing of the data obtained, the correlation of this methodology with other data analysis methods such as immunohistochemistry in tissue microarrays, and the cDNA microarray application in distinct areas of the basic and clinical sciences.

Key words

- Biotechnology
- cDNA microarray
- Gene expression
- Genomics

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Introduction

Genes are hereditary units formed by deoxyribonucleic acid (DNA) sequences organized in chromosomes in the cell nucleus. The intricate process of transcription from these sequences results in the generation of messenger ribonucleic acids (mRNA). These mRNA code for proteins, which are composed of amino acids, and carry out the designated function of the gene (1). Thus, the structural and functional features of cells and tissues are determined by the simultaneous, selective, and differential expression of thousands of genes.

Since the last decade, studies involving gene mapping, cloning, and sequencing have provided significant information for the structural analysis of distinct genomes (2,3). The quantity of information obtained from these

investigations necessitates organization of the acquired data. To satisfy this need, public databases have been created to store millions of gene sequences and expressed sequences tags (ESTs), permitting accession to gene sequences for analysis of the similarities as well as the differences between genomes of distinct species (4).

Structural studies of genomes have raised numerous questions related to the functional and regulatory understanding of gene expression profiles in distinct cell types and tissues from various organisms (3,5,6). DNA microarray technology was developed to study the complex biological processing involving several thousand genes (7,8). One such microarray technology uses single DNA strands or probes (oligomers) which are imprinted onto a glass surface using photolithography (9). Another DNA microarray

methodology, which is the focus of the present review, is complementary DNA (cDNA) microarray technology (10). With these methodologies it is possible 1) to simultaneously compare the dynamic expression pattern of thousands of genes in cells or tissues, 2) to detect molecular differences during distinct stages of cellular processes, e.g., differentiation, proliferation, and the induction of apoptosis, 3) to identify transcriptional differences between non-diseased and pathological conditions, 4) to identify changes in cells due to a drug response, and 5) to identify novel biomarkers for potential use in the diagnosis, prognosis and clinical therapy of diseases.

The principles of cDNA microarray technology

A classical methodology used for the detection and quantification of mRNA in a given cell is called Northern blot analysis (11). The main principle of Northern blot analysis is the hybridization of a radiolabeled gene-specific probe to mRNA bound to a filter, in order to determine if that gene is present. Another method used to detect expression of a specific gene is the reverse transcription polymerase chain reaction (RT-PCR). RT-PCR involves the use of gene-specific primers and reverse transcriptase to synthesize a cDNA sequence to the mRNA. The cDNA is then amplified by multiple rounds of polymerase-mediated transcription of this template cDNA (12). There are several types of RT-PCR and the most precise one in terms of quantification is the real-time RT-PCR method, which requires an automated system able to detect fluorescence induced during the RT-PCR reaction (12). During RT-PCR, the expression of a certain gene can be deduced by comparing it to constitutively expressed genes, also known as “housekeeping” genes.

cDNA microarray technology, which analyzes the gene expression levels of thou-

sand of genes simultaneously, is based on the same principles as those for both Northern blot and RT-PCR analyses (7,8). Essentially, cDNA microarray is the hybridization of thousands of genes from cDNA to their corresponding targets on a chip or filter. However, the current challenge of this technology is to analyze the substantial amounts of raw numerical data obtained through the acquisition of hybridization signals (13). Computational and biostatistical analyses are necessary to accurately process significant data for the specific aims of the investigation (Figure 1).

Producing a cDNA microarray matrix

Recently, several companies have designed cDNA microarrays focused on specific gene expression profile systems. These matrices are commercially available by biotechnology companies, including SuperArray Bioscience Corporation and Affymetrix, for use at affordable prices. In this section, we attempt to describe the basic steps in the production of a cDNA microarray matrix.

The first step for producing a cDNA microarray matrix is selection and amplification of total or partial fragments of cDNA sequences to be printed on substrates, which will be subsequently hybridized with target cDNA sequences (14,15). The most common methods for selecting sequences are based on the following resources: 1) gene data banks such as Unigene, dbEST and GenBank, which give information about the specific gene's function and chromosome localization; 2) clone collections or available cDNA sequences in website homepages of commercial enterprises; 3) custom-made constructions of cDNA libraries from target cell or tissue material previously cloned and sequenced for EST identification. Once selected, cDNA fragments are amplified by PCR on multiwell microplates, purified by chemical precipitation, and then examined for quality and quantity by gel electrophore-

sis. The PCR products are printed onto a slide or membrane using a robotic machine, the microarrayer. During the robotic printing, or deposition, of the DNA, capillary tubes receive a constant pressure and serially deposit the DNA on the slide or membrane, thereby creating a "microarray" design (16). Various types of substrates, including glass slides and nylon membranes are pre-treated to augment their hydrophobic charges, thus resulting in an increase in the total adherence of DNA sequences to the substrates (13). Most importantly, data indexing containing the location of thousands of the cDNA clones printed on the substrates is performed by cautious labeling of each microplate using software designed for database analysis. This database also includes the clone identity (clone ID), the gene name, chromosome location, and gene function(s).

Hybridization of the cDNA probe with the target DNA microarray matrix

A crucial factor for successful hybridization of the cDNA probe to the target DNA microarray matrix relies on the quality of the mRNA from cells or tissues. Contaminants

of the probe RNA with genomic DNA, proteins, or detergent residues may result in false-positive/false-negative data.

Labeling the probe during cDNA synthesis by reverse transcription of RNA sequences depends on the type of cDNA microarray being used (16). In general, when cDNA microarray analysis is performed using nylon membranes as the substrate, the probe RNA is radioactively labeled by incorporation of dCTP- P^{33} nucleotides during the process of reverse transcription (7). In the case of a cDNA microarray printed on glass slides, radioactive nucleotides (17) and fluorescent dyes with high efficiency rates of incorporation, such as the cyanines Cy-3 dUTP and Cy-5 dUTP, are used (8). For more information about types of cDNA microarrays and the hybridization process (7,8,17-20), see Table 1.

Analysis tools for the identification of gene expression patterns using cDNA microarray technology

Tools for the analysis of the massive amounts of data generated from cDNA microarrays are still being developed. Currently, investigators analyze cDNA micro-

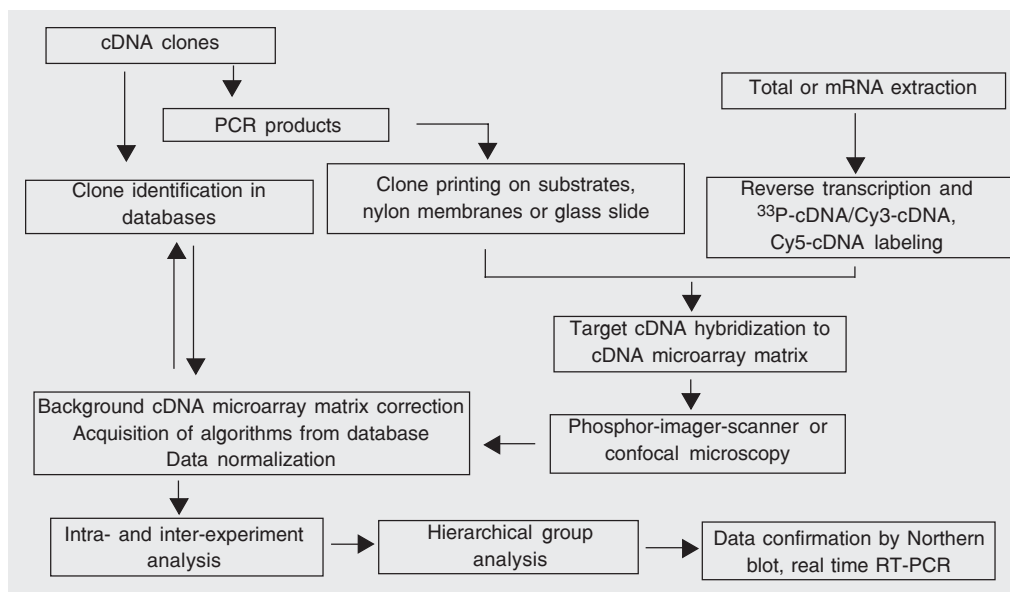


Figure 1. Model of methods used in cDNA microarray technology.

array data using diverse software programs (21-24). This means that there is no single method for analyzing the massive quantity of data obtained using this technology. The use of biostatistical tools has become increasingly important in analyzing the significance of gene expression profiles.

To provide a general idea about some of the types of methods used to analyze cDNA microarray data, we will describe and discuss steps that could hinder the analysis process. Of the utmost importance is the validation of results by completing multiple replicate experiments. After acquisition of the hybridization signals, the substrates are examined visually using computational programs. In this case, the quality of the substrates is considered. For example, fuzzy spot images, dye precipitates, or samples with strong background signals are usually

discarded. This analysis is important to eliminate artifacts, which can result in the detection of false-positive signals (16). Various investigators apply the method of background subtraction of the hybridization signals to the nonspecific signals in the background. In this step, depending on the software used, it is possible to subtract background signals in the following two ways: 1) by the arithmetic mean or median resulting from the signal intensity between the positive signals of spots in the substrate, or 2) by the arithmetic mean or median of the nonspecific signal, such as the signal intensity resulting from the immediate area around each spot corresponding to a specific gene signal. By these two methods, the arithmetic mean or median values are subtracted from the positive signal representing a differentially expressed gene (16). The acquisition of positive signal intensity

Table 1. Types of cDNA microarrays.

Substrate	Quantity of probe RNA	Probe RNA labeling	Hybridization signal detection	Other observations
Glass slide pre-coated with poly-L-lysine (8,18,19)	50-200 µg total RNA or 2-5 µg mRNA (8,18,19)	Fluorescent dyes: Cy-3 and Cy-5 (8,18,19)	By signal intensity fluorescence emitted using Cy-3 and Cy-5, which presents: •Photo stability •Distinct emission and excitation spectra •Signal discrimination by optical filtration using laser confocal microscopy (8,18)	It is possible to hybridize two distinct cDNA sequences, using distinct dyes, in a unique cDNA microarray matrix (8,18,19) Fluorescent arrays allows the comparison of mRNA levels obtained from two different sources (8,18,19)
Glass slide pre-coated with poly-L-lysine (17)	1-10 µg total RNA (17)	Radioisotopes: ³² P or ³³ P (17)	By exposure of the radioactively labeled slides or filters to a photo-sensitive film (7,17,20)	In radioactive arrays it is possible to hybridize only one target cDNA sample to a unique cDNA microarray matrix (7)
Nylon membranes (7,17)	5-15 µg total RNA (7,17,20)	Radioisotopes: ³² P or ³³ P (7,17,20)	Acquired image using a laser phosphor-imager-scanner (7,17,20)	Hybridization to radioactive-based microarrays requires lower hybridization volume and produces lower background of non-specific hybridization than does radioactive hybridization to nylon-based arrays (7,17,20) Higher sensitivity in relation to the fluorescent microarray for hybridization signal detection (17)

References are indicated in parentheses.

in grids of localization created using specific software is completed in arbitrary values known as algorithms. These values can be accessed directly in the database software, which contains previously catalogued information of each gene printed on the substrate.

Due to the enormous quantity of simultaneously analyzed genes and the variability in all of the technical steps of the cDNA microarray technique, it is important to create a factor of adjustment or compensation, which is done through a data normalization process. The existence of nonspecific signals on the substrates, problems related to quantity or quality of the RNA prior to hybridization, or variability in probe incorporation of the RNA can all result in the impairment of sample normalization (14,16). For further meaningful interpretation of the data, the normalization process performs a comparative analysis of the arithmetic mean of the algorithms from an individual group with another, frequently chosen as a control (24,25). There are several types of normalization methods and selecting the most appropriate method can be a challenge. The reason for this is that multiple experimental variables often prevent the accurate identification of differentially expressed genes without false-positive/false-negative results contaminating the evaluation of the data. Mathematical, statistical, and bioinformatic support is critical at this stage in cDNA microarray technology.

Normalization methods include: 1) the logarithmic ratio of measured expression levels between distinct groups based on the mean, median, median differences, or variance obtained from all the positive signals located on the substrates; 2) regression analysis, which is evaluated through the correlation coefficient or Euclidian distance, and is based on the distribution represented by the algorithms corresponding to the signal intensities in the distinct samples of the same group, and 3) statistical tests such as the *t*-test or associative analysis, which consider

multiple paired comparisons based on the significant threshold of $P < 0.05$ and *t*-test threshold significance correction, respectively (25,26). In addition, the locally weighted linear regression (lowess) normalization method, frequently used for fluorescent arrays, accounts for decreasing variations of intensity hybridization signals and spatial location of spotted cDNA on the slide (27).

After normalization, the simplest method to identify differences between patterns of gene expression is to use the ratio difference between a sample and its appropriate control. In this case, an arbitrary limit is established and from this value it is possible to select the differentially expressed genes using database software. For example, it is possible to establish a 2-fold threshold to identify genes with significant changes in expression level. During this process, the genes within this range will be selected and organized in tables located in the database. The genes meeting the selection criteria can be visualized from databases using software that shows self-organizing maps (28,29). Furthermore, these changes in the gene expression profiles between distinct groups can be visualized in dendrograms based on hierarchical clustering analysis (25,29,30). In this case, genes that are down-regulated are generally shown in green and up-regulated genes are generally shown in red. As the gene regulation difference is more intense, so is its respective color. Software programs to analyze cDNA microarray data are available via the Internet.

It is important to note that the analysis of gene expression profiles acquired by cDNA microarray technology involves thousands of genes being analyzed concurrently. Before doing physiologic studies of selected genes, the cDNA microarray results should be confirmed at the RNA level by Northern blot analysis or real time RT-PCR (14). This eliminates the possibility of false-positive cDNA microarray results due to cross-hybridization between genes within the same

gene family. Further confirmation is also highly recommended at the protein level, such as by Western blot analysis, flow cytometry or immunohistochemistry. In this context, it is currently possible to evaluate genes at the protein level using tissue microarray assays (31-33). Tissue microarray assay is a method that allows the evaluation of up to one thousand tissues at once using a specific marker. Basically, small core biopsies of 1 mm or less are isolated from individual paraffin-embedded tissue blocks with a special metallic device or needle, and placed in another paraffin block in an arrayed manner. The advantages of this technique are: 1) the economy and preservation of the original tissue specimens obtained for biopsy to construct the array; 2) to avoid technical problems since the array is constructed in a single glass slide, thus facilitating the washing and staining technical processes, and 3) the speed to perform *in situ* analysis simultaneously in several tissue specimens at one time. Clinically, it is a powerful technique to use conjointly with cDNA microarray analysis to identify novel biomarkers for potential use as diagnostic, prognostic and therapeutic tools against pathological conditions (31,32).

cDNA microarray technology: applications and perspectives

In terms of application, cDNA microarray technology leads to the identification of specific genes and allows researchers to compare the profiles of gene expression in normal versus pathological conditions in various organisms. In basic science, cDNA microarray has been used to identify the role of several genes involved in cellular processes, such as cellular differentiation, through the comparison of gene expression patterns between normal and transfected cell cultures, for example (34). Many research groups have used cDNA microarray technology to study distinct types of cancers as well as the pathogenesis of infectious diseases (35-40).

In this case, identification of novel genes or changes in the gene expression patterns in tissues from non-diseased to diseased state has contributed to better understanding the molecular mechanisms regulating onset and progress of diseases. Additionally, cDNA microarray has been considered a very interesting methodology in pharmaceutical industry. In this context, the analysis of gene expression profile in large scale of cells submitted to distinct drug tests could be relevant to classify potential agents for therapeutic use (41). Several examples of the application of cDNA microarray methodology in basic and clinical sciences (34-48) are described in Table 2.

cDNA microarray analysis is a costly methodology, but the abundance of results generated from this technology is an unquestionable payoff. Once a central research unit is organized for the development of this methodology, various researchers can enjoy the advantage of using previously printed cDNA microarray substrates to investigate differentially expressed genes in both biological and clinical areas. For the organization of cDNA microarray units, the multidisciplinary interaction between mathematical and biological arenas must be considered. A simple hypothetical schematic model demonstrating the infrastructure of a cDNA microarray unit is shown in Figure 2.

Finally, centralized banks containing data of gene expression profiles analyzed by cDNA microarray technology have been created and are available for inspection via the Internet (49,50). In this way, different groups all over the world will be able to compare data of selected gene profiles with others deposited in molecular data banks such as Gene Expression Omnibus or Array Express, designed by the European Bioinformatics Institute (51).

A problem that has restricted a beneficial interaction between various research groups using cDNA microarray technology is the lack of standards for presenting and exchanging

Table 2. Applications of the cDNA microarray technology in basic and clinical sciences.

Life sciences area	Application	References
Endocrinology	Identification of genes involved in cellular differentiation using transfected cells	34
	Analysis of the hormone-regulatory effects on the gene expression in various tissues in the organism	42
Microbiology	From the complete genome sequencing of microorganisms: to analyze their pattern of gene expression to understand their functionality for identification of genes important to the development of drugs and molecular inhibitors of their mechanisms of pathogenesis	35
Immunology	Analysis of the gene expression of the immune system in normal, autoimmune and infectious disease conditions	36,43,44
Oncology	Analysis of the gene expression profiles to identify genes involved in the process of cancer development	37
	Analysis of the gene expression profile for cancer classification and diagnosis	38
	Comparative analysis of the gene expression profiles between benign and malignant tumors	39
	Genomic approaches to the management and treatment of breast cancer	40
Toxicogenomics	Analysis of gene expression profiles of cells exposed to new drugs destined to medicinal use	41
Development	Comparative analysis of gene expression profiles in distinct organisms such as bacteria, yeast, nematodes, <i>Drosophila</i> fly, mice	45
Genetics	Analysis and identification of specific mutations in individual, family or population genomes	46
Gerontology	Comparative analysis of the gene expression profile in tissues from distinct age periods for the identification of genes involved in the processes of aging and age-related diseases	47,48

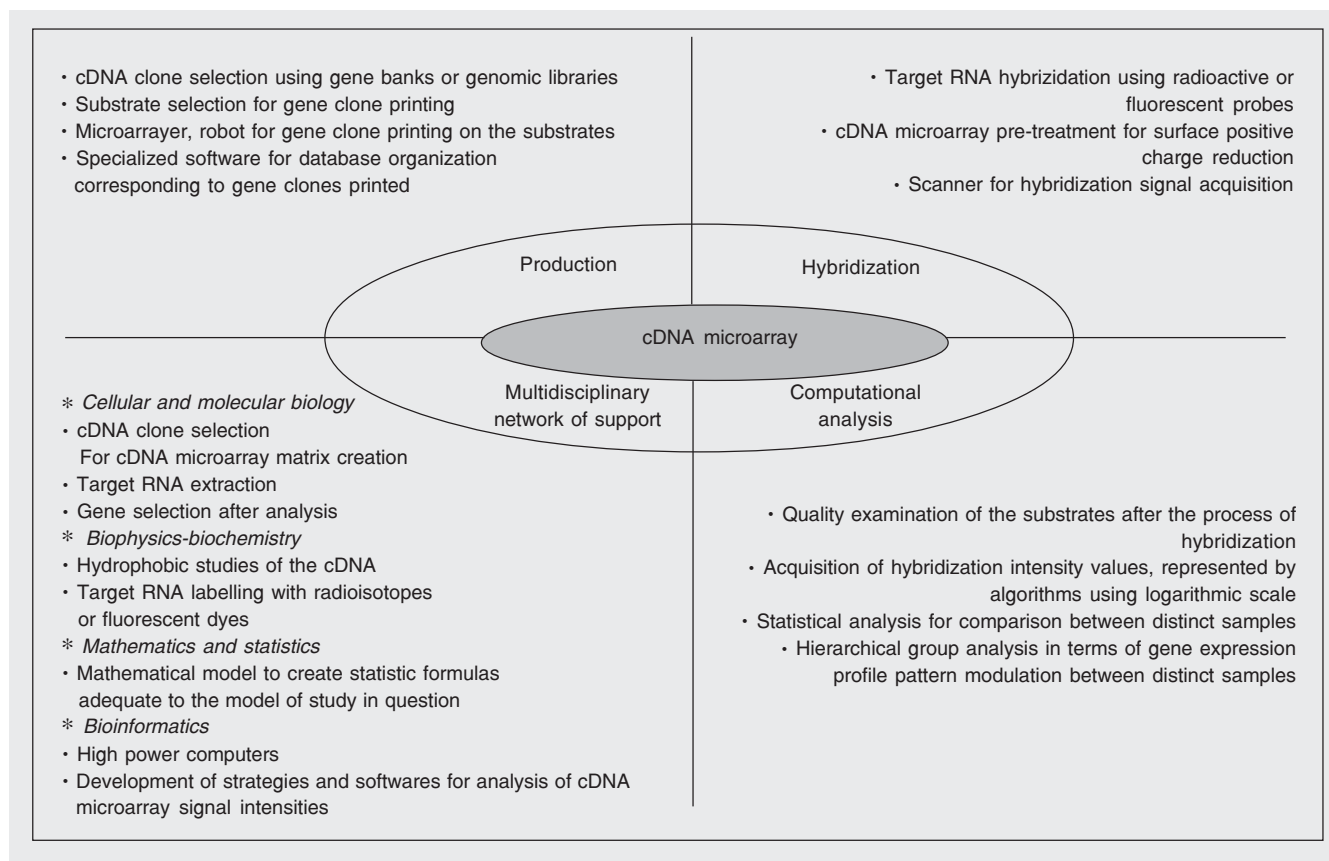


Figure 2. Model of the infrastructural organization of a cDNA microarray unit.

ing results. To compensate for this predicament, the Minimal Information for the Annotation of a Microarray Experiment (MIAME) was created (52). Guidelines leading to the standardization of cDNA microarray experiments have been proposed by the Microarray Gene Expression Data Society (MGED). MIAME is basically composed of six sections: 1) experimental design; 2) array design; 3) samples used, extract preparations, and hybridization; 4) procedures and parameters of hybridization; 5) image, quantification, and parameter measurement, and 6) types, values, and specifications for normalization controls. MIAME has become a requirement to publish cDNA microarray results in many scientific journals. Additionally, distinct groups including MGED, Rosetta, Agilent, and Affymetrix have worked together using bioinformatics to design microarray gene expression (MAGE), which is a common flexible structure platform that permits communication between different research groups using cDNA microarray technology. MAGE is composed of the following: 1) MAGE-ON (object model), a centric data model that uses unified modeling language and implements the MIAME requirements; 2) MAGE-ML (markup language), which documents translation from MAGE-OM to an XML-based data format, and 3) MAGE-SLK, a freely accessible software that defines the application programmers interface to MAGE-OM, thus allowing data export to MAGE-ML and subsequently resulting in the storage of data in a relational database (53).

Recently, nanotechnology has been widely implemented in various arenas, such as industrial, pharmaceutical and governmental applications, to improve modern scientific advancements. Due to the unique physical and chemical characteristics of nanoparticles, these materials have the ability to enhance sensory devices, propulsion additives, tissue remodeling techniques, and drug targeting mechanisms. Furthermore, nanotechnology has been utilized to improve microarray proficiency (54) and to assist in the development of protein nanoarrays (55). Within the laboratory setting, scientists are now beginning to investigate the potential positive/negative impact that nanoparticles have on the induction/inhibition of genes within cells by using the microarray technique (56). Finally, the combination of nanotechnology with microarray technology has even advanced the current knowledge of genes differentially regulated during disease processes (57,58).

Together, these tools can contribute to the understanding of novel gene functions in diverse genomes and of how they correlate with disease pathogenesis in humans. The development and application of cDNA microarray technology have brought a lot of progress to the discovery of drugs, research diagnostics, and potential gene therapy destined to the treatment of various diseases. This fundamental technology assists researchers in understanding the molecular mechanisms involved in multiple and diverse biological processes.

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Affymetrix page [<http://www.affymetrix.com/index.affx>]

Unigene, dBest, GeneBank and Gene Expression Omnibus (GEO) Page [<http://www.ncbi.nlm.nih.gov>]

Microsoft Page [<http://www.microsoft.com/Office/Excel/prodinfo/default.mspix>]

Software programs to analyze cDNA microarray data Pages [<http://www.genome.wi.mit.edu/MPR>];
<http://genome-www5.stanford.edu/>; <http://ep.ebi.ac.uk/EP/>]

Array Express Page [<http://www.ebi.ac.uk/arrayexpress>]

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