# BIOANALYTICAL APPROACHES FOR THE DETECTION OF DIOXIN AND RELATED HALOGENATED AROMATIC HYDROCARBONS

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**Introduction**

Halogenated aromatic hydrocarbons (HAHs), such as polychlorinated dibenzo-p-dioxins (PCDDs), biphenyls (PCBs) and dibenzofurans (PCDFs), represent a large group of compounds which because of their ubiquitous distribution, resistance to biological and chemical degradation, high toxicity and potential for bioaccumulation/biomagnification, can have a significant impact on the health and well being of human and animals (1,2). HAHs have been identified worldwide in a variety of wildlife, domestic and human tissues as well as in food, water, and soil samples. Given these issues, the detection and quantitation of these chemicals in biological, environmental and food samples is of paramount importance. Since HAHs are found not as individual congeners, but as complex HAH mixtures, one problem in the evaluation of risk to HAHs is the identification and quantitation of the toxic/bioactive HAH congeners in a given sample. Although chemical extraction procedures coupled with high resolution gas chromatography-mass spectrometry (HRGC/MS) is considered the gold standard for the identification and quantitation of individual PCDD, PCB and PCDF congeners (3), these procedures are very costly and time-consuming. Accordingly, a variety of rapid and inexpensive screening bioassays capable of detecting and estimating the relative potency of complex mixtures of HAHs have been developed by our lab and others (4-8). These bioanalytical methods are based on the ability of the compounds to be specifically recognized and bound by antibodies (immunoassays) or their ability to transduce a specific biological response in vitro or in cells in culture (bioassays). Current bioassays are based on the mechanism of action of TCDD and related HAHs and utilize the Ah receptor (AhR), a ligand-dependent factor which mediates both the effects of these chemicals (1,2). One major HAH bioassay (**C**hemically **A**ctivated **Lu**ciferase Expression (CALUX)) utilizes a cell line that contains a stably integrated AhR-responsive luciferase reporter gene and this system takes advantage of the ability of the AhR to activate gene expression in a ligand-dependent manner. Exposure of these cells to extracts containing TCDD and/or related HAHs results in the induction of luciferase gene expression in a time-, dose- and chemical specific manner (6). The second is an in vitro assay which measures the ability of a chemical (ligand/agonist) to bind to the AhR and stimulate its transformation and DNA binding (4,5). Both assays are quantitative in that the responses are proportional to the amount of AhR agonist (TCDD/HAH) in the test sample. Although these bioanalytical methods can detect HAHs and related chemicals, each has advantages and disadvantages (7)

**Materials and Methods**

AhR-Dependent Bioassays. The AhR DNA binding assay was carried out as we have previously described in detail (4,5). For the CALUX bioassay, recombinant mouse hepatoma (H1L6.1c3) cells containing the stably transfected AhR-responsive luciferase reporter plasmid pGudLuc6.1 was used (6).

TCDD Immunoassay: Immunochemical analysis of extracts for the presence of TCDD-like PCDDs and PCDFs (i.e. those containing chlorine in the 2,3,7 and 8 positions) was carried out by ELISA using polyclonal antibodies raised against a TCDD-based immunogen (8). The ELISA analysis used conditions were as previously described (9) and 2,3,7-trichloro-8-methyl-dibenzo-p-dioxin (TMDD) was used as the surrogate standard for these experiments.

**Results and Discussion**

Each of these bioanalytical techniques provides us with inexpensive method for the detection TCDD and closely related PCDDs and PCDFs. The TCDD/TMDD-dose response relationship of each assay is shown in figure 1 and these results reveal that the ELISA and DNA binding assays are very similar in their response to TCDC/TMDD. For the detection and quantitation of TCDD, the CALUX bioassay was 7-10-fold more sensitive than the ELISA and DNA binding assays. Comparison of the TCDD detection limits for these assays derived from these and other dose-response curves (Table 1) revealed that each assay is very sensitive (i.e. EC50/I50 values for the assays of between 20-150 pM (0.5 to 1.8 pg/assay well) and minimal detection limits (MDLs) of between 1-12.5 pM (0.03 to 0.2 pg/assay well)).

Table 1. Comparison of the detection limits for TCDD for the HAH bioanalytical techniques.

| Assay Method | EC50 or IC50 | MDL |
|---|---|---|
| CALUX | ~20 pM | ~1 pM |
| DNA Binding | ~150 pM | ~1-5 pM |
| ELISA | ~112 pM | ~12.5 pM |

Although these assays can readily detect TCDD, they are not specific for TCDD alone. The ELISA assay is the most specific of the three systems, but it also can detect any 2,3,7-chloro-substituted PCDD or PCDF. However, this assay fails to detect other related coplanar HAHs, such as the PCBs. In contrast to the ELISA assay, the CALUX and DNA binding assays respond to any chemical that can bind to and activate the AhR and this represents both a major advantage and limitation of these assays. However, we have found through extensive analysis of both pure chemicals and sample extracts that the DNA binding assay not only responds to a much greater spectrum of chemicals (non-HAHs) than the CALUX bioassay, but the magnitude of response to a given extract is generally far greater as well, likely due to its ability to be activated by numerous other chemicals in the mixture. In addition, we have observed that the in vitro AhR DNA binding assay not only responds to chemicals that are solvent extracted from most chromatographic matrices, but that it can respond to contaminants present in most high quality solvents; none of there were positive in the CALUX bioassay. Although the

CALUX bioassay can respond to chemicals in addition to HAHs, we have found that the inclusion of an appropriate clean-up step in the analysis procedure effectively eliminates false positives that arise from these "contaminating" AhR agonists. The high degree of correlation between the TEQs estimated by the CALUX bioassay versus that by HRGCMS for an cleaned up soil sample (Figure 2) demonstrates the removal of non-HAH AhR agonists from the sample extract. Interestingly, even after sample clean-up, false positives are still observed using the in vitro AhR DNA binding assay, presumably due to contaminating AhR agonists from the solvents and/or chromatographic matrices. This difference in response likely results from a direct access of the inducing chemical to the AhR in the in vitro DNA binding assay, while in the CALUX bioassay the inducing chemical must be able to enter the cell and survive metabolic attack. Given the high background activity of the AhR DNA binding assay, we conclude that it is an inappropriate screening bioassay for the detection of TCDD and related HAHs in unknown samples since it would result in an unacceptable number of false positives that would not be obtained using the CALUX bioassay system.

The low level of false positives observed with the CALUX bioassay coupled with the specificity of the immunoassay for TCDD-like PCDDs and PCDFs provides us with a relatively rapid two tiered screening system for TCDD and related HAHs. Because of its somewhat broader range of chemical detection, the CALUX assay can first be used alone to eliminate samples that do not contain HAHs that bind to the Ah receptor. With a positive CALUX result, the sample can be further analyzed directly by HRGCMS procedures or taken to a second tier where it is evaluated by immunoassay. In this scenario, antibodies to TCDD, PCBs or other HAHs can be used to further discriminate positives in the CALUX assay into one of 4 categories: containing TCDD, containing PCBs that react with Ah receptor, containing both TCDD and PCBs or containing an Ah receptor agonist that is neither TCDD or PCB. In samples where only TCDD or PCBs are known to be the primary contaminant either the CALUX or immunoassay can be used alone. Additionally, the CALUX and immunoassays can be used to prescreen large numbers of samples in order to identify those that should be subsequently analyzed by the more costly and time consuming for GC/MS procedures. With proper controls it is very difficult to obtain false negatives with the CALUX and ELISA assays. Thus these techniques can be used to screen out large numbers of negative samples.

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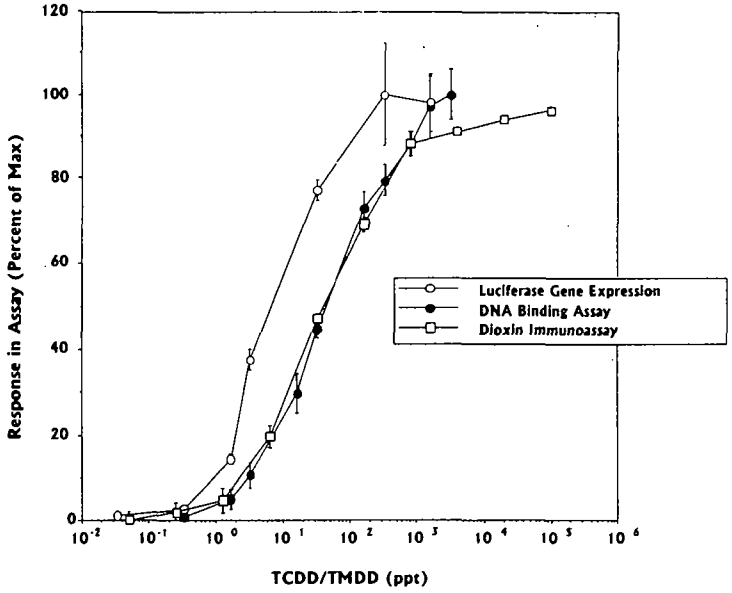


Figure 1. Dose response relationships for the detection of TCDD/TMDD using AhR-based bioassays and the dioxin immuno assay.

Figure 2. Correlation between TCDD equivalents (TEQs) determined by CALUX and GC/MS of soil sample extracts.