

Biomarker-Based Analysis for Contaminants in Sediments/Soil: Review of Cell-Based Assays and cDNA Arrays

PURPOSE: This technical note reviews the existing technology for cell-based biomarker assays and cDNA arrays and explores their potential as rapid, sensitive, and low-cost tools for sediment/soil toxicity screening. The current project extends the application of biomarker-based assays initiated in Technical Note DOER-C1 for dioxins and related compounds to additional sediment/soil contaminants and contaminant modes of action (U.S. Army Engineer Waterways Experiment Station 1998).

BACKGROUND: Presently, sediment evaluations and contaminant monitoring for remediation, confinement, and treatment of high-risk dredged material involve expensive and time-consuming chemical analyses, toxicity testing, and bioaccumulation testing. Sediment chemical analyses are sensitive and specific, but do not necessarily reflect the potential impacts of the contaminants on biota. Tissue residues in exposed organisms provide more relevant information by addressing the questions of bioavailability and the potential for trophic transfer. However, residues are largely limited to individual metals and organic chemicals that bioaccumulate rather than compounds that are metabolized or may be present as complex mixtures. Actual bioassays provide the most interpretable results in terms of survival, growth, and reproduction, but are time-consuming, expensive, and labor-intensive. Application of an appropriate screening assay can reduce the high cost of testing. Good screening tests must be fast and inexpensive to perform while at the same time capable of discriminating between sediments/soils that are unequivocally acceptable or unacceptable for placement in the open environment and those that are questionable and require more definitive evaluation via bioassays.

This research is directed at the further development of screening assays for contaminated sediments using transgenic cell lines and/or copy DNA (cDNA) microarrays that can be applied to extracts of both sediments and organisms living in the sediments. A one-to-one comparison of assay responses to sediment extracts and sediment-exposed organisms will allow discrimination between what is present in the sediments and what is bioavailable. While current cell-based assays offer inexpensive and rapid screening of one endpoint (e.g., dioxin equivalents), cDNA arrays offer the capability of simultaneous screening of multiple endpoints. The activation of genes related to apoptosis, tumor suppression, cell proliferation, cell cycles, cytokines, oxidative stress, and more (including dioxin activity) will be measured in a single exposure. The information provided by cDNA arrays will provide valuable insight into the toxic modes of action of the contaminants present in sediments, which in turn can be utilized to better direct the type of bioassay (e.g., survival, growth and reproduction, genotoxicity) that should be conducted on those sediments/soils requiring fuller evaluation.

CELL-BASED ASSAYS: Many stable cultured cell lines are commercially available, representing various species and organ types. The so-called "immortal" cell lines have the ability to grow and multiply without limitation, unlike normal cells, which can undergo only a limited number of divisions. Given the proper growing conditions (growth media, temperature, humidity, etc.), the immortalized cells can easily be maintained under laboratory conditions. Since the cells do not undergo

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significant alterations in biochemistry between generations, they are ideal for toxicity testing as the lack of change in cell characteristics provides consistent results. Cell cultures provide the basis for a wide variety of rapid screening assays ranging from fairly nonspecific responses (cell death) to highly specific responses (enzyme induction). Recent advancements in molecular biology such as cloning and transgene insertions have allowed the development of highly sensitive cell-based assays for extremely specific endpoints, as discussed in the following example.

P450RGS - A Model Cell-Based Assay. An excellent example of a cell-based screening assay is P450RGS, which is used for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents in sample extracts (Ang et al. 2000; McFarland et al. 1999; U.S. Environmental Protection Agency 1999). The system relies on the interaction of TCDD and TCDD-like compounds (polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and coplanar polychlorinated biphenyls) with a specific receptor, as depicted in Figure 1. In the figure, TCDD diffuses across the cell membrane and interacts with a specific receptor in the cytosol. The TCDD-receptor complex is then translocated into the cell nucleus where it binds to a specific region (dioxin-responsive element, DRE) on DNA. This process leads to the transcription of mRNA, which is then translated into protein synthesis, resulting in the production of DRE gene battery products (e.g., Cyp1A1).

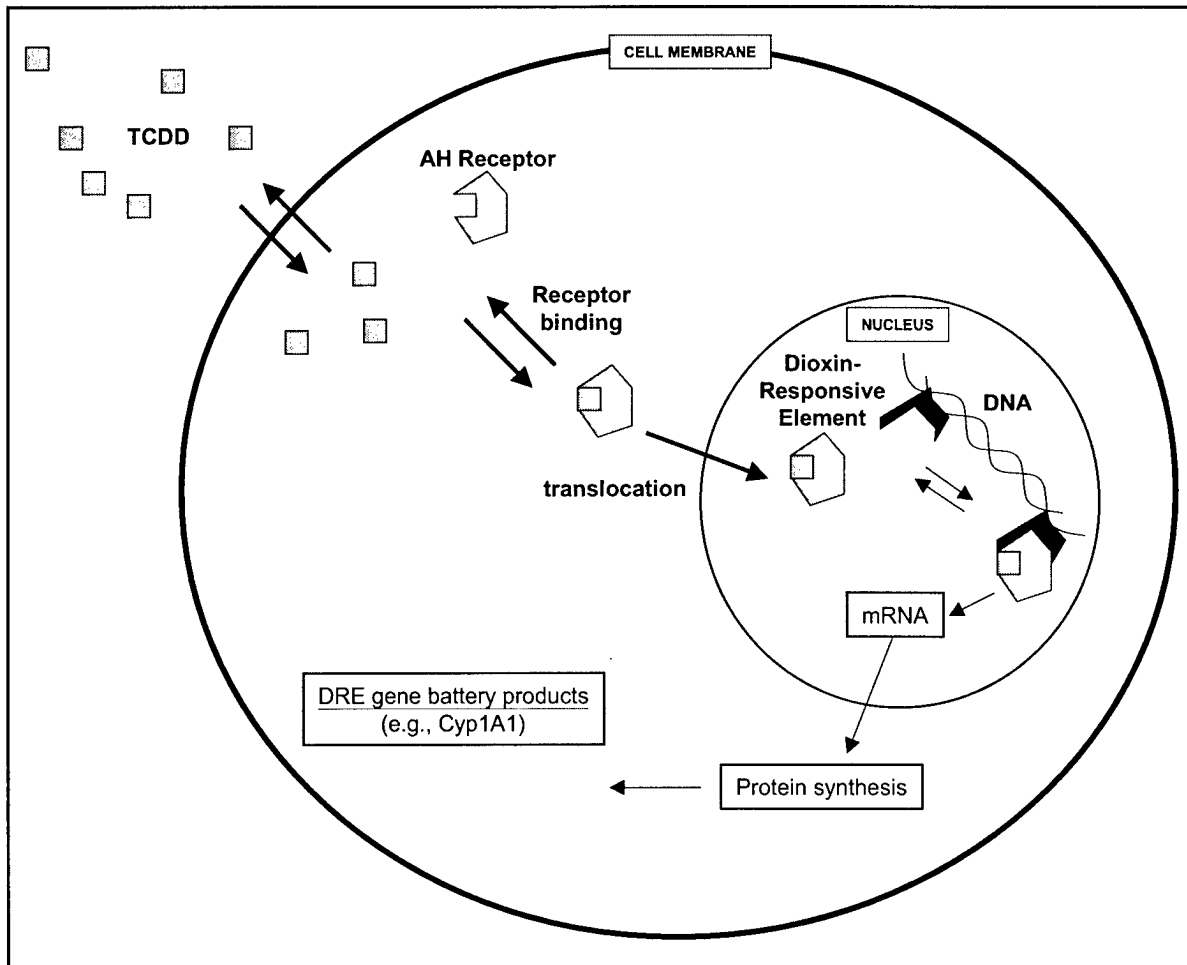


Figure 1. Simplified version of the mechanism of cellular response to TCDD, a model receptor-ligand interaction. See text for details

or DRE) on the DNA. The binding of the complex to the DNA triggers the transcription of several related genes (known as a gene battery), which code for the synthesis of a series of messenger RNA (mRNA), which in turn are translated into proteins. The P450RGS cell line has been modified by the insertion of plasmid DNA that links the DRE region to a transgene, the firefly luciferase gene. Binding of the TCDD-receptor complex to the DRE results in the production of the enzyme luciferase, which can then be quantified in a luminescence assay. The gene insertion allows for an extremely sensitive endpoint, luminescence, compared with the fluorescent endpoint typically measured for the most commonly used native TCDD gene battery product, ethoxyresorufin-O-deethylase (EROD). Although this is an example of TCDD, the general model of specific receptor interaction leading to transcription of specific gene batteries is similar for a wide variety of chemical contaminants.

The P450RGS assay illustrates the potential savings and benefits offered by cell-based assays. Although gas chromatography-mass spectroscopy (GC-MS) is the definitive method for identifying and determining the precise amount of dioxin and dioxin-like compounds present in an environmental sample, the method is expensive (~\$1,200 - \$2,000 per sample). P450RGS is far less expensive at about \$200 per sample, and its use can result in major cost savings when used to screen multiple sediment cores to determine which samples should be confirmed by GC-MS.

A recent field test of the cell-based assay demonstrated the benefits of increased site characterization (McFarland et al., in preparation). In this study, five cores were taken from each of 13 sites in a channel requiring dredging (Figure 2). Each core was sectioned into 3-ft (0.9-m) intervals to provide depth profiles for TCDD contamination (Figure 3). All samples were analyzed in triplicate with the P450RGS assay. At five of the sites, five to ten additional cores were taken and composited for GC-MS analysis. Due to the high cost of GC-MS, no replicates were analyzed. The increased coverage provided by the P450RGS assay (155 samples analyzed in triplicate for cell-based assays versus five samples with no replication for GC-MS) resulted in a detailed and statistically robust characterization of the contamination boundaries within the channel. The P450RGS results shown in Figure 3 indicated that the bulk of the contamination at sites 8, 12, and 13 was in the top layer of the material to be removed. If only the GC-MS data were used to characterize the site, most of the dredged material would be considered contaminated. However, obtaining information of

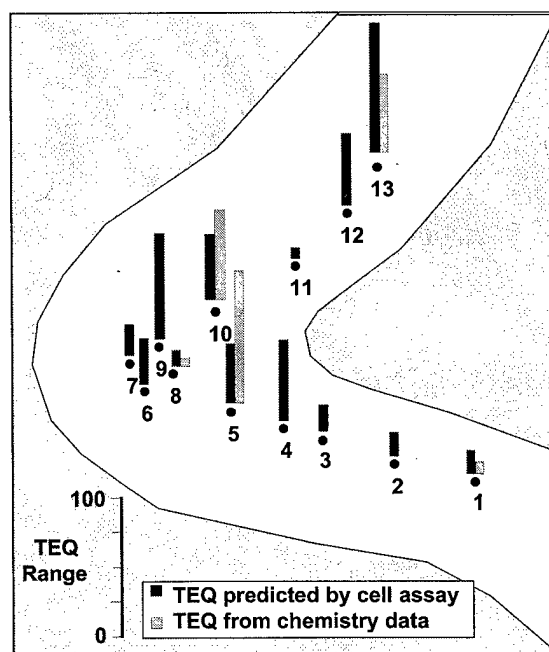


Figure 2. An example of the capabilities of the P450RGS cell-based assay for increasing site characterization. The correlation coefficient for the chemical analysis and cell-based assay data was $r^2 = 0.71$. The TCDD Equivalent (TEQ) from chemical data represents one composite sample, while the TEQ from the cell assay represents a composite of 15 to 60 individual extracts (depending on the depth of the core)

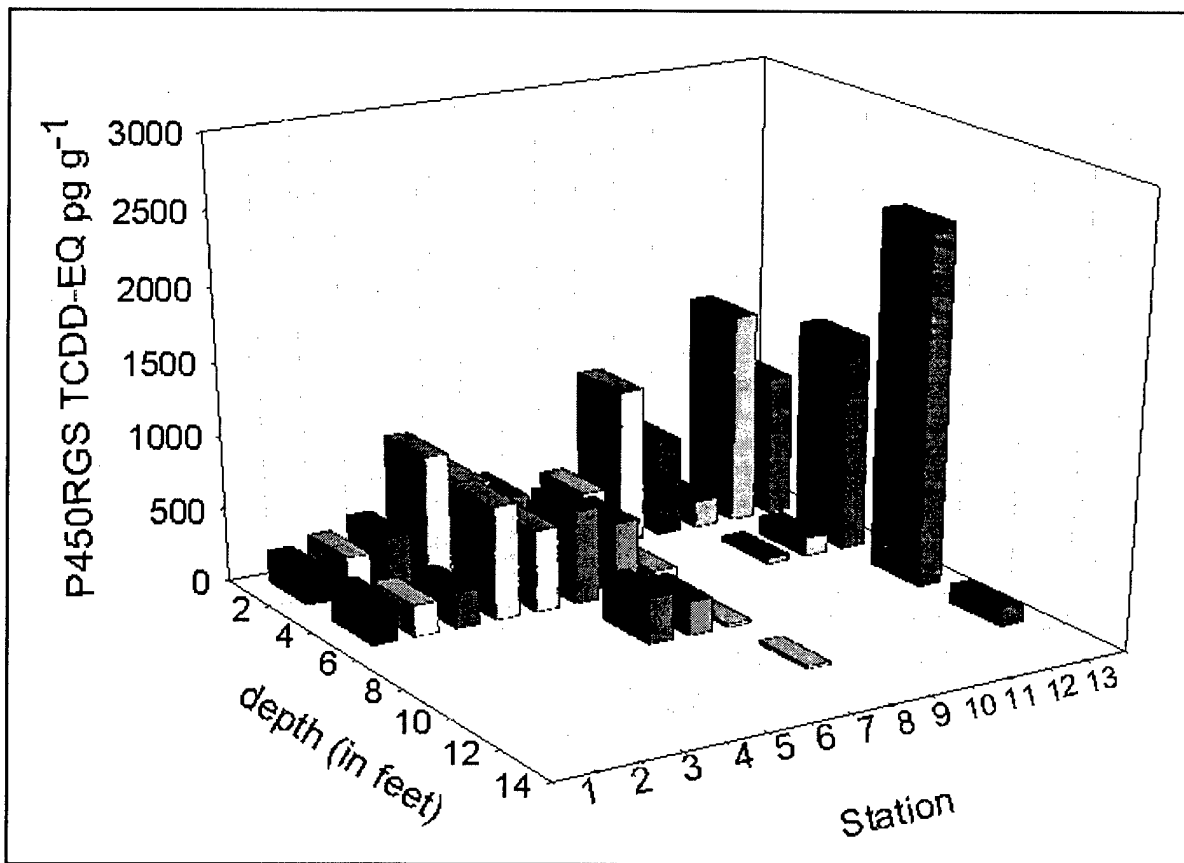


Figure 3. The depth profile of TCDD-EQ at each site as determined by the P450RGS assay. Each bar represents an average from three extracts from each of the five cores (15 samples per depth per site). Note that at several stations the contamination varies with depth; such information could not be obtained from chemical analysis alone due to prohibitively high costs

the detail obtained with this cell-based assay would have been prohibitively expensive using GC-MS, costing at least \$558,000 (155 samples with triplicate extractions, \$1,200 per analysis) compared with the \$31,000 spent on the cell-based assay (\$200 per sample, includes analysis of triplicate extractions).

Cell-based assays such as P450RGS can also be used to determine the bioavailability of contaminants, by analyzing both the media (e.g., water, soil, sediment) and organism (e.g., fish, worm, or clam) of interest. Several currently proposed projects plan on using the cell-based assay to monitor the efficacy of remediation techniques for polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins by exposing organisms to the soil or sediment before, during, and after remediation, then extracting them and analyzing the extracts with the cell-based assay.

Development of New Cell-Based Assays. Cell-based techniques similar to that of the P450RGS assay can also be developed for other contaminant classes. The metal-responsive metallothionein gene is characterized well enough that a cell-based assay can be developed in a manner similar to the P450RGS system (Iyengar, Muller, and Maclean 1996). Another class of

compounds of interest is endocrine disrupters; various cell-based assays already exist for mammalian and invertebrate endocrine disrupters (Routledge and Sumpter 1996; Oberdorster et al. 1999).

By using gene-splicing techniques, it is possible to develop reporter gene systems in which different receptor-ligand interactions trigger the production of different reporter genes. There are many gene products that offer simple assays that can be adapted to produce inexpensive, sensitive, and rapid assays. One of the original reporter gene assays, chloramphenicol acetyl transferase (CAT), is based on comparison of acetylated and nonacetylated products of a radiolabelled substrate. For the most part, this system has been replaced by nonradiolabelled assays that depend on fluorescent, luminescent, or colorimetric endpoints (Figure 4). Each of these endpoints is detected in a different manner. In fluorescence detection the reporter genes produce a protein that absorbs light at one frequency and emits it at a different frequency. In contrast, protein products of luminescent reporter genes produce light via an enzymatically catalyzed chemical reaction when provided an appropriate substrate. Colorimetric assays also require the addition of appropriate substrates, which produce highly colored products via the reaction catalyzed by the reporter gene product. Due to the differences in how

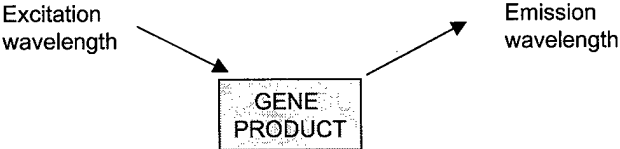
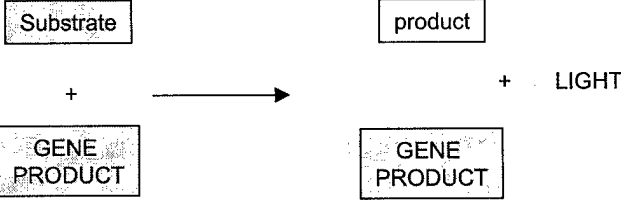
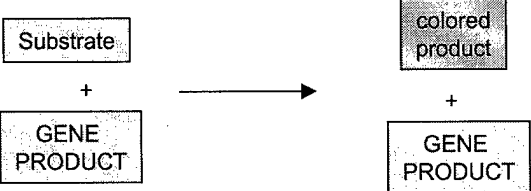
<p style="text-align: center;">Fluorescent</p> 	<p style="text-align: center;">EXAMPLES</p> <p><u>Genes:</u> ECFP, EGFP, EYFP</p> <p><u>Gene products:</u> (Fluorescent proteins)</p> <p>Cyan: ex.= 433/ em=475 nm green: ex.= 488/ em=507 nm yellow: ex.= 513/ em=527 nm</p>
<p style="text-align: center;">Luminescent</p> 	<p style="text-align: center;">EXAMPLE</p> <p><u>Gene:</u> Luc</p> <p><u>Gene product:</u> Firefly Luciferase</p> <p><u>Substrate:</u> luciferin</p>
<p style="text-align: center;">Colorimetric</p> 	<p style="text-align: center;">EXAMPLE</p> <p><u>Gene:</u> lacZ</p> <p><u>Gene product:</u> b-galactosidase</p> <p><u>Substrate:</u> X-gal (forms intense blue color)</p>

Figure 4. Common endpoints for cell-based assays

the gene products can be detected, it is possible to design the assays such that multiple endpoints can be obtained on the same cell sample. Fluorescent endpoints can be run without addition of reagents, and luminescent and colorimetric assays will not interfere with each other if the luminescent assay is conducted first. Thus, it is possible to construct a cell line that can monitor for several classes of contaminants simultaneously.

Current Status. Current research is focused on developing an assay for the detection of compounds that interrupt the hormonal cycle of arthropods (insects and crustaceans). Interference with the interaction between ecdysone (a molting hormone) and its receptor can lead to the inability to grow and reproduce properly, potentially leading to a decline in the population. Since these animals are important components at the base of both the terrestrial and aquatic food chains, a significant decline in population can affect the entire ecosystem. If compounds that adversely affect this hormonal system are present in dredged sediments, they present a potential problem for both dredging and disposal. At this time, no valid assays or tests are available to assess the effects of endocrine disrupters. The current project will lead to the development of a screening assay for ecdysone-disrupting compounds in sediment extracts. A reporter gene system has been obtained (Invitrogen's ecdysone-inducible mammalian expression system), and the genes are being inserted into the hepG2 cell line (the same cell line used in the generation of the P450RGS cell line). At the time this technical note is being written, the first of two plasmids required for this system has been inserted into the cells via electroporation. The gene insertion has been confirmed by both polymerase chain reaction (PCR) of the ecdysone receptor gene and by the zeocin resistance conferred by the plasmid. The second plasmid will be inserted once the transformed cells recover from the electroporation and pass through several generations (to confirm stability of the initial gene). Once the second plasmid is inserted, the response of the cell-based assay will be tested with several compounds known to interact with the system, as well as several environmental contaminants suspected of interfering with the system. In a collaborative effort with other projects at the U.S. Army Engineer Research and Development Center, the assay will also be validated against a whole organism assay (crawfish molting) and a computer modeling program that is designed to determine the potential receptor-ligand interaction of a chemical.

DNA ARRAYS: An alternative approach for monitoring multiple toxicity endpoints simultaneously is the use of DNA arrays. The DNA array technology takes advantage of the recent explosion in biotechnology. Both normal cell function and ability to adapt to changing conditions at a cellular level by increasing or decreasing the production of the various proteins critical to cell survival are controlled at the DNA level. Basically, signals are sent to the DNA to increase or decrease active transcription of genes into various mRNA, which then direct the cell to produce proteins. Theoretically, if one could inventory the mRNA in a cell, a snapshot of all the proteins currently being synthesized within the cell would be obtained. Briefly, a cDNA array consists of complementary DNA (cDNA) of known genes bound to a substrate (the array). These can then be utilized to determine the type and concentrations of the expressed mRNA by essentially extracting the mRNA from the cells and binding them to the array. Knowing what is being synthesized at a given moment would enable the interpretation of how cells are responding to toxicant exposure. Conversely, if how cells respond to different classes of toxicants can be characterized and quantified, then given an unknown mixture such as a sediment extract, the toxicants present in the sample may be able to be identified and quantified.

In order for DNA arrays to be useful as toxicological screening tools, one must know what the various mRNAs are signaling the cell to synthesize. Until recently, this information simply did not exist. However, the human genome project and other genome projects have made great strides in sequencing and identifying the genetic material in organisms, making it possible to identify huge numbers of genes and the products they encode. Although the number is rapidly changing due to the intensive human genome efforts, as of July 2000 over 35,000 human genes have been sequenced and connected to a known gene product, and over 1.7 million Expressed Sequence Tags (ESTs), representing either novel genes or genes that encode for products homologous but not identical to those of known genes, have been identified (UniGene human release statistics, July 2000).

There are various approaches to measuring total expressed mRNA, including microarrays and cDNA arrays. Microarrays, also known as DNA chips, can analyze extremely high numbers of sequences. However, these arrays typically include many nucleotide sequences associated with genes of unknown functions. Since the objective of this work unit (DOER 12B23) was to develop a rapid and inexpensive screening method for sediment toxicity, it was decided to concentrate on the cDNA arrays, which use only genes of known function. A cDNA array is composed of a substrate (nylon membrane, silica chip, or glass slide) upon which nucleotide sequences of known genes are bound to specific locations (Figure 5). Arrays can be created to analyze anywhere from a few genes to over 50,000 (GeneAlbum, made by Incyte Pharmaceuticals). Briefly, to analyze the relative quantities of the various genes being expressed, mRNA is extracted from samples (control and treated), converted into cDNA, and then bound to the arrays (Figure 6). Control and treated samples can then be compared, and the differences in gene expression induced by the treatment can be identified and quantified.

The cDNA Array Assay. Clontech produces a commercially available cDNA array with up to 1,176 known human genes, grouped by their known functions. Their human toxicology array includes 588 genes known to be involved with toxicological responses, for example, genes linked to DNA synthesis/repair, stress responses, and tumor suppression or induction (Figure 5). In order to monitor responses to toxicant exposure with cDNA arrays, the mRNA is extracted from the samples and converted into cDNA (Figure 6) using PCR. The extracted mRNA is placed in a tube along with "primers," or short DNA sequences (oligonucleotides), which bind to complementary sequences on the mRNA targets. An enzyme then binds to the primer/mRNA complex and makes a complementary copy of the mRNA sequence. This step increases the stability of the sample, as cDNA is much more stable than mRNA, which is easily degraded by ubiquitous enzymes (RNases) that are difficult to eliminate from labware. Additionally, the cDNA can be labeled during this step (radiolabel, fluorescent, or chemiluminescent tags), allowing the detection of the sample after it is bound to the array. Once the mRNA has been extracted and converted to cDNA, it is ready to be bound (hybridized) to the cDNA array. The sample is exposed to the array for several hours, during which the cDNA of the sample binds to that of the array in a complementary sequence-specific manner. The amounts of the original mRNAs are then quantified by the amount of the label detected at each spot on the array. Currently, radiolabel (^{33}P) is the most sensitive and most utilized tagging option for membrane-based arrays, although progress is being made for nonradiolabel methods such as fluorescence and chemiluminescence. In the Clontech array system, alterations in the mRNA expression profile are tracked by comparison of two nylon membrane arrays (one control sample, one treated sample).

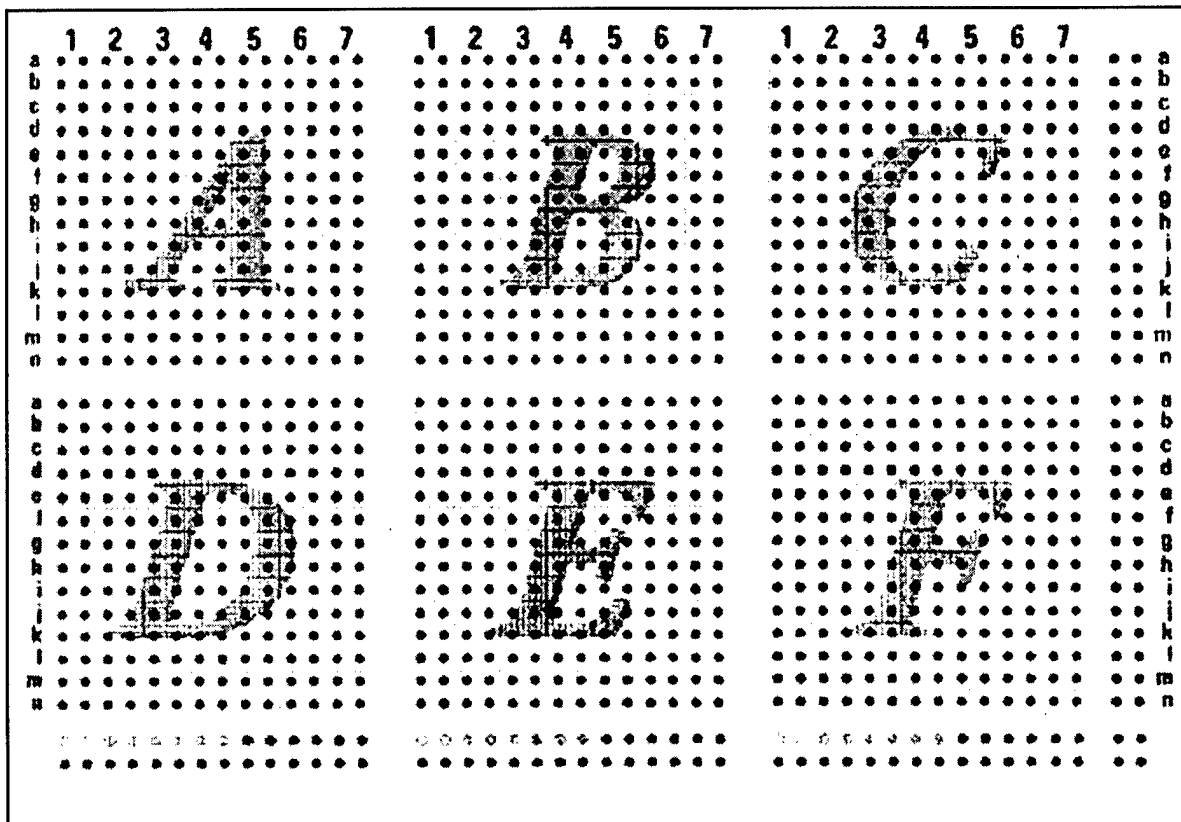


Figure 5. An example of a cDNA array: the Clontech Human Toxicology Array II. Each spot represents a selected toxicologically related gene bound to a nylon membrane. Genes in area A are related to oncogenes, tumor suppressors, cell cycle regulators, and ion channels; area B contains genes related to transporters and signal transduction; area C contains genes related to apoptosis, DNA synthesis, repair, and recombination; area D contains genes related to the nervous system and transcription factors; area E contains genes related to various receptors (e.g., growth factors, hormones), cell adhesion, and general DNA binding proteins; area F contains genes related to stress response, cell signaling/cell-cell communication, and protein turnover

Current Status/Future Directions. At this point, only preliminary trials utilizing the Clontech membrane arrays have been conducted, with initial attempts made to develop a chemiluminescent endpoint rather than the standard radiometric endpoint recommended by the company. Although the results were not promising (high background, low sensitivity), Clontech has recently released a glass array for which chemiluminescent and fluorescent endpoints have been developed. These new developments will be used in conjunction with the hepG2 cell line to develop profiles of genes responding to various common pollutants encountered in dredged materials, as well as the magnitude of the response in relation to the dose. This information, along with the response of the array to dredged material extracts, can eventually be utilized to tailor an array with specific genes of interest that provide data that best fulfill the U.S. Army Corps of Engineers risk assessment needs.

CONCLUSIONS: By analyzing the response of cells to extracts of sediment and/or tissue, cDNA arrays will provide a far better measure of exposure and effect for risk characterization and input to

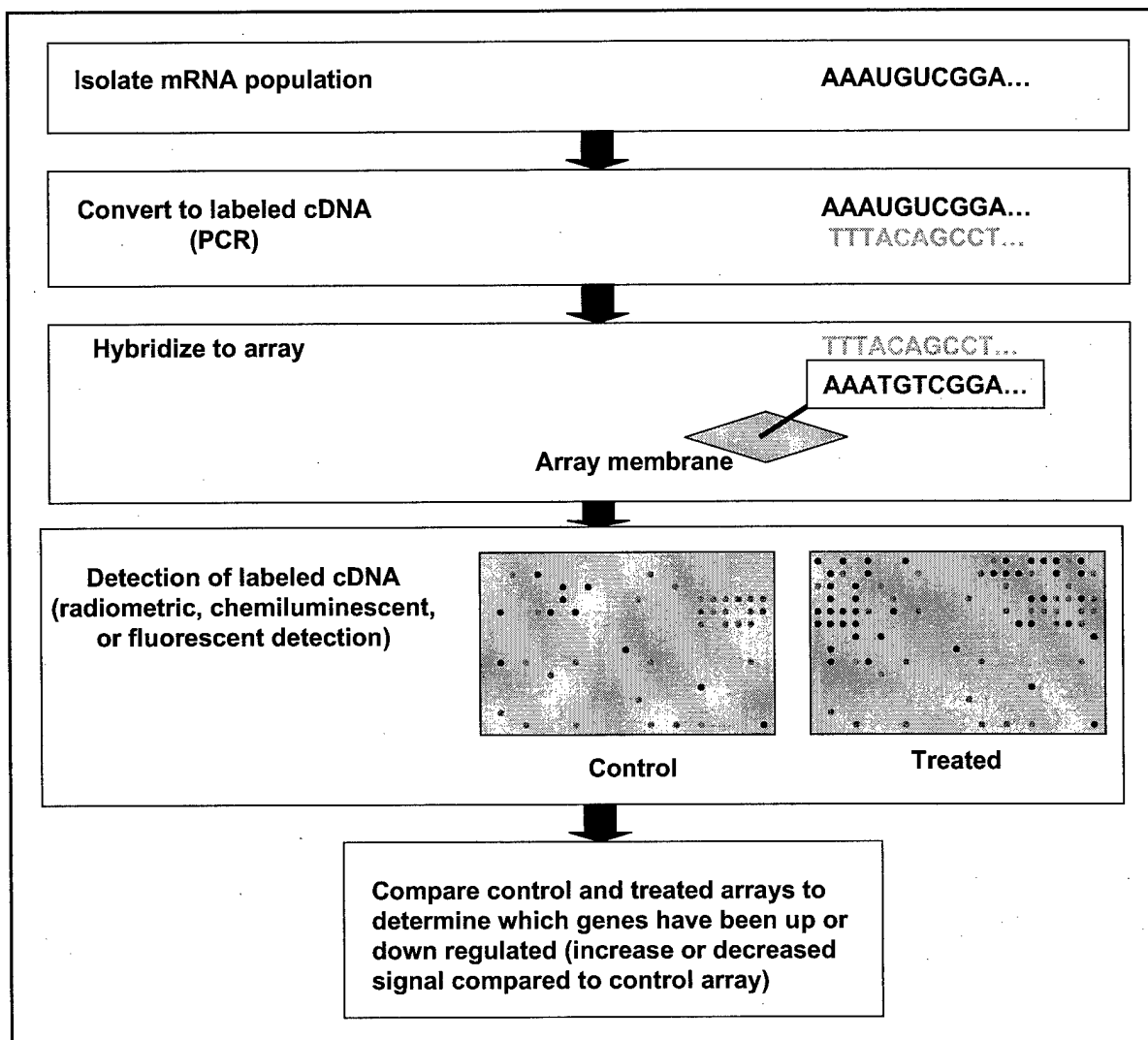


Figure 6. An overview of the cDNA array assay. The mRNA populations are isolated from control and treated cell cultures (a partial sequence shown to the right as an example), converted to tagged cDNA (shown in green), and hybridized to the cDNA bound to the array (one of the genes on the array shown in blue)

models for risk assessment than is presently possible (Corton et al. 1999; Nuwaysir et al. 1999; Rockett and Dix 1999). The mechanistic information obtained from the arrays can also be valuable in determining which samples require further examination with more time-consuming and expensive bioassays, and can direct the type of bioassay (e.g., survival, growth and reproduction, genotoxicity) that should be conducted. Additionally, use of the cDNA arrays to test sediment extracts can help direct the development and/or use of other assays. For example, screening of typical dredged sediments with cDNA arrays may indicate that certain endpoints (e.g., metal-responsive genes) consistently show positive responses and warrant further evaluation with cell-based assays, which can provide high throughput screens due to their compatibility with the 96-well microtiter plate format. Alternatively, initial screening of a site with cDNA arrays may indicate that a certain contaminant class is of concern, whereupon the appropriate cell-based assays can be utilized to

further characterize the extent and distribution of the contamination, in a manner similar to that of the recent field trials of the P450RGS assay.

Finally, by analyzing extracts of organisms living at contaminated sites, both cDNA array and cell-based assays are capable of discriminating between what is present in the media (sediment, soil, water) and what is available to the organisms, thus offering substantial savings over the use of chemical analysis and chronic whole-organism bioassays.

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Inouye, L. S., and McFarland, V. A. (2000). "Biomarker-based analysis for contaminants in sediments/soil: Review of cell-based assays and cDNA arrays," *DOER Technical Notes Collection* (ERDC TN-DOER-C19), U.S. Army Engineer Research and Development Center, Vicksburg, MS. www.wes.army.mil/el/dots/doer

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