Biomarkers *of* ENVIRONMENTALLY ASSOCIATED DISEASE

Technologies, Concepts, *and* Perspectives

Samuel H. Wilson, M.D.
William A. Suk, Ph.D., M.P.H.

LEWIS PUBLISHERS

A CRC Press Company
Boca Raton London New York Washington, D.C.

Library of Congress Cataloging-in-Publication Data

Biomarkers of environmentally associated disease : technologies, concepts, and perspectives / [edited by] Samuel H. Wilson, William A. Suk.
p. cm.
Includes bibliographical references and index.
ISBN 156670-596-7
1. Environmentally induced diseases—Diagnosis. 2. Environmentally induced diseases—Molecular aspects. 3. Biochemical markers. 4. Tumor markers. I. Wilson, Samuel H., 1939- II. Suk, William A. (William Alfred)

RB152.5 .B54 2002
616.9′8—dc21 2002016076
CIP

Visit the CRC Press Web site at www.crcpress.com

International Standard Book Number 156670-596-7
Library of Congress Card Number 2002016076
Printed in the United States of America 1 2 3 4 5 6 7 8 9 0
Printed on acid-free paper

chapter thirty-three

Bioanalytical approaches for the detection of dioxin and related halogenated aromatic hydrocarbons

Michael S. Denison, Scott R. Nagy, Michael Ziccardi, George C. Clark, Michael Chu, David J. Brown, Guomin Shan, Yukio Sugawara, Shirley J. Gee, James Sanborn, and Bruce D. Hammock

Contents

I. Introduction 484
II. Tier 1: CALUX bioassay 485
III. Tier 2: TCDD immunoassay 488
IV. Application of CALUX and TCDD immunoassay 490
Acknowledgments 492
References 493

Abstract Proper epidemiological, risk assessment and exposure analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related halogenated aromatic hydrocarbons (HAHs) requires accurate measurements of these chemicals in the species of interest and in various exposure matrices, i.e., biological, environmental, and food. High-resolution instrumental analysis techniques are established for these chemicals; however, these procedures are costly and time consuming, and as such, they are impractical for large-scale sampling studies, i.e., for epidemiological studies and assessment of areas with widespread contamination. Accordingly, we have developed two

1-56670-596-7/02/$0.00+$1.50

simple, rapid, sensitive, and inexpensive assays (a TCDD immunoassay and a novel recombinant cell bioassay called CALUX) for the detection and relative quantitation of TCDD and related HAHs in extracts of a variety of matrices. These assays can be used individually or in combination as relatively rapid prescreening tools to identify positive samples for subsequent instrumental analysis and to drive chemical fractionation of complex mixtures in order to identify novel chemicals or classes of chemicals which exert dioxin-like activity. The availability of these bioanalytical approaches should greatly facilitate large-scale sampling studies needed for the accurate assessment of exposure to this ubiquitous class of environmental contaminants.

I. Introduction

HAHs, such as polychlorinated dibenzo-p-dioxins (PCDDs), biphenyls (PCBs), and dibenzofurans (PCDFs) represent a large group of compounds which can produce toxic effects at concentrations which can occur in the environment. HAHs have been identified worldwide in a variety of wildlife, domestic and human tissues as well as in food, water, and soil samples. Because of their ubiquitous distribution, resistance to biological and chemical degradation, high toxicity, and potential for bioaccumulation/biomagnification, HAHs can have a significant impact on the health and well-being of humans and animals.[1,2] Exposure to and bioaccumulation of HAHs, including the prototypical and most potent member of this class, TCDD, have been observed to produce a wide variety of species-specific and tissue-specific toxic and biological effects, including lethality, reproductive dysfunction, birth defects, endocrine disruption, liver toxicity, impaired immune function, cancer, and alterations in gene expression.[1–3] Given these issues, the detection and quantitation of these chemicals in biological, environmental, and food samples is of paramount importance. In addition, epidemiological and risk assessment analysis of HAHs/TCDD in humans and animals requires that an accurate measurement of the internal level of exposure be made. However, HAHs are found not as individual congeners, but as complex HAH mixtures, of which the relative and absolute concentrations of individual congeners can vary dramatically. Consequently, one problem in the evaluation of risk to HAHs is the identification and quantitation of toxic/bioactive HAH congeners in biological samples. Although sophisticated cleanup procedures followed by high-resolution gas chromatography-mass spectrometry (GC/MS) can separate, identify, and quantitate individual PCDD, PCB, and PCDF congeners,[4,5] these procedures require highly sophisticated equipment and training, a large amount of sample for analysis, and are also costly and time consuming, particularly when samples may theoretically contain large numbers of different HAH isomers and congeners (up to 209 different PCB, 135 different PCDF and 75 different PCDD congeners). Additionally, the concentration of HAHs in a given sample provides only part of the information necessary to evaluate their potential for biological/toxicological effects in humans and animals. Therefore, from a human and animal health

as well as an environmental standpoint, inexpensive and rapid bioassays capable of detecting and estimating the relative potency of complex mixtures of HAHs would be valuable. Accordingly, we have developed a rapid, inexpensive cell bioassay system that can be used with immunoassays in a two-tiered approach to detect the presence of these chemicals in a variety of biological and environmental matrices. These assays can be used individually or in combination as prescreening tools to allow identification of positive samples for subsequent analysis by GC/MS or other technologies, and they are amenable for large-scale analysis necessary for epidemiological studies. In addition, the bioassay can be used to drive chemical purification of complex mixtures to identify previously unknown components that have a similar mechanism of action as the HAHs and used in combination with chromatographic methods to support identification of HAHs in a sample.

II. Tier 1: CALUX bioassay

The first tier screening bioassay is based on the biochemical mechanism of action of TCDD (Figure 33.1) and related HAHs and involves the Ah receptor (AhR), a ligand-dependent transcription factor which can activate the expression of target genes specifically in response to these chemicals.[6] Following high-affinity binding of TCDD/HAH, the cytosolic ligand–AhR complex undergoes transformation during which it translocates into the nucleus, dissociates from its protein complex, and following its association with the Arnt (AhR nuclear translocator) protein, the AhR complex is converted into its high affinity DNA binding form.[7,8] The binding of the transformed heteromeric ligand–AhR–Arnt complex to its specific DNA recognition site, the dioxin responsive element (DRE), stimulates transcriptional activation of the adjacent gene.[7,9] Taking advantage of the ability of DREs to confer TCDD responsiveness upon an adjacent promoter and gene,[9] combined with the availability of several sensitive reporter genes, we constructed a recombinant TCDD/HAH-inducible expression vector which contain an easily measurable and extremely sensitive reporter gene (firefly luciferase) under HAH/AhR-inducible control of four DREs.[10] Cell lines that have been stably transfected (Figure 33.1) respond to TCDD and related HAHs with the induction of firefly luciferase in a dose- (Figure 33.2a), time-, and AhR-dependent manner.[10] Luciferase activity is readily quantitated by measurement of the amount of light produced in an enzymatic assay. This chemically activated luciferase expression (CALUX) cell bioassay system is inexpensive, rapid (approximately 5 h for complete analysis), sensitive (with a minimal detection limit of about 100–200 ppq TCDD), and has been optimized and streamlined such that the assay can be carried out in a 96-well microtiter format.[11] Because it has been optimized for 96-well formats, the same diluters, dispensers, and readers that are used for enzyme-linked immunosorbent assays (ELISA) can be used with the CALUX system. The system is adaptable in that other reporter systems can be used and also the general approach can be applied to a variety of promoter systems to detect not only HAHs but

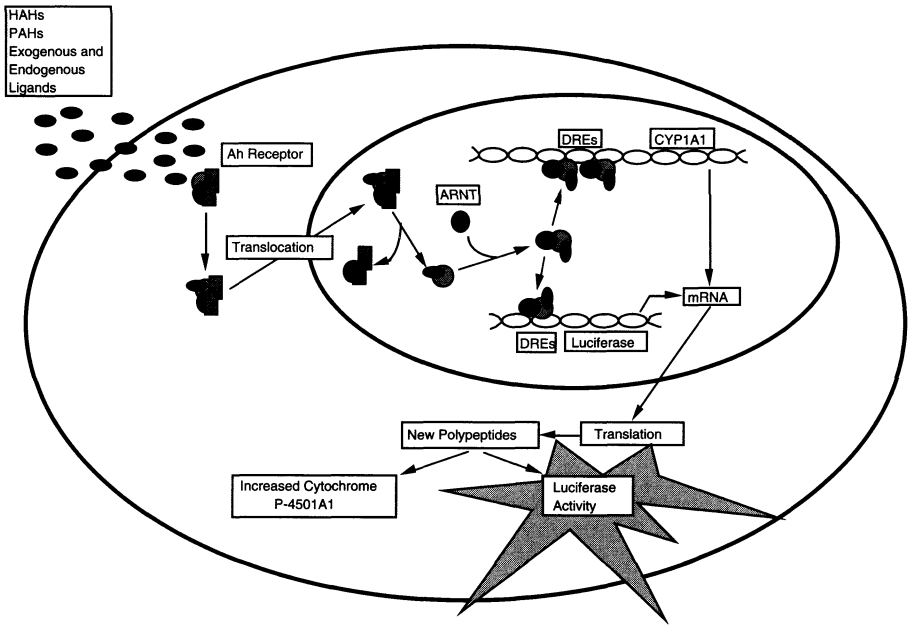
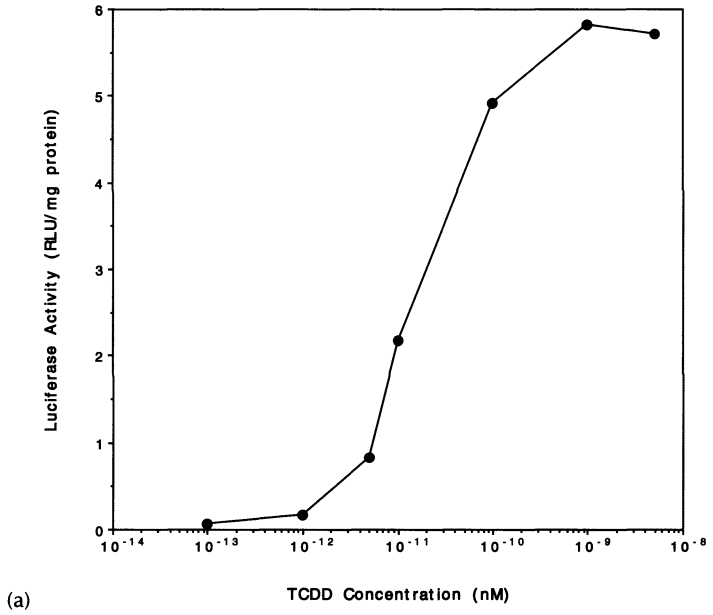


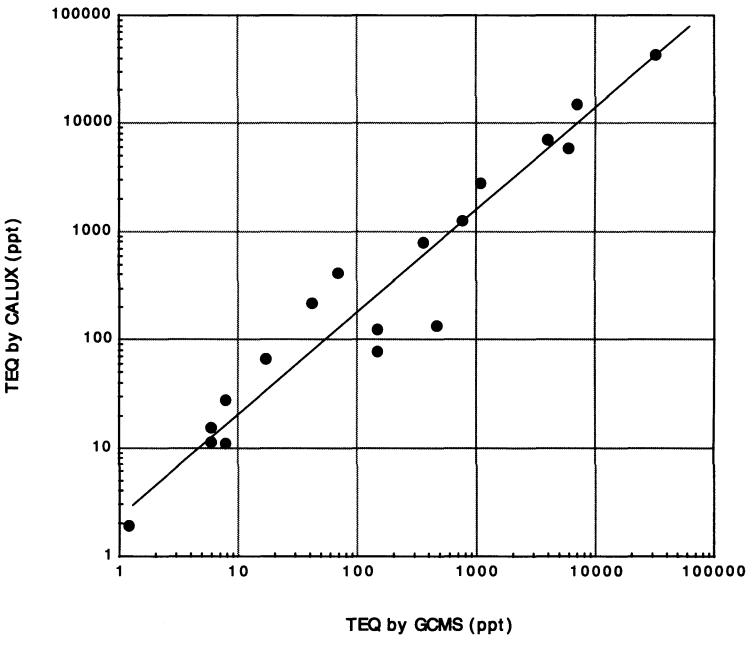
Figure 33.1 AhR-dependent molecular mechanism of TCDD action. The AhR stimulates expression of DRE-dependent endogenous genes as well as the stably integrated DRE-luciferase reporter gene.

estrogens, peroxisome proliferators, and a variety of other compounds with specific receptor systems.[12] In addition, we have recently demonstrated that the CALUX bioassay is also amenable for direct detection of TCDD and TCDD-like chemicals present in whole serum samples, without the need for solvent extraction.[11] Similar to samples which have been solvent extracted, induction of luciferase activity by TCDD-containing serum occurs in a time- and dose-dependent manner and this modification of the bioassay can detect as little as 5–10 ppt TCDD and/or TCDD-equivalents in a 50 µl aliquot of whole serum.[11] This modification of the CALUX assay not only has applications for the screening of samples where only small volumes of blood are available (e.g., infants and endangered and/or small species), but it will allow for large-scale screening of populations for epidemiology studies.

The utility of the CALUX bioassay for detection and relative quantitation of TCDD and related HAHs has been validated in several studies and they demonstrate a high degree of correlation between the CALUX bioassay and GC/MS results.[13–15] The results of an analysis of soil sample extracts by GC/MS and the CALUX bioassay are shown in Figure 33.2b. These data reveal an excellent correlation (R^2=0.932) between the soil extract TCDD equivalents (TEQs) estimated by GC/MS and those equivalents estimated using CALUX bioassay. The CALUX bioassay is simpler, faster and far less expensive than conventional chemical analysis techniques and perhaps more



(a)



(b)

Figure 33.2 Tier I CALUX bioassay. (a) TCDD dose response curve for luciferase induction in stably transfected mouse hepatoma (H1L1.1c2) cells. (b) Relationship between TCDD equivalents (TEQs) determined by CALUX and GC/MS of soil sample extracts.

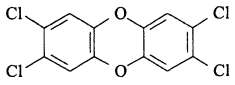
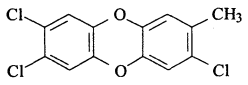
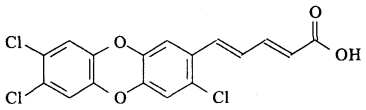
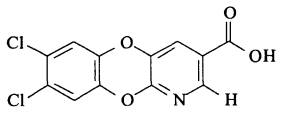
importantly, this assay provides a quantitative measure of the biological potency of the complex mixture, relative to the most potent ligand, TCDD. Given that the CALUX bioassay has the advantage of being able to detect a variety of TCDD-like HAHs and other AhR agonists, it is a perfect first-stage screen. Samples that are positive in this bioassay can undergo congener-specific HAH analysis using GC/MS. However, if confirmation of the presence and relative concentration of TCDD in CALUX-positive extracts is the goal, this can more readily and inexpensively be accomplished using our second tier dioxin selective immunoassay as described below.

III. Tier 2: TCDD immunoassay

Immunoassay, as a rapid, sensitive and selective bioanalytical method, has been widely used for environmental and biological monitoring of small molecular toxins. However, due to the lipophilic properties and the difficulty of the chemistry of dioxins, only a few attempts to detect TCDD by immunoassay have been reported,[16–19] and their sensitivities are not as high as is desirable. Based on careful hapten design and synthesis, a sensitive polyclonal antibody-based ELISA was developed in this laboratory,[20,21] which exhibited an I_{50} value of 12 pg/well (240 ng/L or 0.75 nM), with working range from 2–240 pg/well. Theoretically, an ELISA sensitivity is determined by antibody affinity. The ultimate detection limit of an assay is approximately 10–100 times lower than the K_d of antibody.[22] The K_d of antibody used in our study was measured by accelerator mass spectrometry[23] and found to be 0.1 nM suggesting that a more sensitive immunoassay can be achieved using this antibody. Thus, a series of new coating antigen haptens were designed and synthesized. After extensive screening, a highly sensitive assay was developed with coating Hapten II (Table 33.1). The I_{50} of new assay system was 1.8 pg/well (36 ng/L) (Figure 33.3a) with a lower detection limit of 0.2 pg/well (4.0 ng/L). Because of the restrictions on the use of TCDD, its toxicity, and the high cost of disposal, many laboratories avoid using TCDD as a primary standard. Thus, a TCDD surrogate standard, 2,3,7-trichloro-8-methyl dibenzo-p-dioxin (TMDD), was developed and used in our study.[24] TMDD and related compounds have similar polarity to TCDD and can be used as a secondary analytical standard in a variety of assays including the above bioassay as well as GC/MS. According to the cross-reactivity study[25]), most of the dioxins and PCDFs with a high TEF value (> 0.1) have strong or moderate cross-reactivities in this assay, which suggests that this assay might be a good indicator of toxicity of PCDDs and PCDFs in the test samples.

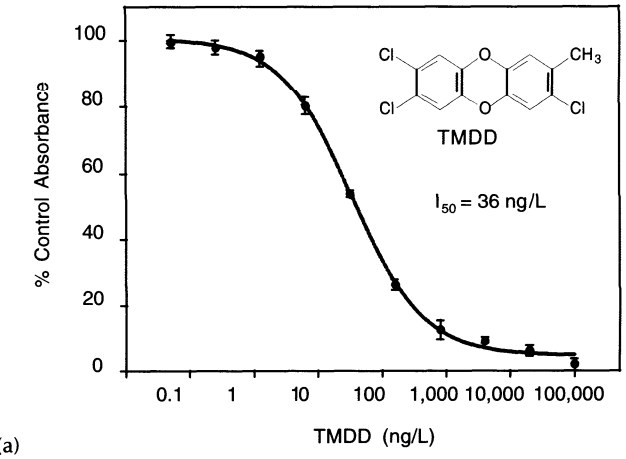
This immunoassay has been validated with extracts from fish and egg samples by GC/MS. A good agreement between GC/MS and ELISA TEQs was obtained from linear regression analysis ($y=1.12x-4.08$, $R^2=0.89$) (Figure 33.3b), and no matrix effects were found for these extracts as prepared. A fairly good correlation between ELISA and TEF values was also observed with these samples ($Y=0.78x+6.37$, $R^2=0.90$). Although there is an overesti-

Table 33.1 Structures of TCDD, Surrogate Standard TMDD and Dioxin Haptens

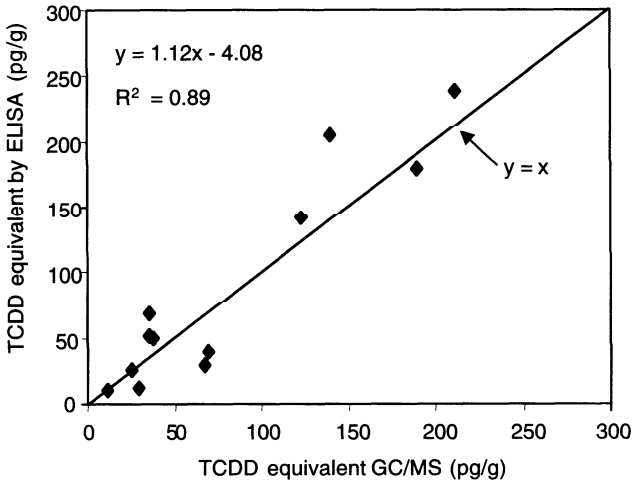
Compound	Structure
TCDD	
TMDD	
Hapten I (immunogen)	
Hapten II	

mation by ELISA in comparison to TEF values, the strong correlation between this ELISA and TEF values indicates that this assay can be used as a TEF screening method for dioxins and PCDFs on its own or sequentially to a more general screen based on the Ah receptor as a tier two method. The method has shown good correlation with spiked samples and with GC/MS results performed by this and other laboratories. For example, in one case, a blank, spiked recovery and positive soil samples were extracted with hexane and analyzed directly by ELISA after evaporation of the hexane with no further clean up. The ELISA data on the crude hexane extract agreed well with ELISA data on the same samples following a Florasil column indicating that for ELISA of soil samples the column was not needed. The ELISA data on the hexane extract agreed well with GC/MS data obtained after a 14-step clean-up and analysis procedure. These data suggest that this ELISA can be used as a rapid, inexpensive screen to detect dioxins in the soil samples.

The antibodies characterized above have a number of other analytical uses. For example, ELISA is of course easy to use as a postcolumn detection system for high-performance liquid chromatography,[26] and the CALUX assay also can be formatted to use in this manner. This approach provides either a confirmation experiment where one can say how much immunoreactivity or CALUX activity has the same retention time as TCDD or as a method to tentatively identify other materials that are positive in the assay but are not TCDD. These and other antibodies can be used for immunoaffinity purification prior to either bioassay or chromatographic analysis such as LC or GC/MS. A simple application of these antibodies is to add them to diluted aliquots of samples which have shown positive in either in CALUX or chromatographic assays. The antibodies will bind TCDD like chemicals



(a)



(b)

Figure 33.3 Tier II immunoassay. (a) TMDD immunoassay inhibition curve. (b) Relationship between TCDD equivalents (TEQs) determined by ELISA and GC/MS of fish and egg sample extracts.

and can be precipitated by a variety of techniques such as magnetic beads coated with goat anti-rabbit antibody or Protein A. If the positive response is removed one can say with high confidence that the assay was detecting a TCDD like chemical.

IV. Application of CALUX and TCDD immunoassay

The CALUX bioassay and the immunoassay provide a two tiered screening system that can be utilized as relatively rapid and inexpensive assays for the detection and relative quantitation of TCDD and TCDD-like chemicals in a wide range of samples and sample extracts (Figure 33.4). The CALUX assay

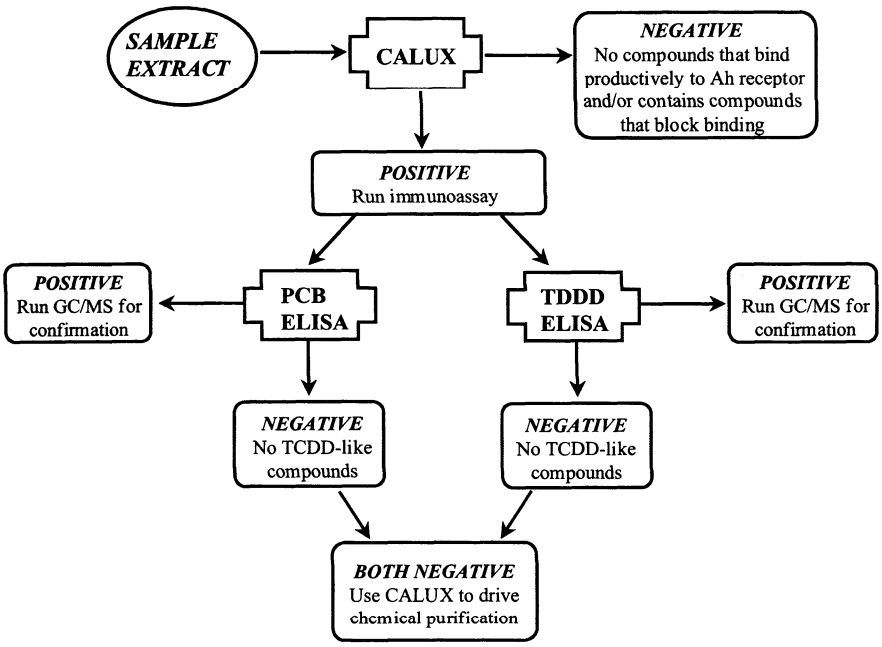


Figure 33.4 Overview of the two-tiered screening system for detection and relative quantitation of TCDD and TCDD-like chemicals in sample extracts.

can be used alone to eliminate samples that do not contain HAHs that bind to the Ah receptor. False negatives at this step could result from the presence of AhR antagonists in a sample; however, the presence of these inhibitory agents can readily be determined by measuring the ability of the sample to reduce the induction response of a known amount of TCDD. With a positive CALUX, the sample can be analyzed directly by chromatographic procedures or taken to a second tier where it is further evaluated by immunoassay. As shown in Figure 33.4, antibodies to TCDD, PCBs or other HAHs can be used to discriminate positives further in the CALUX assay. This second tier will put the sample in one of four categories: containing TCDD, containing PCBs that react with Ah receptor, containing 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 GC/MS procedures. With proper controls, obtaining false negatives with the CALUX and ELISA assays is difficult. Thus, these techniques can be used to screen out large numbers of negative samples. Often the most time-consuming part of GC/MS and LC/MS analysis is cleaning an instrument. The CALUX and immunoassays provide a way to rank samples so that samples with suspected low levels of HAHs can be run on the instrument

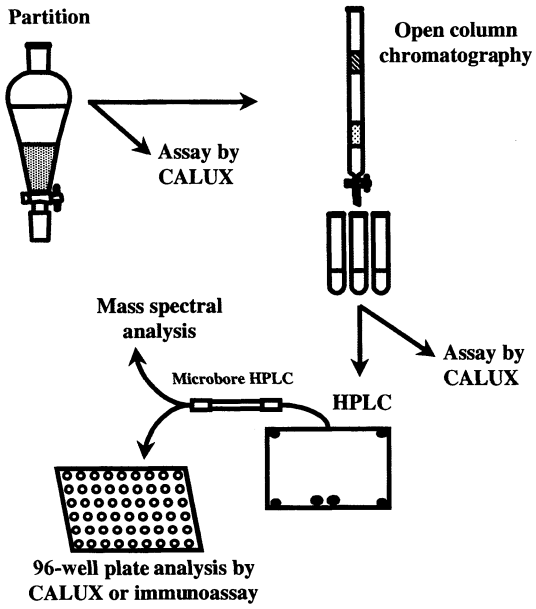


Figure 33.5 Overview of the CALUX bioassay-driven fractionation scheme for the purification and identification of novel AhR agonists.

when the sensitivity is high and samples suspected of high concentrations of HAHs can be either diluted or run subsequently.

Not only have these assays have been used to detect TCDD and related chemicals in variety of biological, environmental and food samples, but the CALUX bioassay provides an avenue to identify and characterize novel chemicals and classes of chemicals which can bind to and activate the AhR signal transduction pathway. Thus, a positive CALUX that is shown by subsequent immunoassay or immunoprecipitation assay (Figure 33.4) to be due to neither TCDD nor PCB must contain a novel AhR agonist. The identify of the activating chemical(s) can be obtained by using the CALUX bioassay to monitor a classical purification scheme based on differential extraction and column chromatography as presented in Figure 33.5.

Acknowledgments

This research was supported by NIEHS/EPA Superfund Basic Research Grant ES04699 and NIEHS SBIR grant R43ES80372.

References

1. Safe, S., Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors, *Crit. Rev. Toxicol.*, 21, 51–88, 1990.
2. Giesy, J.P., Ludwig, J.P., and Tillitt, D.E., Dioxins, dibenzofurans and wildlife, in *Dioxins and Health*, Schecter, A., Ed., Plenum Press, New York, 1994, pp. 249–307.
3. DeVito, M.J. and Birnbaum, L.S., Toxicology of dioxins and related chemicals, in *Dioxins and Health*, Schecter, A., Ed., Plenum Press, New York, 1994, pp. 139–162.
4. Ballschmiter, K., Rappe, C., and Buser, H.R., Chemical properties, analytical methods and environmental levels of PCBs, PCTs, PCNs and PBBs, in *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products*, Kimbrough, R.D. and Jensen, J., Eds., Elsevier Science, Amsterdam, 1989, pp. 239–293.
5. Rappe, C. and Buser, H.R., Chemical and physical properties, analytical methods, sources and environmental levels of halogenated dibenzodioxins and dibenzofurans, in *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products*, Kimbrough, R.D. and Jensen, J., Eds., Elsevier Science, Amsterdam, 1989, pp. 239–293.
6. Denison, M.S., Elferink, C.F., and Phelan, D., The Ah receptor signal transduction pathway, in *Toxicant-Receptor Interactions in the Modulation of Signal Transduction and Gene Expression*,Denison, M.S. and Helferich, W.G., Eds., Taylor and Francis, Bristol, PA, 1998, pp. 3–33.
7. Denison, M.S., Fisher, J.M., and Whitlock, Jr., J.P., Inducible, receptor-dependent protein-DNA interactions at a dioxin-responsive transcriptional enhancer, *Proc. Natl. Acad. Sci. USA*, 85, 2528–2532, 1988.
8. Hord, N.G. and Perdew, G.H., Physicochemical and immunocytochemical analysis of the aryl hydrocarbon receptor nuclear translocator: characterization of two monoclonal antibodies to the aryl hydrocarbon receptor nuclear translocator, *Molec. Pharmacol.*, 46, 618–624, 1994.
9. Denison, M.S., Fisher, J.M., and Whitlock, Jr., J.P., The DNA recognition site for the dioxin-Ah receptor complex: nucleotide sequence and functional analysis, *J. Biol. Chem.*, 263, 17721–17724, 1988.
10. Garrison, P.M. et al., Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals, *Fund. Appl. Toxicol.*, 30, 194–203, 1996.
11. Ziccardi, M.H., Gardner, I.A., and Denison, M.S., Development and modification of a recombinant cell bioassay to directly detect halogenated and polycyclic aromatic hydrocarbons in serum, *Toxicol. Sci.*, 54, 183–193, 2000.
12. Rogers, J.M. and Denison, M.S., Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals, *In Vitro Mol. Toxicol.*, 13, 67–82, 2000.
13. Murk, A.J. et al., Chemical-activated luciferase gene expression (CALUX): a novel *in vitro* bioassay for Ah receptor active compounds in sediments and pore water, *Fundam. Appl. Toxicol.*, 33, 149–160, 1996.

14. Hooper, K. et al., CALUX™ results correlate with GC/MS/MS data from Kazakhstan breast milk samples, *Organohal. Cmpds.*, 45, 236–239, 2000.
15. Van Overmeire, I. et al., A comparative study of GC-HRMS and CALUX™ TEQ determinations in food samples by the Belgian Federal Ministries of Public Health and Agriculture, *Organohal. Cmpds.*, 45, 196–199, 2000.
16. Stanker, L. et al., Monoclonal antibodies for dioxin: antibody characterization and assay development, *Toxicol.*, 45, 229–243, 1987.
17. Vanderlaan, M. et al., Improvement and application of an immunoassay for screening environmental samples for dioxin contamination, *Environ. Toxicol. Chem.*, 7, 859–870, 1988.
18. Langley, M.N. et al., Immunoprobes for polychlorinated dibenzodioxins: synthesis of immunogen and characterization of antibodies, *Food Agricul. Immunol.*, 4, 143–151, 1992.
19. Harrison, R.O. and Carlson, R.E., An immunoassay for TEQ screening of dioxin/furan samples: Current status of assay and applications development, *Chemosphere*, 334, 915–928, 1997.
20. Sanborn, J.R. et al., Hapten synthesis and antibody development for polychlorinated dibenzo-*p*-dioxin immunoassays, *J. Agric. Food Chem.*, 46, 2407–2416, 1988.
21. Sugawara, Y. et al., Development of a highly sensitive enzyme-linked immunosorbent assay based on polyclonal antibodies for the detection of polychlorinated dibenzo-*p*-dioxins, *Anal. Chem.*, 70, 1092–1099, 1998.
22. Jackson, T.M. and Ekins, R.P., Theoretical limitations on immunoassay sensitivity. Current practice and potential advantages of fluorescent Eu3+ chelates as non-radioisotopic tracers, *J. Immunol. Methods*, 87, 13–20, 1986.
23. Shan, G.M. et al., Isotope-labeled immunoassays without radiation waste, *Proc. Natl. Acad. Sci. USA*, 97, 2445–2449, 2000.
24. Shan, G. et al., Surrogates for dioxin analyses: analytical, ELISA and toxicological aspects, *Organohal. Cmpds.*, 45,188–191, 2000.
25. Shan, G. et al., A highly sensitive dioxin immunoassay and its application to soil and biota samples, *Anal. Chim. Acta.*, 444, 169–178, 2001.
26. Krämer, P.M., Li, Q.X., and Hammock, B.D., Integration of liquid chromatography with immunoassay: an approach combining the strengths of both methods, *J. AOAC Int.*, 77, 1275–1287, 1994.