Bioanalytical Tools for Detection and Quantification of Estrogenic and Dioxin-Like Chemicals in Water Recycling and Reuse

Guidance Document for Developing a Standard Operating Procedure

Prepared for:

WaterReuse California

Prepared by:

NWRI
National Water Research Institute

January 2020

Contents

Acknowledgments

The Authors wish to acknowledge all those who have contributed to the effort to produce this SOP guidance document. WateReuse California and its member agency experts recognized the lack of standardized procedures, in the context of California drinking water quality regulations, for commercially available ERα and AhR bioanalytical tools. State Water Board staff recognized the opportunity to work collaboratively with regulated utilities to promote effective implementation of the Recycled Water Policy and improve the data quality for investigatory monitoring of estrogenic and dioxin-like CECs in potable recycled water using bioanalytical screening tools. The quality of stakeholder initiative and engagement is evident in the final work product.

This report was prepared for WateReuse California and the WateReuse Association by the Bioanalytical Implementation Advisory Group (Advisory Group) administered by the National Water Research Institute (NWRI), a 501c3 nonprofit organization and Joint Powers Authority based in Fountain Valley, California.

Bioanalytical Implementation Advisory Group

WateReuse California

The mission of WateReuse California is to promote responsible stewardship of California's water resources by maximizing the safe, practical, and beneficial use of recycled water and by supporting the efforts of the of WateReuse Association at the state level.

Board of Trustees

Mike Markus, Orange County Water District

Richard Nagel, Jacobs

David Pedersen, Las Virgenes Municipal Water District

Paul Sciuto, Monterey One Water

Prabhakar Somavarapu, Sacramento Regional County Sanitation District

Dawn Taffler, Kennedy Jenks

Patricia Tennyson, Katz & Associates

Kim Thorner, Olivenhain Municipal Water District

Mike Thornton, San Elijo Joint Powers Authority

Deven Upadhyay, Metropolitan Water District of Southern California

Robb Whitaker, Water Replenishment District of Southern California

Chapter Trustees

Rhodora Biagtan, HydroScience Engineers

Evelyn Cortez-Davis, Los Angeles Department of Water and Power

Lesley Dobalian, San Diego County Water Authority

Melanie Holmer, Water Reuse Leader – Brown and Caldwell

Joone Lopez, Moulton Nigel Water District

Rob Morrow, Water Systems Consulting, Inc.

John Robinson, John Robinson Consulting

WateReuse Association

The mission of WateReuse is to advocate, educate, and provide leadership for the responsible use of recycled water. Since its founding in 1990, WateReuse has advocated for policies, laws, and funding at the state and federal level to increase the practice of recycling water. Our national office leads the advocacy efforts with the United States Congress and federal agencies, including the Bureau of Reclamation and the Environmental Protection Agency. Seven state sections work with state lawmakers and regulatory agencies to advance state policies on water reuse.

California State Water Resources Control Board

Staff from the California State Water Resources Control Board were instrumental in helping the Advisory Group, the WateReuse Association, and other stakeholders understand the regulatory and technical issues related to using bioanalytical tools to help characterize recycled water. Special thanks to Claire Waggoner, Laura McLellan, and Scott Coffin.

Stakeholders

The following utilities, consulting firms, and laboratories attended the stakeholder workshops and provided comments on the draft report. Their participation was essential in creating this guidance document.

Association of California Water Agencies

BioDetection Systems

Birmingham Water Works

Brown and Caldwell

California Department of Water Resources

Carollo Engineers

Central Valley Clean Water Association

Corona, City of

Eurofins Analytical Labs

HDR, Inc.

Heal the Bay

Inland Empire Utilities Agency

Iontox

LA Sanitation

LADWP

Metropolitan Water District of Southern California

NSF Wastewater

Oceanside, City of

Orange County Sanitation District

Orange County Water District

Pacific EcoRisk Labs

Padre Dam Municipal Water District

Redlands, City of

Regional Water Quailty Control Board, Los Angeles

Riverside, City of

Robertson-Bryan, Inc.

Sacramento Regional County Sanitation District

San Diego, City of

San Diego County Water Authority

San Francisco Bay Regional Water Quality Control Board

South Orange County Wastewater Authority

Southern California Coastal Water Research Project

SPI Engineering

State Water Resources Control Board

The Water Research Foundation

Trussell Technologies

Washington State Department of Health

Water Replenishment District of Southern California

Water Systems Consulting, Inc.

Water UCI

West Basin Municipal Water District

Woodard & Curran

Yucaipa Valley Water District

Peer Reviewers

The Advisory Group appreciates the thoughtful review by three individuals recognized as international leaders in the theory and practice of bioanalytical tools. Their insightful comments were used to improve the technical accuracy and readability of the final report.

Beate Escher, Helmholtz Centre for Environmental Research (UFZ), Germany

Frederic Leusch, Griffith University, Queensland Australia

Shane Snyder, Nanyang Environment and Water Research Institute, Singapore

National Water Research Institute

Staff from NWRI administered and facilitated the stakeholder workshops and supported Advisory Group research and writing activities to produce this document.

Kevin M. Hardy, Executive Director

Suzanne Sharkey, Water Resources Scientist and Project Manager

Mary Collins, Communications Manager

I. INTRODUCTION

A. Scope: What is this document?

This is a guidance document for preparing a standard operating procedure (SOP) for detecting and quantitating estrogen receptor alpha (ERα) and aryl hydrocarbon receptor (AhR) agonists in water samples using recombinant mammalian cell lines containing ERα- or AhR-responsive reporter genes. It also includes elements of the SOP that emphasize performance-based criteria.

The activity of the reporter gene protein induced by chemically activated ERα or AhR complexes measures the total bioanalytical equivalent (BEQ) value of the water sample, which for ERα is expressed as 17β-estradiol (E2)-BEQs and for AhR is expressed as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-BEQs. For detection and relative quantitation of ERα- and AhR-active chemicals/activity, sample extraction is necessary, followed by bioassay analysis and determining BEQ from gene induction results by comparing to reference standard results.

There are many ERα- and AhR-responsive cell bioassays, each with unique characteristics and assay conditions that have been developed from extensive optimization and validation studies. As a consequence, no single bioassay SOP can be established that is appropriate for all current or future ERα- and AhR-based bioassays; it is not the goal of this document to establish such a protocol. Thus, the analysis of water samples by a given bioassay system must use SOPs that have been developed, optimized, and validated for that specific bioassay. To ensure that the results generated using a specific test method are accurate and interpretable, this guidance document also includes and describes necessary QA/QC elements for sample collection and handling, sample preparation methods, data analysis, and reporting.

These methods are applicable to the following water matrices, not all of which must be monitored under the California Water Quality Control Policy for Recycled Water (Recycled Water Policy (1)). Other matrices would require further method development.

- Tertiary treated and disinfected wastewater that complies with Title 22 regulations (final effluent)
- Membrane bioreactor filtrate
- Micro/ultrafiltration filtrate
- Reverse osmosis permeate
- UV and advanced oxidation process product water
- Finished drinking water (from a surface water or groundwater source)

The methods can be used to monitor for ERα- or AhR-active chemicals in potable recycled water, including monitoring required by the Recycled Water Policy. The use of alternative bioanalytical test methods other than those described in Section I-C of this guidance document is not recommended unless validation of an alternative method's ability to detect these chemicals has been established. Validation should use the guidelines presented in this document and available assay performance standards criteria, such as those set forth in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (2). Proper validation of alternative or modified methods will allow for timely amendments and updates to this guidance document after a review and an agreement that performance standards are met.

B. Intent of this Guidance Document

In 2010, the State Water Resources Control Board (State Water Board) convened a Science Advisory Panel (SAP) to provide recommendations for monitoring chemicals of emerging concern (CECs) in recycled water. The SAP noted that due to the ever-growing, expansive, and diverse nature of CECs, non- or semi-targeted methods should be used to complement targeted CEC monitoring, and recommended the use of bioanalytical screening tools to more comprehensively evaluate potential exposures to the breadth of CECs (3). The 2010 SAP recommended the use of *in vitro* (cell-based) bioassays as bioanalytical screening tools that are capable of detecting a wide spectrum of CECs based on a molecular initiating event of a human health-related adverse outcome pathway (3,4). The measurement of quantitative biological activity of a molecular initiating event allows for a sensitive prediction of effect potential that would inform further targeted screening using analytical chemistry to identify known and unknown CECs in recycled water at concentrations that may pose a risk to human health or the environment (3,5).

In 2018, the State Water Board reconvened the SAP to update its recommendations for CEC monitoring in recycled water (5). Based on advancements in the field since 2010, the 2018 SAP recommended the use of ERα- and AhR-based bioassays given their documented inter-laboratory robustness (6), standardization (7,8), use in regulatory capacities in Europe (9,10), as well as their identified utility in recycled water settings (11). Based on these recommendations, on December 11, 2018, the State Water Board adopted Resolution No. 2018-0057 to amend the Recycled Water Policy to include requirements for facilities that produce recycled water for indirect potable reuse via groundwater recharge and reservoir water augmentation to monitor using two bioanalytical screening tools (ERα and AhR) (1).

CEC monitoring pursuant to the Recycled Water Policy is intended to be investigatory and not for regulatory compliance with a specific limit such as a maximum contaminant level or a water quality

objective (1). The monitoring trigger levels (MTLs) in the Recycled Water Policy have been defined as E2-BEQ concentrations of 3.5 ng E2/L and TCDD-BEQ concentrations of 0.5 ng TCDD/L (Table 9 in (1)). For both targeted chemistry and bioanalytical screening tools, the response actions for exceeding the MTLs are to further investigate what is responsible for the exceedance if the magnitude of the exceedance is greater than a factor of 10 higher than the MTL (1). The Recycled Water Policy does not require further investigation if the magnitude of an exceedance is less than 10 times the MTLs because the purpose of the CEC monitoring requirements is to investigate the occurrence and magnitude of CECs in recycled water, and the MTLs are relatively conservative values.

The Recycled Water Policy includes requirements in Attachment A to ensure that data associated with CEC monitoring are of known, consistent, and documented quality and to verify that the laboratory can meet the required reporting limits for the targeted CECs and bioanalytical results. SOPs are integral to ensuring data quality. WateReuse California commissioned a group of experts to develop this SOP guidance document for ERα and AhR bioassays to ensure bioanalytical screening tool data collected pursuant to the Recycled Water Policy are of standard and high quality.

C. Principles of the Test Methods

ERα and AhR are chemical-responsive nuclear receptors that mediate a variety of endogenous physiological responses by their ability to stimulate and/or inhibit the expression of specific genes. Inappropriate activation or inhibition of these receptors by exposure to a variety of exogenous chemicals has been demonstrated to produce adverse health effects in animals and humans (12–18). Although chemical-dependent activation of ERα and AhR leads to distinctly different biological and toxicological responses, the general mechanisms by which these and other nuclear receptors regulate gene expression are very similar, although not identical (15, 19–21). The general mechanism of activation of gene expression by nuclear receptors is shown in Figure 1.

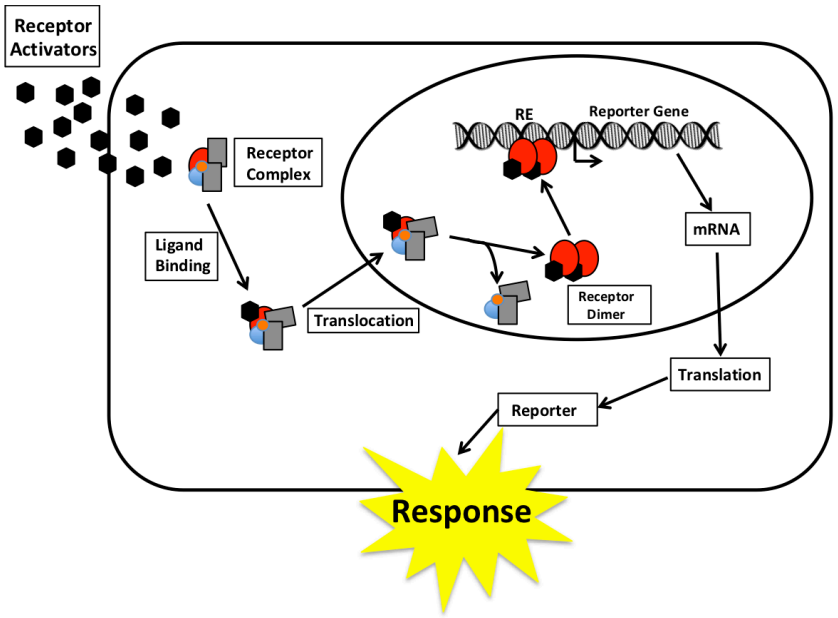


Figure 1. Mechanism of action of nuclear receptor-dependent gene expression and the general principle of the ERα- and AhR-responsive reporter gene cell bioassays. The reporter is a protein, often an enzyme, that can be quantified by substrate turnover.

In the specific case of the ER (estrogen receptor), estrogenic chemicals diffuse into the cell where they can bind to the ER, stimulating its translocation into the nucleus and dimerization with another ligand-activated ER. The binding of the ER:ER dimer to its high affinity DNA binding site (termed an Estrogen Responsive Element (ERE)) adjacent to an estrogen-responsive promoter and gene results in increased transcription and expression of that gene (20,21). There are at least two major subtypes of nuclear ERs, α and β, which are encoded by distinct genes, and each have different biological functions as well as different tissue distributions and ligand binding affinities and specificities (20–22)). Since nuclear ERα mediates the classic estrogenic response and ERβ is not present in most continuous cell lines, most bioassays currently being developed to measure ER activation are specific for ERα.

In a somewhat similar manner, AhR-active chemicals diffuse into the cells and bind to the AhR, stimulating its nuclear translocation. However, once in the nucleus, the ligand-bound AhR dimerizes with a distinctly different, but structurally related protein, called ARNT (Ah Receptor Nuclear Translocator). Binding of the ligand activated AhR:ARNT complex to its specific DNA binding site (termed a Dioxin Responsive Element (DRE)) next to an AhR-responsive promoter and gene leads to increased expression of that gene (15,19,23). Given that the adverse effects of ERα and AhR agonists are mediated via alterations in gene expression, aspects of these receptor pathways have been used to develop bioassays for the detection of ERα and AhR activators and inhibitors (24–31).

The bioassay methods described in this document use ERα or AhR/ARNT-containing mammalian cell lines that contain a stably or transiently transfected ERα- or AhR-responsive reporter gene plasmid in which EREs or DREs have been inserted immediately upstream of a promoter and reporter gene (Figure 1). Numerous reporter genes have been used for such assays and include luciferase, green fluorescent protein, placental alkaline phosphatase, LacZ and others (32–33). Exposure of these transfected cells to 17β-estradiol (E2) or another ER agonist (for ER-responsive cells) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) or related dioxin-like/AhR-active chemical (for AhR-responsive cells) stimulates ERα/AhR nuclear translocation, receptor dimerization, binding of the ligand-activated receptor dimer to an ERE/DRE adjacent to the reporter gene, and stimulation of reporter gene transcription. The resulting mRNA from the reporter gene is translated into the reporter protein and its associated activity is subsequently measured. Induction of reporter genes in these transfected cell lines occurs in a time-, chemical concentration-, and receptor-dependent and chemical-specific manner (7,8,24–26,34). Additionally, the amount of induced reporter gene activity in these bioassays has been shown to be directly proportional to the amount of activated ERα/AhR and ultimately the concentration and potency of the inducing chemicals (i.e., ERα or AhR activators) to which the cells have been exposed. As such, a measure of the potency of the sample extract can be determined from the results of these assays and is expressed as bioanalytical equivalents (BEQs) relative to that of the reference standard chemical.

II. OVERVIEW OF SAMPLE PREPARATION AND BIOASSAY SCREENING PROCEDURES

One-liter water samples for bioassay testing and matrix spike recoveries are collected in amber glass bottles at each of the desired times and sampling locations. Samples are stored on ice and must be transported to the testing laboratory as soon as possible after collection and extracted within 14 days after collection. Solvent extracts of each test sample, matrix spike samples, a field blank sample and a method blank sample are prepared using a solid phase extraction (SPE) method and the final solvent extracts are exchanged into dimethylsulfoxide (DMSO). Extracts can be stored frozen at –20°C/–80°C, but should be analyzed in the bioassays within 45 days after extraction. ERα and AhR cell bioassays are incubated with an aliquot of each sample and then reporter gene activity is measured. Assays are validated using method and solvent blanks, reference standards, and test run acceptability criteria. Induced reporter gene activity is compared to a positive reference standard for the particular bioassay to determine the E2-BEQs and TCDD-BEQs of the sample extract. The BEQ value is then compared to the MTL established for ERα or AhR bioassays and the resulting ratio is used to determine the specific response actions for an associated BEQ level response (see Table 10 of the Recycled Water Policy (1)).

If the BEQ to MTL ratio for the AhR bioassay is greater than 10 (see Table 10 of the Recycled Water Policy, response actions C and D (1)), then an additional cleanup step must be performed to determine if toxic dioxin-like chemicals are present. As described in Section II-B-2, additional samples must be collected from the same site as the original sample and subjected to a more extensive extraction and cleanup procedure to isolate toxic from nontoxic AhR-active chemicals (13,15,19,23,26,35–37). A positive AhR bioassay result from the cleaned up extract indicates the presence of toxic dioxin-like chemicals (such as halogenated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs)) in the sample.

This SOP guidance document presents the application of both ERα- and AhR-based cell bioassays for detection and quantitation of chemicals in water samples. Since currently available SOPs and guidance documents are only focused on one type of cell bioassay (ERα or AhR), this document was developed from numerous SOPs, guidance documents, technical reports, and scientific references (1,2,5,7,8,26,31,34,38). A tabular overview of the recommended test guidelines described in detail throughout this guidance document is presented in Appendix 2.

A. Sample Collection and Storage

1. Selecting Sampling Bottles and Materials

Use new unused or cleaned used one-liter amber glass bottles (borosilicate glass) preferably with polypropylene caps or polytetrafluoroethylene (PTFE (Teflon)) lined caps. To avoid photo-degradation of compounds of interest, amber glass bottles are preferred. If amber glass bottles are not available, wrap clear glass bottles in aluminum foil or store them in a dark container.

Bottles that have been previously used for water collection should be thoroughly cleaned as soon after use as is practical using the following procedure prior to reuse. After routine cleaning with detergent, rinse bottles and caps five times with tap water and five times with ultrapure filtered water (or equivalent). Then rinse the clean bottles and caps three times with a minimal amount of methanol, then rinse once with ultrapure water. Bake the bottles in a furnace/oven at 400° to 500°C overnight and cap them after cooling. Note that excessive baking of glassware can produce sites on the glass surface that irreversibly bind chemicals (39).

2. Collecting samples

a. Sample collection

Use disposable nitrile gloves during sampling and avoid skin contact with the sample. Minimize the use of hand cream, sunscreen, cosmetics, or other personal care products before sampling, because they may contain ER- or AhR-active chemicals. If any additional equipment is needed to collect or process the sample, use only material made of cleaned glass, PTFE, aluminum, or stainless steel. Additional protocols for avoiding contamination during sample collection have been described in documents such as the USGS National Field Manual for the Collection of Water-Quality Data (40).

b. Sample numbers

For each sampling location, collect the following:

1. One one-liter water sample for combined ERα/AhR analysis.

For each sample batch (20 samples or less, not including QA/QC samples), collect the following QA/QC samples:

2. One additional one-liter sample, to serve as a duplicate (field replicate). Collect the duplicate from a randomly selected sampling event/location as close as possible in time and space as the original one-liter sample for combined ERα/AhR analysis. This field replicate sample will be used to assess

the precision of sample preparation and analysis and will provide additional QA/QC to identify any sampling or bioanalytical/bioassay variability.

3. Four additional one-liter water samples: two one-liter samples for the ERα matrix spike analyses and two one-liter samples for the AhR matrix spike analyses collected from a randomly selected sampling event/location. If different water matrices are being tested as part of the sample batch, then a set of water samples for each distinct matrix should be collected for ERα and AhR matrix spikes. The matrix spikes will allow determination of the reference standard precision and recovery from the water sample.
4. One liter of ultrapure water from a randomly selected collection site to serve as a field blank sample. This field blank should replicate the sample collection procedure as closely as possible and is used to assess possible field contamination of samples at the sample collection site.
5. One liter of ultrapure water in the laboratory for use as a method blank sample to determine if active chemicals from materials (such as SPE sorbent, solvent) used in the method are affecting results.

Table 1 presents a sampling scenario indicating the number and types of samples collected per sample batch. For example, if sampling was carried out by a utility at six locations and had six samples total, the following samples and controls would need to be collected for extraction and bioassay analysis. In this example, the sixth water sample was randomly selected for the QA/QC field replicate and matrix spike sample.

Table 1. Erα and AhR bioassay water sampling scenario with six test samples.

Type of Sample	Liters for Bioassay Analysis
Field Blank for Combined ERα and AhR	1 (ultrapure water)
Method Blank for Combined ERα and AhR	1 (ultrapure water)
Samples 1–5 for Combined ERα and AhR	1/sample (5 total)
Sample 6 for Combined ERα and AhR	1
Sample 6 Replicate for Combined ERα and AhR	1
Sample 6 Matrix Spike and Matrix Spike Duplicate for ERα (E2) Recovery	2
Sample 6 Matrix Spike and Matrix Spike Duplicate for AhR (TCDD) Recovery	2
Total Liters For Analysis	13

In the event that any AhR-positive water sample analysis detects a TCDD-BEQ greater than 10 times the MTL, resample the site. Collect two additional one-liter water samples from the same site as the original positive sample. Use one sample to determine total AhR activity and the other sample to determine the presence/absence of toxic dioxin-like chemicals (DLCs) as described in Sections II-B-1 and II-B-2. Table 2 presents a resampling scenario for those sample sites in which the bioassay analysis detects a TCDD-BEQ 10 times greater than the MTL. This table indicates the number and types of samples that must be collected for detection of toxic DLCs.

Table 2. Water resampling scenario for five samples when analysis detects a TCDD-BEQ more than 10 times the MTL.

Type of Sample	Liters for AhR Bioassay	Liters for DLC Detection
Field Blank	1 (ultrapure water)	1 (ultrapure water)
Method Blank	1 (ultrapure water)	1 (ultrapure water)
Sample 1–4	4	4
Sample 5	1	1
Sample 5 Replicate	0	1
Sample 5 Matrix Spike for AhR (TCDD) and Matrix Spike Duplicate	0	2
Total Liters For AhR Analysis	7	10

c. Sample treatment and holding before transport

Prepare all one-liter sample bottles with sodium azide (1 gram per liter) to preserve the sample, and sodium thiosulfate (80 mg per liter) to quench any residual chlorine. The bottles for samples and blanks should be prepared identically. As soon as possible after sample collection, store the samples in coolers with ice packs to achieve a temperature of $\leq 6^{o}C$. Wet ice is acceptable as long as bottles are adequately sealed (for example, with Parafilm™ and placed in sealed bags). Extra care should be taken to protect the bottles from breaking in the cooler by using bubble wrap. Refrigerate samples at holding site at $\leq 6^{o}C$ while awaiting transport.

3. Transporting Samples

Deliver the samples to the testing laboratory as soon as possible after sampling. During transport, keep the samples in a closed cooler with ice packs to minimize exposure to light, temperature increases, and external contamination. Sample temperature must not exceed $6^{o}C$ during the first 14 days after collection and must be confirmed to be at or below $6^{o}C$ when received at the testing laboratory. If there is insufficient time for sample temperature to be reduced to $\leq 6^{o}C$, it must be demonstrated that the samples

are on ice and in the process of cooling. After samples arrive, store them at ≤6°C and protect them from light until they are extracted.

4. Storing Samples and Sample Extracts

While samples should be extracted as soon as possible, they can be stored up to 14 days at ≤6°C before extraction, and must not be frozen. The duration and conditions of sample storage must be recorded for each sample. Sample extracts can be stored frozen at –20°C/–80°C, but should be analyzed within 45 days after extraction.

5. Tracking Chain of Custody

Sample information, including the date, time, location, name of the person who collected the sample and any other relevant information is recorded at the time of collection on a Chain of Custody (COC) form and forwarded with the sample. Shipment information (date, time, temperature, shipping conditions, etc.) is added to the COC form when it is sent to the analysis laboratory (outside or in-house testing laboratory) and receipt information is added to the COC form (date, time, condition, temperature, etc.) and the name of the individual receiving the samples. Transfer of the samples through storage, extraction, and disposal is also tracked and relevant information noted. Custody tracking should include all chronological documentation that records the sequence of collection, custody, control, transfer, analysis, and disposition of the sample.

B. Sample Preparation

1. Extracting Samples for Detection of ERα- and AhR-Active Chemicals

Water samples (test samples, matrix spike samples, field blank samples, and method blank samples) should be prepared using solid phase extraction (SPE). The final extract from this method is used directly in both the ERα and AhR bioassays (Figure 2). Reagent-grade solvents and chemicals must be used in all procedures.

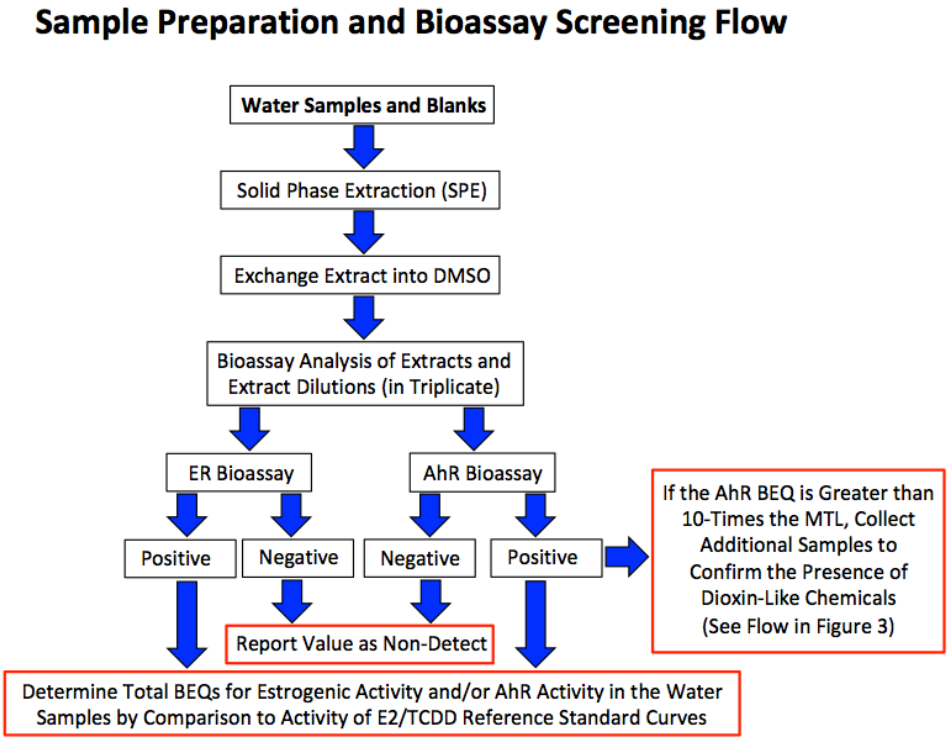


Figure 2. Overall water sample extract preparation and ERα/AhR bioassay analysis.

SPE cartridges (such as Oasis HLB 6 cc, 200 mg) are preconditioned by passing 2 x 3 mL of dichloromethane (DCM), 2 x 3 mL of methanol, and 2 x 3 mL of ultrapure water, in that order. Larger SPE cartridges can be used (such as Oasis HLB 6 cc, 500 mg), but require larger volumes of solvent for preconditioning and eluting. Extract the non-filtered, one-liter samples using a preconditioned SPE cartridge with a vacuum manifold (or equivalent) not exceeding a flow rate of 10 mL/min. Allow the SPE cartridge to dry on the manifold for 30 min and the dried cartridge can be stored at –20°C (wrapped in foil) until eluted.

Elute the cartridge with 2 x 5 mL of methanol followed by 2 x 5 mL of DCM. For each sample, combine the methanol and DCM elution fractions and evaporate down to ~0.5 mL under a gentle stream of high-purity grade nitrogen gas (do not evaporate to dryness). Rinse the walls of the tube with 1 mL of 50:50 methanol:DCM to ensure that any material adsorbed to the side of the tube is collected, evaporate down to ~0.5 mL, then add DMSO (0.1 mL) and evaporate the remaining methanol:DCM under nitrogen gas. If a precipitate is observed, dilute the sample further with a minimal amount of DMSO (up to 0.25 mL DMSO) to dissolve the precipitate. The walls of the tube can be rinsed with the DMSO when it is added to ensure that any material adsorbed to the side of the tube is collected. SPE methods using different solvent combinations and cartridges as well as other methods have also been described for the preparation of

water extracts for ERα and/or AhR bioassay analysis, and are alternative methods that could be used (6,34,38,39,41,42). While the DMSO sample extract can be stored frozen at –20°C/–80°C for an extended period, extracts should be analyzed in the ERα and AhR bioassays within 45 days after extraction.

2. Extracting Samples for Detection of Toxic AhR-Active Dioxin-Like Chemicals (DLCs)

For every AhR-positive water sample extract with an AhR-BEQ greater than 10 times the MTL, collect two additional one-liter water samples from the same site (see Table 2) and separately extract each sample by SPE as described in Section II-B-1. One of the sample extracts (for detection of DLCs) is stored frozen at –20°C/–80°C, while the other extract is dried and exchanged into DMSO as described in Section II-B-1 and tested in the AhR bioassay. If the calculated AhR-BEQ for this sample is less than 10 times the MTL, it indicates that the sample no longer contains high enough levels of AhR-active chemicals to warrant further processing and analysis of the remaining frozen sample extract for DLCs. However, if the calculated AhR-BEQ for this sample is greater than 10 times the MTL, it confirms the presence of high levels of AhR activity at this sample site, and the remaining frozen sample extract is thawed and further cleaned up as described in the following paragraph to remove nontoxic AhR activators before analysis. Analysis of these two sample extracts will allow determination of total AhR activity and detection of any toxic DLCs present in the samples (Figure 3).

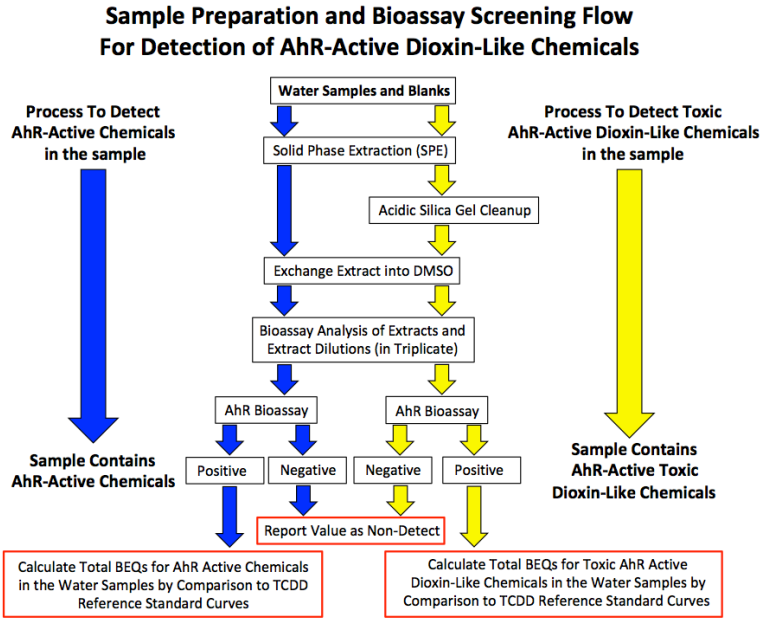


Figure 3. Overall sample preparation and cleanup of AhR bioassay positive water samples for determination of total AhR activity and the absence or presence of toxic DLCs.

Sample cleanup for analysis of toxic DLCs involves passage of the methanol:DCM sample extract over an acidic silica gel column to remove unwanted nontoxic AhR-active chemicals. Sulfuric acid (55%)-impregnated silica gel cartridges for sample cleanup are commercially available from Fujifilm/Wako Pure Chemicals (Presep Cartridges Cat#293-35581) (described in reference 31). Before loading the sample, the column is rinsed with 20 mL of n-hexane. Mix the extract of the DLC confirmation water sample, the field blank sample, and the method blank sample with 2 mL of n-hexane and apply to separate cleanup columns. Dioxin-like toxic AhR-active chemicals are eluted from the column with 18 mL of n-hexane, the eluate is evaporated down to ~0.5 mL under a gentle stream of nitrogen (do not evaporate to dryness), followed by the addition of DMSO (0.1 mL) and evaporation of the remaining n-hexane. The development and application of this extraction/cleanup method for detection of dioxin-like toxic AhR activators using cell bioassays is described by Suzuki et al. (31).

Sulfuric acid (55%)-impregnated silica gel is also commercially available (Fujifilm-Wako Pure Chemicals (cat. 197-13811) for those who prefer to pack their own columns. Other prepackaged multilayer acidic silica gel cartridges and columns specifically designed for dioxin sample cleanup are also commercially available (such as GL Sciences (1050-24011 and 1050-24301), Supelco (52732-U and 21267-U), and Fujifilm Wako (295-41651)), but include 22% to 44% sulfuric acid-impregnated silica gel and/or more complex chromatography methods, and require different solvents and elution conditions (38,42). A number of commercially available systems for automated dioxin cleanup can be used for this process, and additional details on dioxin cleanup methods can be found in USEPA method documents (8,38,39). The final DMSO sample extract can be stored frozen at –20°C/–80°C but should be analyzed in the AhR bioassays within 45 days after extraction.

3. Analyzing Matrix Spike Recovery

Recovery analysis is very important for quantitative bioanalytical methods, so including matrix spikes in duplicate water samples collected at the same time as the test samples allows determination of the percent recovery of the desired ERα and AhR agonists through sample processing.

It is recommended that E2 be used as the reference standard spike for chemicals detected by the ERα bioassay, and it should be spiked at a concentration of 3.5 ng/L. For the AhR bioassay, it is recommended that TCDD be used as the reference standard spike for chemicals detected by the AhR bioassay and it should be spiked at a concentration of 0.5 ng/L (water solubility is reported to be 8–19 ng/L (43,44)).

E2 and TCDD used as reference standards and matrix spike recovery standards for bioassay analysis must be certified for chemical composition and concentration by instrument analysis either by the vendor or the bioassay analysis laboratory.

Duplicate matrix spike water samples (two one-liter samples for ERα and two one-liter samples for AhR) are needed for each extraction batch of the same sample matrix type. Do not combine the ERα and AhR spike to the same one-liter matrix spike because of potential interference between ERα and AhR activators in the bioassays. The water sample that was spiked may contain ERα/AhR-active chemicals, so determination of the matrix spike recovery must be corrected. To do this, the total ERα/AhR activity of the one-liter matrix spike sample (BEQ (water+spike)) minus the total ERα/AhR activity in the unspiked one-liter water sample (BEQ (water)) measures the recovered activity of the reference spike. Comparing this concentration to the activity of the original spiked amount of E2 or TCDD (BEQ (spike)) allows determination of the overall percent recovery of the reference spike (45). The equation to calculate the E2/TCDD spike recovery is:

$$\text{E2/TCDD spike recovery} = \frac{\text{BEQ (water+spike)} - \text{BEQ (water)}}{\text{BEQ (spike)}}$$

III. BIOASSAY ANALYSIS

A. Overview

Water sample extracts prepared as described above are analyzed in ERα and AhR cell bioassays using the SOPs specifically developed, optimized, and validated by the bioassay producers/manufacturers. These bioassay SOPs should be followed as closely as possible and the SOPs should be provided by the test kit manufacturer, cell line developer, and/or commercial laboratory that conducts the analysis. Despite the different bioassays that are available, for all bioassays there are specific, required QA/QC criteria and test method components that ensure the results generated using a specific test method are accurate and interpretable. The common criteria and test method components necessary for inclusion in all ERα and AhR cell bioassays are presented below.

The overall flow for sample preparation and ERα/AhR bioassay analysis of water samples is outlined in Figure 2. ERα/AhR-responsive cells are incubated with an aliquot of each sample for 18–24 hours (depending on the specific bioassay). Measure reporter gene activity and subtract the background reporter gene activity in method/solvent blank-treated cells from the activity of the sample-treated cells. All sample extracts, dilutions, and controls are analyzed in the bioassay in at least triplicate wells of the assay plate.

While the highest concentration of each extract is generally used in the bioassay, based on the maximal DMSO concentration allowed in the assay, the final concentration of the sample in a test well should be equivalent to at least 10 to 20 times that of the original collected water sample. Typically 1 or 2 dilutions of the sample extract are tested in each bioassay. Comparison of the induced reporter gene activity above that of the method blank to the activity obtained with the positive reference standard for the particular assay, after subtraction of the background reporter gene activity of the solvent blank sample, allows determination of the relative potency (BEQ) of the sample to activate the ERα- or AhR-dependent cell bioassay. Results should be reported in ng E2-BEQ or TCDD-BEQ/L.

Samples are considered negative in the bioassays and reported as non-detects if the E2- or TCDD-BEQs are below the current reporting limit, while samples exhibiting positive activity have E2- or TCDD-BEQs greater than or equal to the current reporting limit (see the current reporting limits (Table 3) and the MTLs in Table 9 of the Recycled Water Policy (1)). For a reporting limit of 0.5 ng/L, a total E2- or TCDD-BEQs <0.5 ng/L is reported as non-detect, while positive activity is observed with E2- or TCDD-BEQs $\geq$0.5 ng/L. If a sufficiently high level of reporter gene activity is obtained with a given sample extract (greater than 15%

of the maximal response elicited by the reference standard compound), then make and analyze three to five serial dilutions of the sample in the bioassay, along with appropriate controls and reference standards, and calculate BEQs for all sample dilutions as a more accurate way to determine its relative potency (BEQs). The dilutions should be made so that several concentrations fall in the linear portion of the concentration response curve (>15% and <85%) of the reference standard, depending on the magnitude of the original response, and the results should show an extract concentration-dependent BEQ-induction response.

Since the AhR can be activated by a wide variety of non-toxic and toxic AhR-active chemicals (15,19,23,35-37), for those water sample extracts confirmed to have an AhR BEQ greater than 10 times the MTL, two additional water samples and associated QA/QC controls must be collected from the site of the original positive sample (see Table 2) and extracted/concentrated by SPE (Figure 3). Subject one of the two water sample extracts to an additional acidic-silica (AS) cleanup step that allows separation of the nontoxic from toxic AhR activators (Figure 3). AhR bioassay analysis of the SPE-prepared sample extract will allow determination of total AhR activity of the sample, while the analysis of the SPE/AS-prepared sample extract will allow determination of the presence or absence of toxic DLCs in the extract (Figure 3). BEQs of AhR active samples can be determined by comparison to the TCDD reference standard curve. Compare the TCDD-BEQ of the total sample extract to the TCDD-BEQ of the AS cleaned up fraction of the sample, which represents AhR activity of toxic DLCs, to determine the relative concentration of toxic and nontoxic AhR activators present in the original sample.

B. Quality Assurance/Quality Control

All reference standards and extracts of water samples, field and method blanks, and matrix spike samples are dissolved in DMSO. Appropriate (serial) dilutions, if required, are prepared in the same solvent. Before being dissolved, all substances should be allowed to equilibrate to room temperature. Stock and working solutions of reference standards, solvent and method blanks, matrix spike solutions, and water sample extracts should not have noticeable precipitate or cloudiness. Reference standard stocks may be prepared in bulk, and final dilutions of reference standards should be prepared fresh before each experiment and used within 24 hours.

C. Controls and Standards

Results with negative and positive controls allow determination of the acceptability of the test run. Solvent/vehicle, field, and method blanks are included to eliminate the contribution of chemicals from the vehicle solvent, field sample collection, sample preparation, and extraction methods and to determine

reporter gene activity induced by aspects of the test procedure itself. These controls must be included in every bioassay run. In addition to test samples, each bioassay plate should contain the following controls and standards: cells with and without DMSO, solvent/vehicle control, method blank control, field blank control, and cells with increasing concentrations of the relevant reference standard. Bioassay reference standards (E2 and TCDD) must be certified for chemical composition and concentration by instrument analysis.

1. Solvent/Vehicle Control

The solvent used to dissolve the test extracts and standards must be included as a solvent control. DMSO, (0.5–1% (v/v) maximum concentration in the assay incubation) is typically used as the solvent/vehicle for ERα and AhR bioassays. The DMSO solvent blank is used to demonstrate that no ERα-/AhR-active chemical is present in the DMSO that was used to resuspend the samples, and it provides a measure of baseline/background reporter gene activity of the bioassay. This activity is subtracted from the reporter gene activity of the reference standards. It is recommended that a lower DMSO concentration be used if DMSO induces reporter gene activity when compared to cells incubated in the absence of DMSO (Table 3).

Table 3. Performance-Based QA/QC guidelines for ERα/AhR bioassays for water quality screening (adapted from 5, 6).

Parameter	Acceptance criteria
Solvent control	Mean response for solvent/vehicle-treated cells should be within 20% RSD of mean response of untreated cells (i.e., cells with no added DMSO).
Calibration	Hill slope and logEC_{50} values for reference standard chemicals should be within the expected range for the given bioassay (LogEC_{50} values should be ± 0.5 log units); R2≥0.95.
Calibration induction ratio	Mean maximal response for the reference standards (E2/TCDD) should be at least four times greater than the mean response of the DMSO control.
Extract cytotoxicity	Cell viability of sample-treated cells should be ≥80% viability compared to cells incubated without DMSO.
Matrix spike	Recovery of spiked activity should be 70 to 130%.
Intra-laboratory precision	RSD/CV of triplicate measurements should be ≤25% for a given sample (including QA and certified reference standard chemical).
Sample measurement (BEQ, ng/L)	Log concentration quantification for (1) samples showing concentration-response (i.e., 3–4 concentrations tested), and (2) concentrations within the linear portion of the log-sigmoidal concentration response curve (i.e., 15% to 85% of the reference standard response). Alternatively, analysis in the initial portion of a linear concentration response curve (0–30%) may be used (47).

2. Method Blank Control

The method blank is an extract of one liter of ultrapure water (or its equivalent) that was prepared in the laboratory and has undergone the identical procedure as the test water samples (including handling, extraction/cleanup, and resuspension in solvent). The method blank is used to determine if these processes generated and/or released any ERα- or AhR-active chemical and if this activity can be subtracted from that of the test sample extract. If elevated reporter gene activity is observed in the method blank sample (i.e., activity above that of the other blanks), consider retesting the sample (a repeat sampling event) after reviewing laboratory procedures to avoid future contamination.

3. Field Blank Control

The field blank is an extract of one liter of ultrapure water (or its equivalent) that is transported into the field in a glass bottle, poured into the same type of glass bottle used for sampling, and subjected to the identical procedure as the test water samples (handling, extraction/cleanup, and resuspension in solvent). The field blank is used to determine if field conditions or handling procedures during sampling introduced any ERα- or AhR-active chemical into the sample. Subtracting method blank activity from this activity measures the contamination from sample collection and handling. If elevated reporter gene activity is observed in the field blank sample (activity above that of the other blanks), consider retesting the sample (a repeat sampling event) after reviewing field sampling and handling procedures to avoid future contamination.

4. Reference Standards

The reference standard for the ERα bioassay is E2 and for the AhR bioassay is TCDD. The concentration range for each reference standard comprises a series of dilutions of six to eight concentrations of each compound that cover the full concentration response curve for induction of reporter gene expression in ERα and AhR bioassays (typically $1x10^{-13}$ M to $1x10^{-8}$ M). Compare results between different runs for a given bioassay by comparing the EC_{50} of full reference standard curves that were included in each run. Comparing results between plates in a given bioassay run requires plate-to-plate standardization, which is accomplished by including full reference standard curve on one plate and partial reference standard curves (fewer dilutions) or full reference standard curves on subsequent plates and comparing the resulting EC_{50} for each. EC_{50} values outside of the laboratory's historical range and/or significant differences in EC_{50} between plates (outside of predefined limits) would indicate excessive variability, in which case the data from the whole plate should be discarded and the test should be repeated.

a. Test Run Acceptability Criteria

Acceptance or rejection of a test run is based on the evaluation of results obtained for the reference standards and controls used for each experiment. Values for the reference standards (EC_{50}) should meet the acceptability criteria as provided for the selected bioassay, and all positive/negative controls should be correctly classified for each accepted experiment. The ability to consistently conduct the test method should be demonstrated by the development and maintenance of a historical database for the reference standards and controls. Standard deviations (SD) or coefficients of variation (CV) for the means of reference standards curve fitting parameters as well as the method detection limit/limit of detection (MDL/LOD) from multiple experiments may be used as a measure of intra-laboratory reproducibility. For bioassays, the MDL/LOD is defined here as the minimum concentration of an ERα- or AhR-active substance (E2 for ERα and TCDD for AhR) that induces reporter gene activity to a level that is significantly greater (with 99% confidence) than the background reporter gene activity in the solvent (DMSO)-treated control cells (28,46). Regardless of whether the bioanalytical analysis is performed by a commercial or in-house laboratory, some general QA/QC guidelines should be followed that address key analytical parameters and their control levels. Table 3 provides some performance-based QA/QC guidelines for the ERα/AhR bioassays; if any of these criteria are not met, then the entire test plate is considered invalid.

b. Sample Run Acceptability Criteria

Each bioassay run must contain at least one full series of dilutions of the reference standard, as well as solvent control and method blank samples. The test sample is determined to have positive ERα/AhR activity if an increase in reporter gene activation is measured which exceeds the reporter gene activity of method blank-treated cells in a statistically significant manner ($p<0.05$ (t-Test or other appropriate method)). The following principles regarding acceptability criteria for the samples should also be met:

1. The relative standard deviation of triplicate assays of a given sample should be <20% (inter-well deviation). If the tested sample concentration shows no response above the limit of detection in all triplicate wells, the relative standard deviation is allowed to be >20 %.
2. No other types of interfering conditions have been observed (such as microbial contamination or cytotoxicity).

If any of these criteria are not met, the sample should be retested.

IV. DATA ANALYSIS AND INTERPRETATION

A. Overall Analysis of Data

The data interpretation procedure developed for each individual bioassay should be used for classifying a positive and negative response within that specific bioassay; however, positive and negative responses relative to the Recycled Water Policy guidelines must be based on ERα and AhR reporting limits (see Section III-A and reference 1). Meeting the test run and sample run acceptability criteria described above (Section III-D and III-E) indicates the test method is operating properly, but it does not ensure that any particular test run will produce accurate data. Accordingly, analysis of random duplicate water sample extracts, analysis of matrix spikes, inclusion of negative controls and concentration-response analysis for the certified reference standards in every assay run, as well as inclusion of the analysis of serial dilutions of any positive test sample extracts, will provide the results necessary to indicate whether accurate data were produced.

B. General Data Interpretation Criteria

Because the literature indicates different approaches for analyzing ERα and AhR bioassay data to calculate the sample result, the interpretation and calculation approach described in this document should be followed for consistency with the Recycled Water Policy. Quantitative (for example, EC_{50}, BEQ) assessments of ERα/AhR-mediated activity should be based on empirical data and sound scientific judgment. Positive results should be characterized by both the magnitude of the effect as compared to the solvent/vehicle control and positive reference standard (E2/TCDD).

C. Calculation of the Bioanalytical Equivalent (BEQ) Concentration of a Sample Extract

For a bioassay run of a single 96-well plate, the plate should include a full (six points minimum, spanning the full response range from 0 to 100%, with at least three points between 15% and 85%) concentration-response curve of the certified reference standard. For a bioassay run of multiple multi-well plates analyzed at the same time, one plate should include a full concentration-response curve (six points minimum, spanning the full response range from 0 to 100%, with at least three points between 15% and 85%) of the certified reference standard (E2 or TCDD) and the other plates in the same run/batch should include a partial concentration-response curve of the certified reference standard (three to four points within the linear range, and must include a maximally inducing concentration to ensure the same maximal activity is obtained between plates). Concentration response analysis results obtained with the reference standard, after subtraction of reporter gene activity of the solvent blank, are expressed as a percent of the maximal induction observed with the reference standard (set at 100%). These results are used to

develop a calibration curve whose characteristics are determined using one of a variety of mathematical modeling methods, such as a least squares/best fit of the sigmoidal logistic standard curve using a two or four parameter Hill Equation formula (with 0% response set to the signal of the unexposed controls and 100% response set to the maximum of the reference standard compounds), a low-effect level linear concentration-response analysis (linear regression) method (47), or other comparable mathematical model.

The induction results obtained for a given concentration of test sample extract, after subtraction of reporter gene activity of the method blank sample, can be inserted into the equation for the reference standard curve and used to calculate the E2-BEQ (for ERα) or TCDD-BEQ (for AhR) of the sample aliquot by direct comparison of the bioassay results to the corresponding activity on the reference standard curve. Graphical examples of such determinations are shown in Appendix 3. BEQ values are reported as the mean ± SD of the results of triplicate analyses of each sample in an individual bioassay.

Typically, one or two dilutions of the sample extract are tested in each bioassay. If a sufficiently high level of reporter gene activity is obtained with a given sample extract (>15% of the reference standard curve), a dilution response curve of induction by three to five serial dilutions of the sample extract can be carried out and BEQs for each dilution determined as a more accurate way to determine its relative potency and to confirm that the slopes of the sample and reference concentration response curves are comparable if a sufficiently robust induction response is obtained.

If the reference standard and sample concentration response curves are not comparable (not parallel), a range of BEQs for the sample may need to be determined from multiple points on the sample concentration response curve for greater accuracy or activity determined from linear concentration response analysis (47,48). The quality of the curve fitting of concentration response curves and calculation of relative activity (BEQs) for test sample extracts can readily be determined with a variety of commercially available statistical software packages. If the quality of the curve fitting is not acceptable (R^2 (goodness of fit) < 0.95), use weighting to account for different variances or choose other mathematical models, for example, a five parametric logistic function or other method. If no acceptable curve fitting is possible for the induction results for the sample extract, then calculation of the E2 or TCDD BEQ for a given sample can only be determined from the concentrations with a positive induction response. BEQ values from triplicate analyses of the aliquot of sample extract (one or two dilutions) are normalized to the original one-liter water sample and final values expressed as ng E2/L for ERα or ng TCDD/L for AhR. For positive activity samples, the ratio of the BEQ to the MTL for the specific bioassay is calculated in order to compare

the resulting BEQ/MTL ratio to the thresholds provided in Table 10 of the Recycled Water Policy (1), which specifies BEQ/MTL thresholds and Response Actions for the ERα and AhR bioanalytical screening tools.

D. Data Analysis Approach for Non-Detects

In the event of a non-detect result for the sample BEQ, the BEQ/MTL ratio must be calculated as described in Sections IV-D-1 and IV-D-2, below, and compared to the thresholds provided in the Recycled Water Policy (1), which specifies BEQ/MTL thresholds and Response Actions. Guidance on identifying a non-detect result is described below in Section IV-D-1. The California Recycled Water Policy provides guidance on how to calculate BEQ/MTL in the event of a non-detect BEQ, which is described below in Section IV-D-2.

1. Definition of non-detect and activity less than the reporting limit (<RL)

A non-detect (ND) is defined as a sample for which the bioassay reporter gene activity is not significantly different from background activity (activity in the method blank). A sample for which the E2- or TCDD-BEQ for reporter gene activity is significantly above background activity (making it a positive response) but below the Reporting Limit (0.5 ng E2 or TCDD/L) set in the California Recycled Water Policy (1) is defined as below the reporting limit (<RL).

2. Calculation of BEQ/MTL in the event of a non-detect BEQ sample result

The California Recycled Water Policy (1) specifies that if a sample BEQ cannot be calculated (if it is a non-detect result or the value is at or below the ERα or AhR bioassay Reporting Limit indicated in the Recycled Water Policy), the Reporting Limit shall be the value used to generate the BEQ/MTL. The MTL values for these assays are specified in the Recycled Water Policy (1). For example, using Reporting Limits and MTL values of 0.5 ng/L and 3.5 ng/L, respectively, for ERα, and 0.5 ng/L and 0.5 ng/L, respectively, for AhR, a non-detect sample result would result in a BEQ sample value of 0.14 for ERα (i.e., BEQ/MTL = 0.5/3.5 = 0.14 ≤ 0.15 threshold, denoting Response Action A) and 1.0 for AhR (i.e., BEQ/MTL = 0.5/0.5 = 1.0 ≤ 1.0 threshold, denoting Response Action A). Note that if a laboratory performing bioanalytical screening tools is able to demonstrate a bioassay MDL that is lower than the Reporting Limit specified in the Recycled Water Policy (1), they can use their MDL as an alternative to the Reporting Limit as either approach will result in BEQ/MTL values below the threshold for Response Action A.

a. Cytotoxicity/Cell Viability

Toxic effects present in the test samples may lead to a reduction of cell viability and, hence, to a reduction of the measured cellular response. Consequently, ERα or AhR responses of a sample may be masked by

acute toxic effects leading to false negative test results or reduced overall response. While cytotoxicity typically results in cells exhibiting an altered morphology (cells typically take on a rounded appearance) and being released from the plate, it does not provide a quantitative measure of cell viability. Accordingly, the cells in each bioassay shall be tested for health/viability to ensure that solvents, standards, test samples and/or methods used in the analysis do not result in cytotoxicity. Accordingly, all bioassay wells used for determination of ERα/AhR BEQs should have ≥80% viability as compared to wells containing untreated cells. A variety of validated cell viability assays (for example, PrestoBlue, Live Cell Multiplex, MTT, and others) can provide the quantitative results necessary to evaluate the impact, if any, of sample extracts on cell viability. If cytotoxicity is produced by a given sample extract, the sample can be diluted and retested at concentrations that produce no cytotoxicity. ERα or AhR activity should not be calculated from any sample that has <80% viability.

V. TEST REPORT

The test report should include the following information:

1. Testing location and contact information for testing laboratory, date of test, and other relevant information on the method and performing laboratory.
2. Identity of the test sample, including a description of the sample, origin and date of sampling, ancillary chemical measurements, if performed, and other characteristics of note.
3. Details of the collection, storage, and preparation of the sample, including storage conditions and time and other manipulations of the sample. Include Chain of Custody sample tracking.
4. Reference (positive control) compound (chemical name/CAS number), source, certification of purity and concentration, batch number or comparable data (if available), and solvent/vehicle (supplier and lot number).
5. Test conditions
    - 5.1. Description of the test method used (details of the method/protocol, kit (if used), and cell line).
    - 5.2. Description of the method used to assess cell viability.
    - 5.3. Enrichment factor of test samples and concentration of spiked chemicals.
    - 5.4. Volume of vehicle and test chemical added to the bioassay and final concentration.
    - 5.5. Duration of incubation.
    - 5.6. Cell density at the start of sample incubation.
    - 5.7. Positive reference standards and certification information.
    - 5.8. Reporter reagents (product name, supplier, and lot).
    - 5.9. Criteria for considering test runs as positive, negative, or equivocal.
6. Assay acceptability check
    - 6.1. Induction responses for each assay plate and whether they meet the minimum required by the test method based on historical controls.
    - 6.2. Actual values for acceptability criteria (EC_{50}, MDL, full concentration response range, Hill slope values for concurrent positive controls/reference standards).
7. Test results
    - 7.1. Raw, normalized (background adjusted), and calculated data for test samples and all standards and controls must be presented in a spreadsheet. Include a measure of error and confidence, such as SD, CV, or 95% CI), and how these values were obtained.

7.2. Include test and sample acceptability criteria and specific acceptability results obtained for all samples and standards.

7.3. Cytotoxicity data with method used and other observed effects (for example, contamination or precipitation).

7.4. Quantitative assessment of the ERα or AhR activity of the sample in BEQs and an overall qualitative assessment (Y/N).

7.5. Concentration-response relationship results for sample extracts, if determined.

7.6. When a BEQ for the sample or sample dilutions is calculated, a description of how these values were obtained must be provided.

8. Discussion of the results
9. Conclusion

VI. ADDITIONAL INFORMATION

A. Laboratory Certifications and Bioassay Proficiency

Rather than limiting water analysis to the few bioassays that have been used extensively, it is recommended that all appropriate ERα/AhR bioassays and laboratories conducting such bioassays can be eligible for inclusion in the proposed water recycling and reuse bioassay analysis. In order to demonstrate their competency in ERα/AhR bioassay analyses for water samples, all laboratories that participate must follow and meet the performance criteria described in this document (for example, positive and negative controls, matrix spikes, and other QA/QC aspects) and provide all required information regarding the characteristics in the test report.

Although there currently is no officially recognized certification process or open round-robin bioassay testing program that documents the performance/proficiency testing competency of individual laboratories to conduct AhR bioassays, one certification process and round-robin testing for water and wastewater using selected ERα cell bioassays has been previously reported in the European Union (34). While ISO or good laboratory practice (GLP) certification can acknowledge quality standards associated with a particular laboratory, and certain ERα/AhR bioassay protocols have received official governmental regulatory approval (7,8,34), performance/proficiency testing and assay validation for most currently available bioassays have been carried out in-house or in limited collaborative studies (28,29,49–53). The OECD *Guidance Document on Good In Vitro Method Practices* (54), provides useful GLP guidelines for *in vitro* test methods for regulatory use in human safety assessment. However, if the specific method has been subjected to an independent validation of the accuracy, precision, and reliability of the procedure by multi-laboratory, multi-user, and day-to-day replication, and/or used in double-blinded validation or round-robin analysis by multiple independent laboratories, this should be described.

B. Safety

This document does not address all safety issues associated with the protocols of these bioassays. Accordingly, the laboratory is responsible for maintaining a safe work environment and a current awareness file of Occupational Safety and Health Act (OSHA) regulations regarding the safe handling of the chemicals listed in this method. A reference file of material safety data sheets (MSDS) should be available to all personnel involved in these analyses.

Safety procedures in compliance with GLPs and OSHA standards should be maintained at all times. Some reagents may contain hazardous materials such as solvents and acids; therefore, the technician should

use caution when using the reagents and avoid contact with eyes, skin, and mucous membranes. All waste materials and solutions should be placed in appropriate containers and disposed of according to all governing state and federal regulations.

The AhR bioassay method uses dilute concentrations of TCDD for the standard curve, positive controls, and matrix spikes. TCDD is an extremely toxic chemical, and the analyst should take the appropriate measures when preparing, handling, and disposing of TCDD and any materials that have come in contact with it. Analysis laboratories must follow all relevant local, state, and federal guidelines when working with and disposing of these chemicals.

Some ERα/AhR cell bioassays use human cancer cell lines, and any materials that have come in contact with or contain these materials are considered biohazardous and must be disposed of as human medical waste. Accordingly, laboratories using human-based cell bioassays must follow all relevant local, state, and federal guidelines in working with and disposing of these materials.

VII. REFERENCES

1. State Water Resources Control Board (2018). Water Quality Control Policy for Recycled Water (inclusive of Attachment A: Requirements for monitoring constituents of emerging concern for recycled water), State Water Resources Control Board, Sacramento, CA.
2. OECD (2005). Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 31), Organisation for Economic Cooperation and Development, Paris.
3. Anderson, P., Denslow, N., Drewes, J.E., Olivieri, A., Schlenk, D. and Snyder, S. (2010). Monitoring Strategies for Chemicals of Emerging Concern (CECs) in Recycled Water: Final Report, California State Water Resources Control Board, Sacramento, CA.
4. Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E. and Villeneuve, D.L. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment, Environ Toxicol Chem 29: 730–741.
5. Drewes, J.E., Anderson, P., Denslow, N., Jakubowski, W., Oliveri, A., Schlenk, D. and Snyder, S. (2018). Monitoring strategies for constituents of emerging concern (CECs) in recycled water, Southern California Coastal Water Research Group (SCCWRP), Technical Report 1032.
6. Mehinto, A.C., Jia, A., Snyder, S.A., Jayasinghe, B.S., Denslow, N.D., Crago, J., Schlenk, D., Menzie, C., Westerheide, S.D., Leusch, F.D.L. and Maruya, K.A. (2015). Interlaboratory comparison of in vitro bioassays for screening of endocrine active chemicals in recycled water, Water Res. 83, 303–309.
7. OECD (2016). Performance-Based Test Guidelines For Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists (for TG 455). Environment, Health and Safety Publications, Series on Testing and Assessment (No.173.), Organisation for Economic Cooperation and Development, Paris.
8. USEPA (2014). SW-846 Test Method 4435: Screening for dioxin-like chemical activity in soils and sediments using the CALUX bioassay and toxic equivalents (TEQs) determinations, USEPA, Washington, DC. https://www.epa.gov/sites/production/files/2015-12/documents/4435.pdf
9. Commission Regulation (EU) (2014). No 589/2014 of 2 June 2014 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 252/2012 (food).
10. Commission Regulation (EU) (2014). No 709/2014 of 20 June 2014 amending Regulation (EC) No.152/2009 as regards to the determination of the levels of dioxins and polychlorinated biphenyls (feed).
11. Leusch, F.D.L. and Snyder, S.A. (2015). Bioanalytical tools: half a century of application for portable reuse, Environ. Sci. Water. Res. Technol. 1, 606–621.
12. Tyler, C.R., Jobling, S. and Sumpter, J.P. (1998). Endocrine disruption in wildlife: A critical review of the evidence, Crit. Rev. Toxicol. 28, 319–361.
13. Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. and Peterson, R.E. (2006). The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol. Sci. 93, 223–241.

14. White, S.S. and Birnbaum, L.S. (2009). An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. J. Environ. Sci. Health, pt. C. Environ. Carcin. Ecotoxicol. Rev. 27, 197–211.

15. Denison, M.S., Soshilov, A.A., He, G., DeGroot, D.E. and Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor, Toxicol. Sci. 124, 1–22.

16. Bergman, K., Heindel, J.J., Jobling, S., Kidd, K.A. and Zoeller, R.T. (2013). State of the science of endocrine disrupting chemicals—2012: An assessment of the state of the science of endocrine disruptors. World Health Organization, United Nations Environment Programme. Geneva, Switzerland.

17. De Coster, S. and Van Larebeke, N. (2012). Endocrine-disrupting chemicals: Associated disorders and mechanisms of action, J. Environ. Public Health, 2012: 713696.

18. McLachlan, J.A. (2016). Environmental signaling: from environmental estrogens to endocrine-disrupting chemicals and beyond, Andrology 4, 684–694.

19. Beischlag, T.V., Luis Morales, J., Hollingshead, B.D., and Perdew, G.H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression, Crit. Rev. Eukaryot. Gene Expr. 18, 207–250.

20. Aranda, A. and Pascual, A. (2001) Nuclear hormone receptors and gene expression, Physiol Rev. 81, 1269–1304.

21. Yasar, P., Ayaz, G., User, S.D., Gupur, G. and Muyan, M. (2016). Molecular mechanism of estrogen-estrogen receptor signaling. Reprod. Med. Biol. 16, 4–20.

22. Arnal, J.F., Lenfant, F., Metivier, R., Flouriot, G., Henrion, D., Adlanmerini, M., Fontaine, C., Gourdy, P., Chambon, P., Katzenellenbogen, B. and Katzenellenbogen, J. (2017). Membrane and nuclear estrogen receptor alpha actions: From tissue specificity to medical implications, Physiol. Rev. 97, 1045–1087.

23. Denison, M.S. and Nagy, S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals, Ann. Rev. Pharmacol. Toxicol. 43, 309–334.

24. Rogers, J.M. and Denison, M.S. (2000). 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 & Molec. Toxicol. 13, 67–82.

25. Behnisch, P.A., Hosoe, K. and Sakai, S.-I. (2001). Bioanalytical screening methods for dioxins and dioxin-like compounds – a review of bioassay/biomarker technology, Environ. Int. 27, 413–439.

26. Denison, M.S., Zhao, B., Baston, D.S., Clark, G.C., Murata, H. and Han, D.-H. (2004). Recombinant cell bioassay systems for the detection and relative quantitation of halogenated dioxins and related chemicals, Talanta 63, 1123–1133.

27. Sonneveld, E., Riteco, J.A., Jansen, H.J., Pieterse, B., Brouwer, A., Schoonen, W.G. and van der Burg, B. (2006). Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities, Toxicol. Sci. 89, 173–187.

28. Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Hecker, M., Giesy, J.P., Engwall, M., van Bavel, B. and Hollert, H. (2014). In vitro bioassays for detecting dioxin-like activity – Application potentials and limits of detection, a review, Sci. Total Environ. 487, 37–48.

29. Kunz, P.Y., Simon, E., Creusot, N., Jayasinghe, B.S., Kienle, C., Maletz, S., Schifferli, A., Schönlau, C., Aït-Aïssa, S., Denslow, N.D., Hollert, H., Werner, I. and Vermeirssen, E.L.M. (2017). Effect-based tools for monitoring estrogenic mixtures: Evaluation of five in vitro bioassays, Water Res. 110, 378–388.

30. Otarola, G., Castillo, H. and Marcellini, S. (2018). Aryl hydrocarbon receptor-based bioassay for dioxin detection: Thinking outside the box, J. Appl. Toxicol. 38, 437–449.

31. Suzuki, G., Nakamura, M., Michinaka, C., Tue, N.M., Handa, H. and Takigami, H. (2017). Separate screening of brominated and chlorinated dioxins in field samples using in vitro reporter gene assays with rat and mouse hepatoma cell lines, Anal. Chim. Acta 975, 86–95.
32. Gui, Q., Lawson, T., Shan, S., Yan, L. and Liu, Y. (2017). The application of whole cell-based biosensors for use in environmental analysis and in medical diagnostics, Sensors 17, 1623.
33. Schenborn, E. and Groskreutz, D. (1999). Reporter gene vectors and assays, Molec. Biotechnol. 13, 29–44.
34. ISO19040-3 (2018). Water quality - Determination of the estrogenic potential of water and waste water – Part 3: In vitro human cell-based reporter gene assay, International Organization for Standardization, Geneva, Switzerland. https://www.iso.org/standard/66297.html
35. Denison, M.S., Seidel, S.D., Rogers, W.J., Ziccardi, M., Winter, G.M. and Heath-Pagliuso, S. (1999). Natural and synthetic ligands for the Ah receptor, in: Molecular Biology Approaches to Toxicology, Edited by Puga, A. and Wallace, K.B., pp. 393–410, Taylor & Francis, Philadelphia.
36. Nguyen, L.P. and Bradfield, C.A. (2008). The search for endogenous activators of the aryl hydrocarbon receptor, Chem. Res. Toxicol. 21, 102–116.
37. DeGroot, D.E., He, G., Fraccalvieri, D., Bonati, L., Pandini, A. and Denison, M.S. (2011). AhR Ligands: Promiscuity in binding and diversity in response. In: The Ah Receptor in Biology and Toxicology, Edited by Pohjanvirta, R., pp. 63–79, John Wiley & Sons, Inc. Hoboken, NJ.
38. USEPA (2007). SW846 Test Method 8290A: polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS), USEPA, Washington, DC. https://www.epa.gov/sites/production/files/2016-01/documents/sw846method8290a.pdf
39. USEPA (1994). Test Method 1613: Tetra- through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS, USEPA, Washington, DC. https://www.epa.gov/sites/production/files/2015-08/documents/method_1613b_1994.pdf
40. USGS (2006). Collection of water samples, In: National Field Manual for the Collection of Water-Quality Data (Book 9), Handbooks for Water-Resources Investigations, U.S. Geological Survey, Reston, VA.
41. Daniels, K.D., VanDervort, D., Wu, S., Leusch, F.D.L., van de Merwe, J.P., Jia, A. and Snyder, S.A. (2018). Downstream trends of in vitro bioassay responses in a wastewater effluent-dominated river, Chemosphere 212, 182–192.
42. Sato, M., Takigami, H., Hayakwa, K. and Sakai, S. (2010). Water-quality monitoring technique for dioxins during dredging using on-site phase extraction with graphitic carbon and analysis with DR-CALUX, J. Environ. Sci Health, Pt. A, 45, 867–874.
43. Adams, W.J. and Blaine, K.M. (1986). A water solubility determination of 2,3,7,8-TCDD, Chemosphere 15, 1397–1400.
44. Marple, L., Brunck, R. and Throop, L. (1986). Water solubility of 2,3,7,8-tetrachlorodibenzo-p-dioxin, Environ. Sci. Technol. 20, 180–182.
45. Neale, P.A., Brack, W., Ait-Aissa, S., Busch, W., Hollender, J., Krauss, M., Maillot-Maréchal, E., Munz, N.A., Schlichting, R., Schulze, T., Vogler, B. and Escher, B. (2018). Solid-phase extraction as sample preparation of water samples for cell-based and other in vitro bioassays, Environ. Sci. Process. Impacts 20, 493–504.
46. USEPA (2016). Definition and procedure for the determination of the method detection limit, revision 2, EPA 821-R-16-006, USEPA, Washington, DC. https://www.epa.gov/sites/production/files/2016-12/documents/mdl-procedure_rev2_12-13-2016.pdf

47. Escher, B., Neale, P.A. and Villeneuve, D. (2018). The advantages of linear concentration-response curves for in vitro bioassays with environmental samples, Environ. Toxicol. Chem. 37, 2273–2280.

48. Villeneuve, D.L., Blankenship, A.L. and Giesy, J.P. (2000). Derivation and application of relative potency estimates based on in vitro bioassay results, Environ. Toxicol. Chem. 19, 2835–2843.

49. Long, M., Laier, P., Vinggaard, A.M., Andersen, R., Lynggaard, J. and Bonefeld-Jorgensen, E.C. (2003). Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line, Toxicol. 194, 77–93.

50. Leusch, F.D., de Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., Tremblay, L.A., Wilson, V.S. and Chapman, H.F. (2010). Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters, Environ. Sci. Technol. 44, 3853–3860.

51. Van Langenhove, K., Croes, K., Denison, M.S., Elskens, M. and Baeyens, W. (2011). The CALUX bio-assay: Analytical comparison between mouse hepatoma cell lines with a low (H1L6.1c3) and high (H1L7.5c1) number of dioxin response elements. Talanta 85, 2039–2046.

52. Jarosova, B., Blaha, L., Giesy, J.P. and Hilscherova, K. (2014). What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe?, Environ. Int. 64, 98–109.

53. Kase, R., Javurkova, B., Simon, E., Swart, K., Buchinger, S., et. al. (2018). Screening and risk management solutions for steroidal estrogens in surface and wastewater, Trends Anal. Chem. 102, 343–358.

54. OECD (2018). Guidance Document on Good In Vitro Method Practices (GIVIMP), OECD Series on Testing and Assessment, No. 286, OECD Publishing, Paris, https://www.oecd.org/env/guidance-document-on-good-in-vitro-method-practices-givimp-9789264304796-en.htm

APPENDICES

1. List of abbreviations.

2. Recommended sample preparation and bioassay test guidelines.

3. Graphical examples of bioassay BEQ determination.

APPENDIX 1. List of Abbreviations.

AhR – Ah Receptor

ARNT – Ah Receptor Nuclear Translocator

AS – Acidic Silica

BEQ – Bioanalytical Equivalent

CAS – Chemical Abstract Service

CECs – Chemicals of Emerging Concern

CI – Confidence Interval

COC – Chain of Custody

CV – Coefficient of Variation

DCM – Dichloromethane

DMSO – Dimethylsulfoxide

DLC – Dioxin-Like Chemical

DRE – Dioxin Response Element

E2 – 17β-Estradiol

E2-BEQ – 17β-Estradiol Bioanalytical Equivalent

EC_{50} – Effective Concentration Producing 50% Response

ER – Estrogen Receptor

ERα – Estrogen Receptor α

ERE – Estrogen Response Element

GLP – Good Laboratory Practice

ISO – International Organization for Standardization

L – Liter

LOD – Limit of Detection

MDL – Method Detection Limit

mg – Milligram

mL – Milliliter

MSDS – Material Safety Data Sheet

MTL – Monitoring Trigger Level

ng – nanogram

OECD – Organization for Cooperation and Development

OSHA – Occupational Safety and Health Act

PCBs – Polychlorinated Biphenyls

PCDDs – Polychlorinated Dibenzo-p-dioxins

PCDFs – Polychlorinated Dibenzofurans

PTFE – Polytetrafluoroethylene (Teflon)

QA/QC – Quality Assurance/Quality Control

RL – Reporting Limit

RSD – Relative Standard Deviation

SAP – Science Advisory Panel

SD – Standard Deviation

SOP – Standard Operating Procedure

SPE – Solid Phase Extraction

TCDD – 2,3,7,8-Tetrachlorodibenzo-p-dioxin

TCDD-BEQ – TCDD Bioanalytical Equivalent

USEPA – United States Environmental Protection Agency

USGS – United States Geological Survey

UV – Ultraviolet

APPENDIX 2. Recommended sample preparation and bioassay test guidelines.

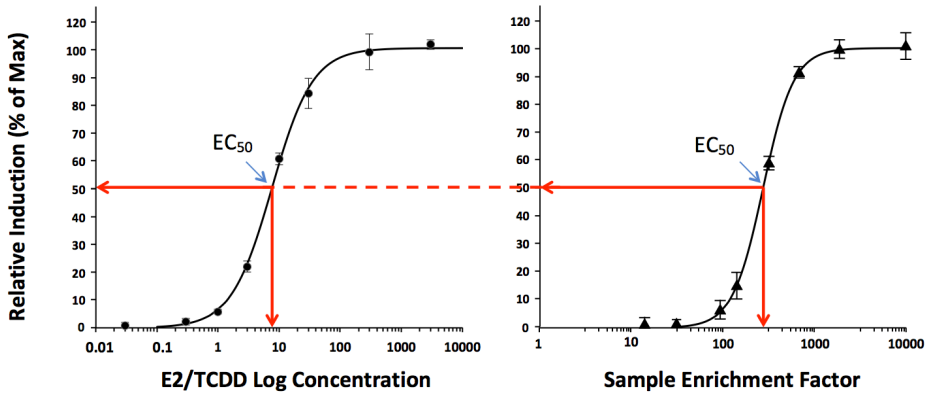
Component	Guideline
Sample collection and preservation	1L water samples in amber glass bottles containing sodium thiosulfate (80 mg/L) (only if sample was chlorinated) and sodium azide (1 g/L)
Sample storage	Up to 14 days at ≤6°C before extraction Up to 45 days at –20°C or –80°C after extraction before analysis
Sample extraction	Solid phase extraction using Oasis HLB cartridges (or equivalent) Elution using 5 mL methanol and 5 mL dichloromethane (if using a 200 mg Oasis HLB SPE cartridge) Final extract in 0.1 – 0.25 mL DMSO
Extraction QA samples	One method blank and one field blank Duplicate matrix spike samples (spiked with assay-specific reference chemical at EC_{50} – EC_{80} level) One replicate field sample
Assay-specific reference standard chemical	17β-Estradiol for ERα assay 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) for AhR assay
Calibration	Full 6–8 point concentration-response curve on one plate Partial curve (3–4 point) on subsequent plates within the same run/batch, with one concentration producing a maximal induction response to allow interplate comparisons
Enrichment factor and test sample concentration factor	Extracts are prepared in DMSO and the highest concentration of test sample in the assay wells must be at least 10–20X higher than that of the original sample (unless dilution is required due to cytotoxicity)
Bioassay measurement	All samples tested at least in triplicate
Bioassay QA samples	Cells in media (for cytotoxicity evaluation) Cells in media with DMSO (vehicle control) Certified reference standard (for inter-laboratory comparison)
Data interpretation	Expressed as bioanalytical equivalent concentration (BEQ, ng reference compound/L) BEQs derived using the linear portion of a sigmoidal log concentration-response curve or using a linear concentration-response curve

APPENDIX 3. Graphical examples of BEQ determinations.

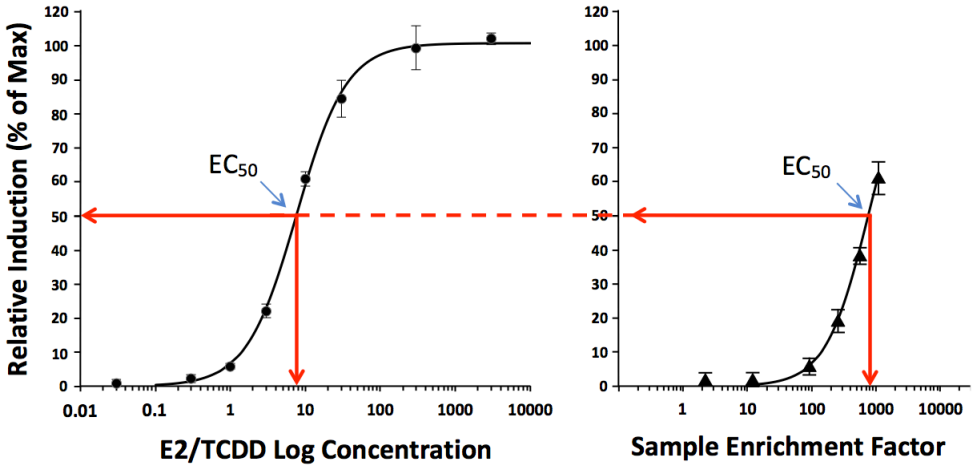
Concentration response analysis of the reference standards (E2 and TCDD) is utilized to develop a calibration curve whose characteristics are determined using one of a variety of mathematical modeling methods (such as a least squares best fit of the sigmoidal logistic standard curve using a two or four parameter Hill Equation formula (7,8), a low-effect level linear concentration-response analysis (linear regression) method (47), or other comparable mathematical model). The induction results obtained for a given concentration of test sample extract, after subtraction of reporter gene activity of the method blank sample, is inserted into the equation of the reference standard curve and used to calculate the E2 (for ERα) or TCDD (for AhR) BEQ of the sample aliquot essentially by direct comparison of the bioassay results to the corresponding activity on the reference standard curve. Final sample BEQs (ng E2-BEQs/L or ng TCDDBEQs/L) are determined after normalization of results to the original one-liter water sample volume.

For illustration, graphical examples of determinations of BEQ activity from sigmoidal log plots of reference standard concentrations or sample dilutions producing a range of reporter gene activity responses are presented below. The reporter gene activity is shown as Relative Induction, with values expressed as a percent of the maximal activity of the reference standard (set at 100%), which allows direct comparisons between assay plates and results on different days. The sample concentration is expressed as Sample Enrichment Factor, which expresses sample enrichment relative to the original water sample. For example, an enrichment factor of 100 means the sample in the bioassay is 100-fold more concentrated than the original water sample, while an enrichment factor of 1 means the sample in the bioassay is in the same concentration as the original sample.

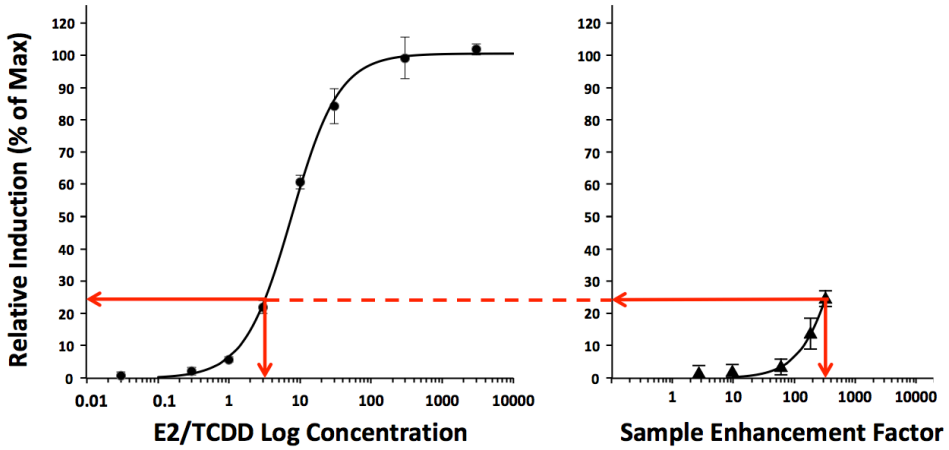
Case 1. Bioassay analysis of the test sample extract (right panel) resulted in a full concentration response curve with a maximal activity comparable to that of the reference standard. For such results, the EC_{50} value of the sample is the same as the EC_{50} value of the reference standard and is the optimal response level used to calculate the E2-/TCDD-BEQ for the sample aliquot.



Case 2. Bioassay analysis of the test sample extract (right panel) resulted in a partial concentration response curve with a maximal activity (~61% of that of the reference standard response) that is greater than the EC_{50} value of the reference standard (left panel). For such results, the EC_{50} value of the sample is the response level used to calculate the E2-/TCDD-BEQ for the sample aliquot.



Case 3. Bioassay analysis of the test sample extract (right panel) resulted in a partial concentration response curve with a maximal activity (~25% of that of the reference standard response) that is less than the EC_{50} value of the reference standard (left panel). For such results, the highest activity of the sample extract is the response level used to calculate the E2-/TCDD-BEQ for the sample aliquot.



Case 4. Bioassay analysis of the test sample extract (right panel) resulted in a partial concentration response curve with a maximal activity (~8% of that of the reference standard response) that is much less than the EC_{50} value of the reference standard (left panel). As with Case 3, the highest activity of the sample extract is the response level used to calculate the E2-/TCDD-BEQ for the sample aliquot. Since dilution analysis of a sample extract is only recommended for those samples whose induction response levels greater than 15% of the maximal activity of the reference standard (see Sections III-A and IV-C), dilution of a sample that gives only a 7% response would not need dilution response analysis.