METHOD FOR TOXIC EQUIVALENTS (TEQS) DETERMINATIONS FOR DIOXIN-LIKE CHEMICAL ACTIVITY WITH THE CALUX® BIOASSAY

SW-846 is not intended to be an analytical training manual. Therefore, method procedures are written based on the assumption that they will be performed by analysts who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

In addition, SW-846 methods, with the exception of required method use for the analysis of method-defined parameters, are intended to be guidance methods which contain general information on how to perform an analytical procedure or technique which a laboratory can use as a basic starting point for generating its own detailed Standard Operating Procedure (SOP), either for its own general use or for a specific project application. The performance data included in this method are for guidance purposes only, and are not intended to be and must not be used as absolute QC acceptance criteria for purposes of laboratory accreditation.

1.0 SCOPE AND APPLICATION

1.1 Method 4435 is a bio-analytical screening procedure for dioxin-like compounds in soils/sediments. This method is based on the ability of dioxin and related chemicals to activate the Ah receptor (AhR), a chemical-responsive DNA binding protein that is responsible for producing the toxic and biological effects of these chemicals. Measurement of the level of activation of AhR-dependent gene expression by a chemical or chemical extract provides a measure by which to estimate the relative potency and toxic potential of these chemicals and/or extracts with resulting values expressed as Toxic Equivalents (TEQs). Information on a commercially available genetically engineered cell line that contains the firefly luciferase gene under trans-activational control of the AhR (Ref. 41) can be found at the following website: http://www.dioxins.com/

This cell line can be used for the sensitive detection and relative quantification of AhR agonists and agonist activity of complex mixtures. The in vitro assay is designated as the Chemical-Activated Luciferase Expression or CALUX® assay. The most widely studied class of compounds that activate this system is the polychlorinated diaromatic hydrocarbons (PCDH), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD, dioxin). The relative toxic and biological potency of many PCDH compounds are quantified and expressed relative to that of 2,3,7,8-TCDD, since this is one of the most potent activators of AhR-mediated effects, including gene transcription. This relative quantification approach generates overall potency values known as Toxic Equivalents (TEQs) and the results obtained from using this method provide a measure of TEQs in a sample.

1.2 By using the sample processing procedures in this method and an affinity column (Ref. 42) polychlorinated biphenyls (PCBs) can be separated from chlorinated dioxins/dibenzofurans (PCDDs/PCDFs) making it possible to determine what portion of the total TEQs of a sample is due to each of these classes of compounds. This is the Dioxin/Furan- and PCB-specific (DIPS) analysis or the DIPS-CALUX bioassay for dioxin-like chemicals.

1.3 The AhR-dependent mechanism of the toxic and biological effects of dioxin-like chemicals and the basis of the CALUX® bioassay measurement and estimate of TEQ is shown in Figure 1 (Ref. 13). The AhR receptor complex is capable of binding dioxins, furans, PCBs

and other dioxin-like compounds. Once these chemicals bind to the AhR, the complex migrates into the nucleus where it specifically binds to the ARNT protein. The resulting chemical: AhR:ARNT complex then binds to a specific DNA sequence, the Dioxin Responsive Element (DRE), which is present upstream from many genes including that of CYP1A1, and this binding stimulates expression of the adjacent gene. In the case of the CALUX® assay, a plasmid containing four DREs immediately upstream of the firefly luciferase reporter gene was stably transfected into the mouse Hepa1c1c7 cell line to produce the recombinant cell line H1L6.1c3 (6.1 cells). This transformed cell line responds to toxic PCDDs, PCDFs and PCBs, and high molecular weight polynuclear aromatic hydrocarbons (PAHs) with the dose-dependent induction of firefly luciferase (Refs. 13-16 and 34). Comparison of these results to a 2,3,7,8-TCDD standard curve for induction allows for determination of the TEQs in a given sample.

1.4 By using sample processing procedures in this method, it is possible to separate polyhalogenated biphenyls from polyhalogenated dioxins/dibenzofurans present in the same sample. Using this DIPS-CALUX® bioassay it is possible to determine the portion of the total TEQ activity in a given sample that is due to each of these classes of compounds (Ref. 6).

1.5 Toxic Equivalents (TEQs):

The concept of Toxic Equivalents (TEQs) has been promulgated by the World Health Organization to provide a means of quantifying for risk assessment purposes the toxicity of a family of chemicals with a similar overall mechanism of toxicity (Ref. 38). The family of dioxin-like chemicals (PCDHs) within this group includes 7 chlorinated dibenzo-p-dioxin congeners with 4 to 8 chorines on the molecule, 10 chlorinated dibenzofuran congeners with 4 to 8 chlorines on the molecule, and 12 chlorinated biphenyls with 4 to 10 chlorines on the molecule. A list of the dioxin-like chemicals along with their assigned toxic equivalency factors (TEF) to scale their toxicity relative to the most toxic congener 2,3,7,8-tetrachlorodibenzo-p-dioxin is shown in Table 1. The REP values shown on this table and their significance will be discussed in Sec. 11.

1.6 Limits of Detection

Limits of detection can be adjusted to meet the needs of the project. As the sample size increases the detection limit will decrease. Sample sizes of 2 – 10 g will typically give a detection limit of less than1 pg/g. Limits of detection are determined based on the y-intercept from the Hill Equation and the standard deviation of the DMSO blanks from the bioassay. The limit of detection for the plate in relative light units is defined as the greater of either 2.5 times the standard deviation of the DMSO blanks, or the y-intercept plus 2.5 times the standard deviation of the DMSO blanks. The limit of detection for the plate in picograms of 2,3,7,8-TCDD is determined from the relative light unit limits of detection using the Hill Equation. Limit of detection for each sample is determined based on the amount of sample used, the portion of the sample extract used and the recovery for that type of sample.

1.7 A Tiered Approach Is Recommended for Quantification of TEQs in a Sample

1.7.1 Range Finding Analysis, The first step:

This step can be performed on all of the three sample processing procedures outlined in Sec. 2.2. The first step is to screen the samples by conducting a range finding analysis on the sample to determine the proper dilution. (See Sec. 4.2) This provides an estimate of the concentration of dioxin-like chemicals in the sample that is in the linear portion of the 2,3,7,8-TCDD calibration curve, for both the dioxin/furan and PCB fractions. The optimal results are obtained with a dilution of the sample that produces an induction response from the cells that falls close to EC_{50} (EC_{50} is the

equivalent concentration at 50% of the maximum response of the 2,3,7,8-TCDD standard) value for the 2,3,7,8-TCDD standard curve run on each plate. This completes the sample screen procedure. For more comprehensive sample analysis refer to Sec. 1.7.2.

1.7.2 Quantification Analysis, The second step:

The second step for comprehensive analysis is to analyze the three individual extracts of the sample with a positive response at the optimum dilution (determined as described in Sec. 2.2), this allows accurate estimation of the TEQ value of dioxin-like chemicals present in a given sample and allows mean and standard deviation calculations.

1.8 This method is intended to be performed by trained analysts who are familiar with organic/analytical sample processing techniques and cell culture techniques. Training on the CALUX® bioassay system can be obtained from the manufacturer. Instructions are provided in the form of standard operating procedures (SOPs), which are detailed and thorough. The manufacturer (http://www.dioxins.com/) also provides comprehensive training which is included with licensing the technology. This technology has several steps where attention to detail is critical to generating acceptable sample results. This includes careful processing of samples through the extraction and cleanup procedures, pipetting small volumes, and accurately weighing out samples.

2.0 SUMMARY OF METHOD

2.1 This method is a relatively rapid screening method capable of estimating the Toxic Equivalents (TEQs) concentration for dioxin-like chemicals in a sample. The sample is extracted in an organic solvent and fractionated through the sample processing procedure (See Sec. 11). An extract that contains the halogenated dioxins/furans is separated from an extract containing the halogenated biphenyls. These extracts are applied to monolayers of H1L6.1c3 cells and the amount of luciferase induction is measured after 20 to 24 hours. A standard dilution series of 2,3,7,8-TCDD is included on each plate of cells. Estimation of dioxin/2,3,7,8-TCDD-like TEQ activity present in the sample extract is performed by extrapolation to the 2,3,7,8-TCDD standard curve by least squares estimates with the 4 parameter Hill Equation.

2.2 There are three modes by which the DIPS-CALUX bioassay is performed. These are the screening mode with historical recovery, screening mode surrogate recovery, and the semi-quantitative mode. The screening mode involves the analysis of a single aliquot of the sample and recovery is estimated from the mean of historical recoveries that have been obtained for soils/sediment samples. This is considered to be acceptable as the variability of recoveries for soils/sediment samples has been relatively small (76.2 +/- 8.5%). Using this mode would indicate whether a sample needed to be further analyzed by either the semi-quantitative mode or by chemical analysis. The screening mode surrogate recovery, involves processing two aliquots of the sample, the first for analysis in the DIPS-CALUX bioassay and the second used for the surrogate spike with radiolabeled 2,3,7,8- TCDD to estimate recovery. The semi-quantitative mode involves analyzing three aliquots of the sample in the DIPS-CALUX bioassay and a fourth aliquot of the sample used for determination of recovery with radiolabeled 2,3,7,8-TCDD. The cost of sample analysis is dependent upon which mode of the DIPS-CALUX bioassay is used for estimation of the levels of sample contamination.

3.0 DEFINITIONS

Refer to Chapter One and the manufacturer's instructions for definitions that may be relevant to this procedure. In addition, see Method 4000 for a glossary of basic bioassay terms.

4.0 INTERFERENCES

4.1 Chemical interference

The chemicals listed in Table 2 are relatively weak agonists for the Ah receptor and induce poorly. The results in the table demonstrate the percent recovery of each compound in the clean-up method and the amount of carry-over into the dioxin and PCB fractions. (Refs. 7, 33, 35 and 37)

4.1.1 PAH interference

PAHs pose little interference in the method since the extraction and cleanup methodology employed typically removes virtually all PAHs using an acid silica gel column. In addition, chrysene, benzo(b)fluoranthene, indeno(1,2,3c,d)pyrene and acenaphthylene are the only PAHs we have found to bleed through the clean-up procedure with approximately 5 % - 26 % (see Table 2) passing through the processing steps. For the analysis of samples that are expected to have extremely high levels of PAHs, users should subject the sample to an additional acidic-silica cleanup step. Testing the relative activity of the sample after each round of cleanup would reveal whether additional AhR active chemicals (i.e. PAHs) are removed with additional cleanup. Other materials such as silver nitrate, florsil and alumina can be used in additional cleanup steps, due to the rarity of these compounds in the environment these additional materials are rarely used.

4.1.2 AhR Receptor interference: Compounds that can activate the AhR receptor could result in potential interference with the determination of TEQ due to dioxins/furans and PCBs. The PAH class of potential interferences do not typically pass through the cleanup system and should not be potential interfering agents. The cleanup methodology is outlined in Secs. 11.3 and 11.4. The remaining classes of potential interfering compounds are other halogenated (i.e. brominated and fluorinated) dibenzo-p-dioxins/furans and biphenyls, and halogenated naphthalene’s and these appear to have similar toxicological properties as their chlorinated derivatives. Isotope specific dilution methods using high-resolution mass spectroscopy high-resolution gas chromatography (HRGC/MS) for quantifying 2,3,7,8-TCDD do not measure these structurally similar halogenated dibenzodioxins/furans and biphenyls and this may be one of the primary reasons that estimates of TEQ of dioxin-like chemicals by this method are slightly higher than HRGC/MS generated estimates of TEQ.

4.2 Cell Toxicity:

Extremely high concentrations of PCDD/PCDF or PCB are not cytotoxic to cells. However, other contaminants that may be present in sample extracts could potentially be cytotoxic. The manufacturer has generally found that the sample processing procedures used in this method result in removal of most potentially cytotoxic compounds. The manufacturer as a standard operating procedure monitors the cells after chemical treatment to determine whether cytotoxicity has occurred and which could lead to potential false negative results for dioxin-like TEQ. Cell toxicity is identified if cells are found to be detached from the culture plate or whether they exhibit a major change in morphology. Generally, both cell toxicity and

chemical insolubility can be overcome by sample dilution. In this method typically 6 dilutions are run to reduce potential cell toxicity and overcome any insolubility issues the sample may have (1:4, 1:10, 1:100, 1:500, 1:1000 and 1:10,000). For samples that are suspect to solubility the dilutions are increased (1:50,000, 1:100,000, 1:200,000, 1:500,000, 1:1,000,000 and 1:2,000,000).

5.0 SAFETY

5.1 This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA regulations regarding the safe handling of the chemicals listed in this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in these analyses.

5.2 Safety procedures in compliance with good laboratory practices (GLP) 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.

5.3 This method employs the use of dilute concentrations of 2,3,7,8-TCDD for the standard curve and quality control solutions contain PCB 126, and a solution comprised of all 17 chlorinated dioxin/furan congeners for which the WHO has established dioxin-like TEF values. The analyst should take the appropriate measures when preparing, handling, and disposal of these standards.

6.0 EQUIPMENT AND SUPPLIES

Refer to Table 3 and to the manufacturer's website (http://www.dioxins.com/) for a comprehensive list of recommended equipment and supplies.

7.0 REAGENTS AND STANDARDS

7.1 Reagent grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 Refer to Table 4 and to the manufacturer's website (http://www.dioxins.com/) for a comprehensive list of recommended reagents and standards.

8.0 SAMPLE COLLECTION AND STORAGE

Sample collection, preservation, and storage requirements may vary by EPA program and may be specified in the regulation or project planning document that requires compliance monitoring for a given contaminant. Where such requirements are specified in the regulation, follow those requirements. In the absence of specific regulatory requirements, use the following

information as guidance in determining the sample collection, preservation, and storage requirements.

8.1 Sample Collection

This bioassay testing process employs very small sample volumes. Therefore, sample collection procedures must focus on the amounts and procedures necessary to ensure that the sample is representative of the source.

8.2 Sample Receiving and Tracking

Samples can be shipped directly to the manufacturer, received and logged in via the manufacturer's electronic tracking system. Temperature of the samples and the condition of samples are noted immediately. Any instructions from the client are also noted. The samples are then placed in the appropriate storage conditions prior to processing.

8.3 Sample and Extract Storage

Biological samples are generally stored in a –70°C freezer. Food products are stored at 0°C – 4°C depending on the samples individual needs. Feed, soils and other non-perishable samples are usually held at room temperature unless the sample requires or the client requests other storage conditions.

Samples may be extracted and analyzed at the same laboratory or samples may be extracted and then shipped to the manufacturer for analysis. If extracts are shipped to the manufacturer, the manufacturer performs the sample cleanup to separate the PCDD/PCDF and the PCBs from the extract. If a cleanup was already performed on the extracts, no further work need be done on the samples prior to shipping. The extracts must be shipped in sealed vials.

Storage of extracts can be at room temperature. Extracts must also be covered with clean Teflon lined caps to reduce evaporation or clean aluminum foil, if extracts are dry.

8.4 Holding Times

No maximum holding times for extracts have been established. However, PCDDs, PCDFs, and PCB have an extremely long half-life and are very stable in environmental matrices and are essentially nonvolatile. Clients may request specific holding times for project-specific applications.

9.0 QUALITY CONTROL

9.1 Refer to Chapter One for guidance on quality assurance (QA) and quality control (QC) protocols. Each laboratory should maintain a formal quality assurance program. The laboratory should also maintain records to document the quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection. When inconsistencies exist between QC guidelines, method-specific QC criteria take precedence over both technique-specific criteria and those criteria given in Chapter One, and technique-specific QC criteria take precedence over the criteria in Chapter One.

9.2 Parts of the quality control criteria outlined in this method are based on methods for GC/HRMS such as Methods 8290 and 1613. For a more detailed description of the QC process please refer to Ref. 3, Appendix B available from the manufacturer's website (http://www.dioxins.com/).

9.3 Monitoring of solvents used in sample preparation is conducted on a regular basis. Each lot of solvent is tested by evaporating a 5 mL aliquot of the solvent into 4 μL of DMSO. The DMSO solution is suspended in cell culture medium and exposed to the cells. All solvents are tested for reactivity in the assay prior to use and a relative light units (RLU) induction of less than 2 times background must be obtained for the solvents to be used. A solvent blank is also included in each batch of samples. The solvent blank is treated in the same way as the samples and serves as a control to monitor for contribution of activity from any of the solvents or column matrices used in sample preparation.

9.4 Reference samples were prepared from lake sediment materials that were finely ground (solids) and analyzed twice by GC/HRMS for dioxin TEQ (Average GC/MS TEQ = 13.6 ± 6.8 pg/g). The lake sediment material was then spiked with 30 pg/g 2,3,7,8-TCDD to provide a final dioxin TEQ concentration equivalent to the action level. The reference material was shaken or stirred for three days, aliquoted and an aliquot was analyzed by GC/HRMS. The un-spiked reference sample is included in each sample batch and is prepared and analyzed using the same method as the unknown samples. Figures 2 and 3 show the QC charts for the un-spiked lake sediment reference sample for 2003 and 2004 respectively. Figure 4 depicts the QC chart for the spiked lake sediment reference sample.

9.5 Quality Control (QC) charts are maintained for all reference samples as well as for a standard solution of PCB 126 and a mixture of the 17 dioxin/ furan congeners (those congeners assigned TEF values for dioxin-like activity by the WHO) that are analyzed on each plate (each of these standard solutions produces a response near the middle of the dose response curve). These figures are generally reported as a three-month average, however the data for these samples can be monitored over longer time periods to insure against longer-term variation in the assay (Figures 5-8). In QC charts, the results for the standard mixtures are reported as a ratio relative to the 6.25 pg/g point of the 2,3,7,8-TCDD standard curve (near middle of linear range) and the reference materials are reported as the TEQ estimate determined from the standard curve. If the reference material or either of the standard mixtures differs by more than two standard deviations from the moving average or a reference material is below the limits of detection the plate is declared invalid and all samples on the plate are reanalyzed. Figures 5 and 6 depict the QC charts for the PCDD/PCDF standard mixture for 2003 and 2004 respectively. Figures 7 and 8 depict the QC charts for the PCB 126 for 2003 and 2004 respectively. Figures 9 and 10 depict the 2,3,7,8-TCDD standard curves for 30 samples in 2003 and 2004 respectively.

9.6 Quality Control Criteria for Use of This Method as a Semi-quantitative Estimate of Dioxin TEQ:

In addition to the quality controls used in the screening assay, the 2,3,7,8-TCDD standard curve is modeled to a sigmoid curve described by the four parameter Hill Equation using a least squares best fit. Estimation of TEQ values for sample extracts are conducted based on the derived Hill Equation with the following limitations listed (i.e. PCB fraction response must be less than 50% of 2,3,7,8-TCDD maximal response and dioxin/furan fraction response must be less than 75%). Any samples that exceed these limits, or are below the limits of detection are reanalyzed using appropriate sample dilutions.

9.7 Recovery Determination

Recovery determination (as described in Sec. 10.2 for 1234-TCDD and ^{14}C labeled 2,3,7,8-TCDD recoveries) is conducted using a duplicate sample that has been spiked with either congeners that are radioactively labeled, or with a known amount of an equimolar mixture of the unlabeled 17 congeners (see Figure 1). Using the radioactively labeled spike the

recovery is the percent of the recovered spike versus the amount added to the sample as determined by liquid scintillation. For the unlabeled spike, subtracting the TEQ for the sample from the TEQ for the spiked sample and dividing the result by the TEQ for the spike determines the recovery. For a more detailed description of the QC process please refer to Ref. 6, Appendix B available from the manufacturer's website (http://www.dioxins.com/).

9.8 Limits of Detection:

Limits of detection are determined based on the y-intercept from the Hill Equation and the standard deviation of the DMSO blanks from the bioassay. The limits of detection for the plate in relative light units is defined as the greater of; 2.5 times the standard deviation of the DMSO blanks, or the y-intercept plus 2.5 times the standard deviation of the DMSO blanks. The limit of detection for the plate in pg of 2,3,7,8-TCDD is determined from the relative light unit limits of detection using the Hill Equation. Limit of detection for each sample is determined based on the amount of sample used, the portion of the sample extract used and the recovery for that type of sample.

9.9 Standards, reagents, solvents and any other materials used in the extraction, clean up, and analysis, must be properly stored and must never be used beyond the manufacturer's and/or supplier's expiration dates. A standard expiration time of one year is utilized for all solvents and reagents.

10.0 CALIBRATION AND STANDARDIZATION

Calibration and standardization of each assay is very important to ensure accuracy of any measurement of any system. The following is a description of the necessary calibration and standardization procedures for this method.

10.1 Calibration and Standardization of each Assay:

Calibration and standardization is performed with each assay. An eleven point standard curve of 2,3,7,8-TCDD (100 pg – 0.0977 pg) is run on each 96 well plate analyzed. Each plate also contains 7 additional QC calibration points consisting of 4 DMSO solvent controls, 1 media blank QC, 1 dioxin QC standard (contains all 17 dioxin congeners recognized by the World Health Organization), 1 PCB QC standard (80.5 ng/ml PCB 126). For internal recoveries 2 additional QC samples are added to estimate recovery. The dioxin and PCB QC points are compared to the 6.25 pg point on the standard curve and entered into their respective QC charts. See Sec. 10.2 for a full explanation of the percent recovery points. This gives this method 20 QC calibration points.

10.2 Recovery Determinations:

Analyses of recovery determinations are very important for quantitative bio-analytical methods for dioxins/furans and PCBs. Bioassays do not differentiate between isotopically labeled and unlabeled analytes. Therefore, recovery determinations in bioassays can be accomplished with a surrogate sample spiked with a radiolabeled congener of dioxin. It has been demonstrated that 1,2,3,4-TCDD, a biologically inactive congener of the dioxin family of chemicals, can be used as an internal spike to determine recoveries of dioxin-like chemicals. Samples were spiked with ^{14}C labeled 2,3,7,8-TCDD or 1,2,3,4-TCDD and submitted to extraction and clean up using the cleanup method in Sec. 11.3.2. The cleanup column is differentially eluted to yield a PCB and PCDD/PCDF fraction. The 1,2,3,4-TCDD spiked samples were resuspended in toluene containing four PCB injection standards, and recoveries determined by gas chromatography with electron capture detection or scintillation counting.

Average recoveries determined by 1,2,3,4-TCDD with paired samples spiked with ^{14}C- 2,3,7,8-TCDD indicated that the recoveries determined by the two methods were very similar, 88.5% (± 1.2%) and 87.2% (± 2.4%), respectively (see Figure 1). Recovery determinations were also verified by HRGC/HRMS. This procedure allows for quantitative determination of dioxin-like chemicals in various sample matrices. For a more detailed description of the QC process please refer to Ref. 6, Appendix B available from the manufacturer's website (http://www.dioxins.com/).

11.0 PROCEDURE

This section describes the procedures used in this method. This includes: growth and storage of the mouse H1L6.1c3 recombinant cell line; preparation of 96 well plates, extraction and cleanup of samples, dosing plates and analysis of the data.

NOTE: Always use sterile techniques when working with cells.

11.1 Cell Culture:

11.1.1 Culturing the H1L6.1c3 Cell Line:

The recombinant H1L6.1c3 mouse cell line (Ref. 41) is grown at 37°C in 5% CO_2 and 100% humidity. Cells are grown in plastic cell culture flasks containing RPMI 1640 media supplemented with 8% fetal calf serum (FCS) and 1% Penicillin / Streptomycin. No external selective pressure (i.e. Geneticin - G418) is needed to maintain the stable integration of the DRE-reporter plasmid in the cell line.

11.1.2 Cell Storage:

Cells are stored in liquid nitrogen. After growth the cells are placed in a freezing media, consisting of RPMI supplemented with 10% FCS, 1% Penicillin/Streptomycin, and 8% DMSO. Cells are stored at 1,000,000 – 2,000,000 cells per mL in 2 mL cryogenic vials. After the cells and freezing media are placed in the vials, they are stored at –70°C for 24 hours and then transferred to the liquid nitrogen Dewar for long-term storage.

11.1.3 Thawing Cells:

Cells are stored in a liquid nitrogen Dewar in 2-ml cryogenic vials. These vials are removed from the Dewar and the screw cap loosened slightly to release any excess gas. The cap is then tightened and the vial is thawed quickly by placing it in either a water bath or incubator at 37°C until thawed. The cell and freezing media mixture is then transferred to a 50 mL centrifuge tube and 10 to 20 mL of RPMI 1640 is added and centrifuged at 2000 rpm for 10 min. The media is removed from the cells and 5 mL of fresh RPMI 1640 is added. The cell pellet is disrupted by repeated pipetting in the media and placed in a 25cm^2 culture flask for growth.

11.1.4 Growth and Splitting Cells:

The cells are first grown in 25cm^2 flasks to 80% confluence before transfer to a 75cm^2 flask, which is also grown to 80% confluence. The media is removed from the flasks and cells are washed with PBS. Two mL of the trypsin is then added to the flask and allowed to incubate at 37°C for 5 to 10 min. The flasks are then washed with PBS to remove all of the cells from the growth surface and pipetted into a 50-mL centrifuge tube,

containing 5 mL of RPMI 1640 medium, and centrifuged at 2000 rpm for 10 min. The cells are then transferred to two 75cm^2 flasks containing 10 mL RPMI 1640 each.

11.1.5 Counting Cells:

After trypsinizing and centrifugation of the cells, cells are counted using a hemocytometer. After centrifuging the trypsinized cells, the cells are re-suspended in 30 ml of RPMI 1640. Fifteen microliters of this solution is then placed on the hemocytometer for counting. Four of the quadrants on the hemocytometer are counted: the upper left, upper right, lower right, and lower left. Determine the average of the 4 counts. The volume of each square is 10^{-4} mL, therefore: cells/ml = (average number per large square) x 10^4/ml. x 1/(dilution). This count is used in the plating of cells (see Sec. 11.2.1)

Example: Starting dilution: 30 mL
Total count of cells for all four grids: 468
Average of four grids: 117
Average / 75 (equivalent of 75 x $10^{4)}$ = 1.56
1.56 x starting dilution (30 mL)= 46.8
Add 16.8mls (to the original 30 mL dilution) for a total of 46.8mls.
On average 20 mL are needed for one 96 well plate.

11.2 Preparation for Testing:

First determine the number of samples, standards, blanks and QC to be tested per batch and prepare plates with sufficient number of wells to accomplish the test.

11.2.1 Plating Cells:

Remove a 96-well plate from sterile package. Using the eppendorf multi-pipettor, pipette 200 µl of cell/media solution to each well (See Sec. 11.1.5). Label plate with date and time of plating and cell concentration. Incubate plate(s) at 37^{o}C in an atmosphere supplemented with approximately 5% carbon dioxide. Cells incubate for minimum of 24 hours before dosing.

11.2.2 Dosing Cells: Preparation of 2,3,7,8-TCDD standard curve:

1) A stock solution of 50 ng/mL 2,3,7,8-TCDD is used to generate an eleven point standard curve.
2) Place 4 *u*L of dimethyl sulfoxide (DMSO) to each of the eleven test tubes.
3) Add 4 *u*L of the 50 ng/mL 2,3,7,8-TCDD solution to the first tube and thoroughly mix (25 ng/mL solution).
4) Transfer 4 *u*L of the solution in the first tube to the second test tube and mix using a vortex.
5) Continue the process of producing two fold dilutions until you have added 4 *u*L of the mixture in tube 10 to tube 11. Mix tube eleven, and discard 4 *u*L of the mixture from tube 11.
6) This produces a 2,3,7,8-TCDD standard curve composed of the final concentrations listed in Table 7.

NOTE: The standard curve should be treated the same way as the samples. The same lot of hexane used to prepare the sample

extracts should also be used to add 1 mL of hexane to each tube in the standard curve.

11.2.3 Dosing Cells: Prepare Samples for Dosing:

1) Add 4 μL of DMSO to 13 mm tube.
2) Add appropriate sample amount.
3) If sample is less than 1 mL add hexane to sample until 1ml is reached.
4) Concentrate samples for 6 minutes, then an additional two minutes to remove any remaining solvent.
5) Add 400 μL of RPMI 1640 cell culture medium (supplemented with 8% fetal calf serum and 1% penicillin/streptomycin solution to each sample tube and vortex for fifteen seconds.

11.2.4 Dosing Plate:

1) Remove the 96-well plate of cells that have been incubated for 20-24 hours, at 37 ^{o}C in an atmosphere of approximately 5% carbon dioxide, from the incubator.
2) Place a piece of absorbent paper in the bio-hood. Remove plate lid and invert the plate and tap it on paper to remove medium.
3) After visually inspecting the plate, add 200 μL of sample to be tested to each well. When adding the sample, place the tip of the pipettor on the wall of the well and pipette slowly. This is done so that the cells layer is not disturbed.
4) Once all samples have been added, record date/time on plate and place the plate in the incubator for required time. Cell line H1L6.1c3 is incubated with a chemical or extract for 20-24 hours.

11.2.5 Cell Lysis and Luciferase Activity Measurement:

After the appropriate incubation time (see Sec. 11.2.1) the plates are removed from the incubator and the media removed. Each well is rinsed with 50 μL PBS. After which each well is inspected for cell viability, noting any damaged, morphologically changed or missing cells. White backing tape is then applied to the bottom and 30 μL of Promega Lysis buffer is added to each well. The 96 well plate is then shaken for 1 minute before inserting the plate into the microplate luminometer. For analysis of luciferase activity, 50 μL of luciferase substrate (luciferin) is automatically injected into each well, the mixture is allowed to incubate for 5 seconds following by quantitation of luciferase activity (light production) over a 15 second time period. The light produced from the cleavage of luciferin by luciferase is expressed as relative light units (RLUs). These RLUs are then analyzed using an Excel spread sheet that calculates the TEQ for the dioxin/furan and PCB fractions.

11.3 Experimental Set-Up, Sample Extraction and Clean-Up:

11.3.1 Experimental Set Up:

Samples are first logged in to the manufacturer tracking system and assigned project number and individual sample numbers. Next the number of samples, solvent blanks, QC's and recoveries are calculated to determine the number of extractions necessary.

11.3.2 Sample Extraction and Clean-Up:

Samples are extracted using a modification of the Method 8290 extraction method (for soil, sediment, and ash). Three dried aliquots of each sample are ground (when possible) and two to ten gram aliquots are placed in solvent cleaned glass vials fitted with PTFE lined caps. For the screening process, one aliquot of the sample is used, and another aliquot is processed for recovery purpose if historical recovery data is not used. The samples are extracted with a 20% solution of methanol in toluene, then twice with toluene. Each extraction of sample with solvent is incubated in an ultrasonic water bath for approximately 10 minutes. The three extracts from each sample are filtered, pooled and concentrated by vacuum centrifugation. The sample extract is then suspended in hexane and prepared for the clean-up method. The clean-up system consists of two piggy-backed columns. The first column is an acid silica gel chromatography column used to remove PAHs. The second column is the XCARB clean-up column. The XCARB column is an affinity column that binds halogenated dioxins/furans and biphenyls and these chemical classes can be differentially eluted first with a mixture of hexane, toluene, ethyl acetate solution to elute the PCB fraction. Next the column is inverted and rinsed with toluene to elute the dioxin/furan fraction. The eluate from the clean-up method is concentrated under vacuum. This is then brought up in hexane for dilution analysis in the bioassay.

11.4 Preparation of Cleaned-Up Samples for Analysis:

After the samples are passed through the clean-up system and re-suspended in 4 mL of hexane (see Sec 11.2.2), a range finding is performed on the samples by diluting them (1:4; 1:10; 1:100; 1:500; 1:1000; and 1:10,000). For suspected high level samples, where higher chlorinated dioxin/furan insolubility may be present, range finding dilutions are increased (1:50,000, 1:100,000, 1:200,000, 1:500,000, 1:1,000,000 and 1:2,000,000) (see Sec 11.2.5). This allows for a range finding estimation of the dioxin/furan and PCB content and to look for any problems with insolubility of the dioxins/furans and PCBs in the sample. The sample aliquots are then concentrated under vacuum into DMSO and re-suspended in 400 μL of RPMI 1640 and dosed on the plated cells (see Sec. 11.2.2). The cells are incubated in a humidified CO_2 atmosphere for 16-24 hours and then assayed for luciferase activity.

11.5 Alternative Sample Extraction for Detection of PAH content:

The experimental set up is identical to the dioxin and PCB analysis (see Sec. 11.3.2). The only differences from the previous method in Sec. 11.3 are that there is no clean-up step performed and that the bioassay is only incubated for 4 hours instead of the standard 16-24. The sample is extracted, pooled and concentrated and then dosed without clean-up as in Sec. 11.2.2. This will give a total TEQ score for the PAHs in addition to the dioxins/furans, PCBs and any other AhR active chemicals in the sample extract. To calculate the estimate of PAH the sample is run through the full process and the two results are compared. The PCDD/PCDF and PCB results are subtracted from the previous results. The result would be the estimate of PAHs within the sample.

12.0 DATA ANALYSIS AND CALCULATIONS

Data analysis and calculations are performed in an Excel spreadsheet using the RLU data produced by the luminometer. The following is a description of the procedure for data analysis and calculations (Also see Figures 11-14).

12.1 Exporting RLU Data:

A Microsoft Excel spread sheet is used to analyze the data.

12.1.1 Luminometer Data:

Data from the Luminometer is exported to a Microsoft Excel spreadsheet ("raw results") at the completion of the Standard Luciferase Assay. This Excel worksheet, then graphs the data as CALUX® activity in RLUs versus concentration of the test compound (Figures 11 – 14).

12.1.2 Background Subtraction:

The data collected by the Luminometer, ("raw results"), includes contributions from induced expression of luciferase caused by the presence of the test compound, as well as contributions by background production of luciferase and the "dark current" from the photomultiplier tube. In order to identify the component contributed by the induced expression of luciferase, the results from a blank well is subtracted from the "raw results". This blank well contains cells that were exposed to cell culture medium dosed with 1% (v/v) DMSO (see Figure 11).

12.1.3 Standard 2,3,7,8-TCDD curve:

A standard curve for 2,3,7,8- TCDD is included in each experiment. Refer to Sec. 11.2.2 for solution makeup (See also Figures 11 - 14).

12.1.4 Four Parameter Hill Equation Formula:

Results are calculated using TEQ values for the sample based on a least squares best fit of the standard curve of 2,3,7,8-tetrachlorodibenzo-p-dioxin (See Sec.12.3, and Figures 13 and 14).

12.2 EC_{50} Determination:

The first step in determining the EC_{50} of a standard curve is to look at the curve for the compound and determine if it has a sigmoid shape. If it does not, the standard curve cannot be used for determining the results of the assay.

12.2.1 Standard Curve:

At low concentrations a sigmoid curve should remain near the background level or slowly increase with increased concentration until a threshold is reached. At this point the dose response curve should become steeper and remain linear with increasing concentration. Eventually, the response should reach a maximum. And increasing concentrations will not result in increased response, as the dose response curve flattens out.

The response at the higher concentrations of 2,3,7,8-TCDD results in saturation of the RLU response. Generally, the linear portion of the curve is used to perform a regression analysis with the concentration of 2,3,7,8-TCDD plotted versus Relative Light Units (RLUs). The linear portion of the curve generally includes RLU responses between 300 and 8000 RLU units.

12.2.2 Linear Portion of Standard Curve:

Generally the linear portion of the sigmoid curve will cover a 50 to 100-fold range of concentrations. In order to make a good estimate of the EC_{50} it would be preferable to have at least four data points within this linear region (2 or 3-fold dilution series) (See Figures 13 and 14).

12.2.3 Calculating EC_{50}:

The EC_{50} for a sample is calculated using Microsoft Forecast Function. First the midpoint of the linear portion of the standard curve is determined. Then the forecast function is applied to the 3 data points surrounding the midpoint.

Excel forecast formula: FORECAST (x,known_y's,known_x's)

12.3 Calculation of TEQ: Least Squares – Best Fit:

Least Squares, best fit is used to predict the best fit for the data using a four variable Hill Equation with the natural log of the 2,3,7,8-TCDD concentration in femptograms/tube plotted versus Relative Light Units (RLU).

The response of a sample is compared to the response of 2,3,7,8-TCDD, corrected for dilution of the unknown and assigned a relative concentration to 2,3,7,8-TCDD (TEQ) (see Figures 11 - 14).

13.0 METHOD PERFORMANCE

13.1 Performance data and related information are provided in SW-846 methods only as examples and guidance. The data do not represent required performance criteria for users of the methods. Instead, performance criteria should be developed on a project-specific basis, and the laboratory should establish in-house QC performance criteria for the application of this method. <u>These performance data are not intended to be and must not be used as absolute QC acceptance criteria for purposes of laboratory accreditation.</u> In the case of this method, any test kits used must be able to meet the performance specifications for the intended application. Also, follow the manufacturer's instructions for quality control procedures specific to the test kit used.

13.2 Field Studies:

Field studies have been conducted at an EPA sponsored SITE study in Saginaw, MI (April 26 – May 1, 2004) using this method: and a mobile lab was set up for analysis of soil/sediment and extracts in the field. In addition, pre-site soil/sediment and extracts were tested at the manufacturer's facility and the data for the pre-site samples is included in this report (see Figures 15 - 17). Ninety-six well assay plates containing mouse 6.1 cell line were sealed at the manufacturer and transported to the site in an airtight box. Extraction and clean-up columns were also pre-made at the manufacturer and transported to the SITE study location. Samples were extracted and cleaned up on site in the mobile lab and analyzed using the sealed

plates produced at the manufacturer. The plates had been sealed for 7 days prior to use. An airtight box was used in the field to see if it could be used in place of a standard humidified CO_2 incubator. The analyst can breathe into the specially made valves giving the cells the gas concentrations needed, or use a 5% CO_2 tank if desired. The airtight box can then be placed in a 37°C water bath for 16-20 hours to control temperature during incubation. The comparison results between the airtight box and the incubator are included in this report (see Sec. 12 and Figure 17).

13.3 This section demonstrates the correlation between this method and GCMS determinations. Figures 15 – 17 represent data from the EPA pre-SITE study samples. Figures 18 – 22 represent samples from the EPA, Waste Sites, and Asian Companies. Figure 23 represents the standard solutions from an international cross lab validation study. Figure 24 represents the Correlation Determination for all Sample Matrices.

13.4 Figure 15 represents the dioxin / furan TEQ comparison of this method to GC/MS for soil samples for pre-field testing during the EPA SITE field study. These samples were processed in March of 2004 using the screening technique with a surrogate recovery. These TEQs were calculated using the WHO 1997 REP values.

13.5 Figure 16 represents the PCB TEQ comparison from this method to GC/MS for soil samples for pre-field testing during the EPA SITE field study. These samples were processed in March of 2004 using the screening technique with a surrogate recovery. These TEQs were calculated using the WHO 1997 REP values.

13.6 Figure 17 represents the dioxin/furan TEQ comparison of this method using the 37°C water jacketed incubator to the results obtained using an air tight chamber kept at 37°C for soil samples for pre-field testing during the EPA SITE field study. These samples were processed in March of 2004 using the screening technique with a surrogate recovery. These TEQs were calculated using the WHO 1997 REP values.

13.7 Table 8 outlines the TEQ-pg/g for the soil samples for pre-field testing during the EPA SITE field study. This table represents the dioxin / furan TEQ comparison of this method using the 37°C water jacketed incubator to the results obtained using an air tight chamber kept at 37°C. These samples were processed in March of 2004 using the screening technique with a surrogate recovery. These TEQs were calculated using the WHO 1997 REP values.

13.8 Figure 18 depicts the dioxin/furan TEQ correlation between this method and GC/MS for soil samples. The GC/MS data was generated by 3 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Table 9 for individual sample data correlations.

13.9 Figure 19 depicts the dioxin/furan TEQ correlation between this method and GC/MS for Ash samples. The GC/MS data was generated by3 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Table 11 for individual sample data correlations.

13.10 Figure 20 depicts the dioxin/furan TEQ correlation between this method and GC/MS for Exhaust extract samples. The GC/MS data was generated by 3 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Table 13 for individual sample data correlations.

13.11 Figure 21 depicts the dioxin/furan TEQ correlation between this method and GC/MS for Soil Extract samples. The GC/MS data was generated by 3 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Table 10 for individual sample data correlations.

13.12 Figure 22 depicts the dioxin/furan TEQ correlation between this method and GC/MS for Ash Extract samples. The GC/MS data was generated by3 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Table 12 for individual sample data correlations.

13.13 Figure 23 depicts the dioxin/furan TEQ correlation between this method and GC/MS for Standard Solution samples. The GC/MS data was generated by 3 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Table 14 for individual sample data correlations.

13.14 Figure 24 depicts the dioxin/furan TEQ correlation between this method and GC/MS for all samples. This shows a high over all correlation between this method and GC/MS for all samples with an R^2 value of 0.9631. The GC/MS data was generated by 5 different companies. Please see Appendix A (available from the manufacturer's website http://www.dioxins.com/) for the data table comparing data from this method to GC/MS for each individual sample and company. See Sec. 13.0, for individual sample data correlations.

13.15 Table 9 depicts the Method 4435 dioxin/furan data results with the GC/MS results for all soil samples. See Figure 18 for the associated graph.

13.16 Table 10 depicts the Method 4435 dioxin/furan data results with the GC/MS results for all Soil Extract samples. Extracts were part of a double-blinded study, in which the original sample weight was unknown. Original data generated using this method is shown as well as the adjusted data per 10 gram sample size. See Figure 21 for the associated graph.

13.17 Table 11 depicts the Method 4435 dioxin/furan data results with the GC/MS results for all Ash samples. See Figure 19 for the associated graph.

13.18 Table 12 depicts the Method 4435 dioxin/furan data results with the GC/MS results for all Ash Extract samples. Extracts were part of a double blinded, in which the original sample weight was unknown. Original data generated using this method is shown as well as the adjusted data per 10 gram sample size. See Figure 22 for the associated graph.

13.19 Table 13 depicts the Method 4435 dioxin/furan data results with the GC/MS results for all Exhaust Extract samples. Extracts were part of a double blinded, in which the original sample weight and volume of air was unknown. Original data generated using this method is shown as well as the adjusted data per cubic meter. See Figure 20 for the associated graph.

13.20 Table 14 depicts the Method 4435 dioxin/furan data results with the GC/MS results for all solution samples. See Figure 23 for the associated graph.

14.0 POLLUTION PREVENTION

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity and/or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operations. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions consult *Less is Better: Laboratory Chemical Management for Waste Reduction* available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th St., N.W. Washington, D.C. 20036, http://www.acs.org.

14.3 This method is an analytical assay which does not produce any air pollution and minimal waste pollution. This method can be used and is currently used in remediation projects around the world to reduce both air and ground pollution. This is done through monitoring and testing sites suspected of being contaminated with dioxin like compounds. GLP and OSHA guidelines are followed at all times to prevent any pollution of the environment.

14.4 This method produces minimal waste. This waste is contained in biohazard containers and disposed of in compliance with all state, local and federal regulations. The method is also used in projects around the world to manage waste problems. Samples heading for a waste site can be tested for dioxin like compounds prior to disposal to determine if any further processing needs to be done before disposal. GLP and OSHA guidelines are followed at all times when performing this method to prevent any pollution of the environment.

15.0 WASTE MANAGEMENT

The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* available from the American Chemical Society at the address listed in Sec. 14.2.

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17.0 TABLES, DIAGRAMS, FLOW CHARTS, AND VALIDATION DATA

The following pages contain the tables and figures referenced by this method.

TABLE 1

WHO TEF AND METHOD 4435 REP VALUES

Compound	WHO TEF	CALUX REP Based on EC50 Values	REP Range (EC20 - EC50)
Dioxins			
TCDD	1	1.00 +/- 0.01	
12378-PeCDD	1	0.73 +/- 0.10	0.44 to 1.02
123478-HxCDD	0.1	0.075 +/- 0.014	0.034 to 0.137
123678-HxCDD	0.1	0.098 +/- 0.017	0.043 to 0.183
123789-HxCDD	0.1	0.061 +/- 0.012	0.028 to 0.114
1234678-HpCDD	0.01	0.031 +/- 0.008	0.015 to 0.058
OCDD	0.0001	0.00034 +/- 0.00008	0.00025 to 0.00049
Furans			
2378-TCDF	0.1	0.067 +/- 0.010	0.040 to 0.104
12378-PeCDF	0.05	0.14 +/- 0.04	0.14 to 0.15
23478-PeCDF	0.5	0.58 +/- 0.08	0.37 to 0.78
123478-HxCDF	0.1	0.13 +/- 0.02	0.07 to 0.20
123678-HxCDF	0.1	0.14 +/- 0.03	0.10 to 0.19
123789-HxCDF	0.1	0.11 +/- 0.02	0.05 to 0.18
234678-HxCDF	0.1	0.31 +/- 0.06	0.31 to 0.31
1234678-HpCDF	0.01	0.024 +/- 0.007	0.019 to 0.031
1234789-HpCDF	0.01	0.044 +/- 0.010	0.032 to 0.059
OCDF	0.0001	0.0016 +/- 0.0005	0.0003 to 0.0058
PCBs			
PCB 77	0.0005	0.0014 +/- 0.0004	0.0012 to 0.0017
PCB 81	0.0001	0.0045 +/- 0.0012	0.0022 to 0.0085
PCB 114	0.0005	0.00014 +/- 0.00002	0.00014 to 0.00017
PCB 126	0.1	0.038 +/- 0.007	0.037 to 0.042
PCB 156	0.0005	0.00014 +/- 0.00002	0.00013 to 0.00019
PCB 169	0.01	0.0011 +/- 0.0003	0.0007 to 0.0017

TABLE 2

CHEMICAL INTERFERENCES

Compound	Acid silica only % Recovery	Dioxin fraction % Recovery	PCB fraction % Recovery
benzo(a)pyrene	0.002%	0.02%	ND
chrysene	28%	0.2%	ND
acenaphthylene	ND	0.2%	ND
benzo(a)anthracene	0.05%	0.005%	ND
benzo(b)fluoranthene	26%	5.7%	<0.001%
benzo(k)fluoranthene	<0.001%	<0.001%	ND
creosote	ND	ND	ND
p-cresol	ND	ND	ND
dibenzo(a,h)anthracene	<0.001%	ND	ND
1,2-diphenylhydrazine	ND	ND	ND
Fluorene	ND	ND	ND
ideno(1,2,3 cd)pyrene	0.6%	2.5%	0.005%
perylene	ND	ND	ND
2-phenylindole	ND	ND	ND
o-cresol	ND	ND	ND
tryptamine	ND	ND	3%

TABLE 3

RECOMMENDED EQUIPMENT AND SUPPLIES

Supply Item	Supplier	Cat #	Substitution
Micropipettor, 0.5-10 µL range	VWR	40000-200	or equivalent
Micropipetor 2-20 ml range	VWR	40000-202	or equivalent
Micropipettor, 20-200 µL range	VWR	40000-204	or equivalent
Micropipetor 100 – 1000 ml range	VWR	40000-208	or equivalent
Multipipettor, repeating - syringe type	Fisher	21-380-8	or equivalent
EDP2, 10-100ul Electronic Pipette	VWR	E2-100 EDP2	or equivalent
Drummond diaphragm pipettor	VWR	53498-708	or equivalent
10 ml pippet tips	Denville Scientific	P-1095-CP	or equivalent
200 ml pippet tips	Denville Scientific	P4114	or equivalent
1000 ml pippet tips	Denville Scientific	P4026	or equivalent
1.0 ml multipipettor syringes, case of 100	Fisher	21-381-337	or equivalent
10.0 ml multipipettor syringes, case of 100	Fisher	21-381-340	or equivalent
10 ml sterile pipettes, plastic, case of 200	Denville Scientific	P1096-CP	or equivalent
10 ml graduated glass pippets	VWR	53283-776	or equivalent
25 cm2 Tisue Culture Flasks	VWR	15708-096	or equivalent
75 cm2 Tissue Culture Flasks	VWR	29186-080	or equivalent
scintillation vials, case of 500	Fisher	03-340-129	or equivalent
scintillation vial caps, teflon liner, case of 500	Fisher	03-340-131	or equivalent
25 ml drying tubes, case of 100	VWR	17453-142	or equivalent
10 ml drying tubes, case of 200	VWR	17453-140	or equivalent
13 x 100 mm Test Tubes	VWR	60825-414	or equivalent
16 x 125mm Test Tubes	VWR	60825-630	or equivalent
50 ml centrifuge Tubes	VWR	21020-695	or equivalent
15 ml plastic centrifuge tubes, sterile	Denville	C-1018	or equivalent
50 ml plastic centri. Tubes	VWR	21008-951	or equivalent
13mm test tube racks	Fisher	14-809-22	or equivalent
13mm test tube racks for dosing	Fisher	14-810-54A	or equivalent
16 mm test tube racks	Fisher	14-809-24	or equivalent
50 ml test tube racks	Fisher	14-809-28	or equivalent
9" Pasteur pipettes	VWR	53283-915	or equivalent
pipette bulbs, 2 ml capacity, pack of 72	VWR	56311-062	or equivalent
96 well plates	VWR	29444-010	or equivalent
Backing Tape	Perkin Elmer	6005199	or equivalent
latex gloves	Marsh Bio	L6003PF	or equivalent
glass wool, 8 micron	Fisher	11-388	or equivalent
Benchtop paper, 2 rolls of 20" x 300"	VWR	14672-200	or equivalent
Tubing 12mm, CS-25lb	VWR	32814-227	or equivalent

TABLE 3
(cont.)

RECOMMENDED EQUIPMENT AND SUPPLIES

Equipment Item name	Supplier	Cat #	Substitution
Class II biological safety hood and stand	Fisher	16-108-99	or equivalent
Water Jacketed Cell culture incubator, with CO_2 and temp. control	Fisher	11-689-4	or equivalent
Centrifuge, low speed, tabletop with swinging bucket rotor	Fisher	04-978-50	or equivalent
Centrifuge concentrator with vacuum pump with cold trap	Fisher	16-315-45	or equivalent
Shaker for 96 well plates	Fisher	14-271-9	or equivalent
Microscope, inverted	Fisher	12-561-INV	or equivalent
Microscope	Fisher	12-561-3M	or equivalent
Hemocytometer, cell counter	Fisher	02-671-5	or equivalent
Hand tally counter	Fisher	07-905-6	or equivalent
Vortex – mixer	Fisher	12-814	or equivalent
sonicating water bath	Fisher	15-335-30	or equivalent
Vacuum pump with liquid trap (side arm erylenmeyer flask)	Fisher	01-092-29	or equivalent
Refrigerator/freezer	Fisher	13-986-106A	or equivalent
-70 celcius freezer	Fisher	13-989-187	or equivalent
Liquid Nitrogen dewar	Fisher	11-675-92	or equivalent
Luminometer Berthold and dedicated computer	Berthold		or equivalent
Combustion test kit, CO_2 monitoring	Fisher	10-884-1	or equivalent
250^{o}C Oven	VWR		or equivalent
Autoclave	VWR	58618-009	or equivalent
High-Capacity top loading Balance 0.01g-4200g	VWR	14216-518	or equivalent

TABLE 4

RECOMMENDED REAGENTS AND STANDARDS

Solvent / Reagent Item	Supplier	Cat #	Substitution
Phosphate buffered saline 1x	VWR	45000-446	or equivalent
Trypsin	VWR	MT 25-054-C1	or equivalent
pen/strep solution	VWR	45000-650	or equivalent
Fetal Calf serum	Atlanta Biological	S11150	or equivalent
RPMI 1640 1X (MOD.) with L-Glutamine medium	VWR	45000-396	or equivalent
Cell Culture Lysis Reagent	Promega	E1531	or equivalent
Luciferase Substrate Solution	Promega	E1501	or equivalent
70 % ethanol, for cleaning and as coolant for cold trap	VWR	VW3609-4	or equivalent
Sodium hydroxide	VWR	JT3722-4	or equivalent
DMSO	Sigma-Aldrich	27,043-1	or equivalent
Sulfuric acid, concentrated, 2.5 L, ACS reagent	Fisher	A300-212	or equivalent
Hexane, optima grade, case of 4-4L bottles	Fisher	BJ216-4	or equivalent
Toluene, optima grade, case of 4-4L bottles	Fisher	T291-4LC	or equivalent
Methanol, case of 4-4L bottles	Fisher	A454-4	or equivalent
Acetone, case of 4-4L bottles	Fisher	A929-4	or equivalent
Ethyl acetate	VWR	JT9282-3	or equivalent

TABLE 5

EXAMPLE UNLABELED 17 CONGENERS INTERNAL SPIKE RECOVERIES

Sample	TEQ	Spike sample minus sample pg	% Recovery
spiked sample	4.65	1.28	80%
unspiked sample	3.37	-	-
equimolar spike	1.61	-	-
spiked sample	10.52	8.66	84%
unspiked sample	1.86	-	-
equimolar spike	10.28	-	-
spiked sample	5.22	5.05	72%
unspiked sample	0.17	-	-
equimolar spike	6.98	-	-
spiked sample	11.09	10.82	94%
unspiked sample	0.27	-	-
equimolar spike	11.45	-	-

TABLE 6

COMPARISON OF 1,2,3,4-TCDD RECOVERIES DETERMINED BY GC/ECD AND ^{14}C LABELED 2,3,7,8-TCDD RECOVERIES DETERMINED BY SCINTILLATIONS COUNTING

Sample	1234-TCDD by GC/ECD	^{14}C 2,3,7,8-TCDD by scintillation counting
1	76.3% ± 1.1%	91.2% ± 0.9%
2	95.4% ± 1.7%	84.1% ± 2.9%
3	91.6% ± 1.8%	89.9% ± 2.4%
4	90.6% ± 0.3%	83.5% ± 3.4%

TABLE 7

EXAMPLE STANDARD CURVE CONCENTRATIONS

TCDD Concentrations pg/ml	Picograms/Tube
250	100
125	50
62.5	25
31.25	12.5
15.625	6.25
7.81	3.13
3.91	1.56
1.95	0.78
0.977	0.391
0.488	0.195
0.244	0.098

TABLE 8

FIELD TEST COMPARISON of Standard INCUBATOR TO AIRTIGHT BOX

XDS ID #	TEQ-pg/g Incubator PCDD / PCDF	Airtight box PCDD / PCDF
Site Sample #1	0.13	0.14
Site Sample #2	46.27	55.58
Site Sample #3	0.13	0.15
Site Sample #4	0.13	0.13
Site Sample #5	523.29	711.11
Site Sample #6	169.79	184.15
Site Sample #7	0.34	0.58
Site Sample #8	97.57	86.67
Site Sample #9	193.02	349.01
Site Sample #10	70.56	142.44
Site Sample #11	95.05	97.51
Site Sample #12	0.56	0.75

TABLE 9

COMPARISON OF METHOD 4435 AND GC/MS RESULTS FOR SOIL SAMPLES

Company	Sample Matrix	Sample ID	TEQ-ppt mean (pg/g)	std error	Percent std error	GC/MS (pg/g)
Company-2 Asia	Soil	Soil-1	2.56	0.30	12%	0.19
Company-2 Asia	Soil	Soil-2	28.33	3.09	11%	3.80
Company-2 Asia	Soil	Soil-3	25.63	3.47	14%	12.00
Company-2 Asia	Soil	Soil-4	352.32	30.53	9%	120.00
Company-2 Asia	Soil	Soil-5	4034.97	339.88	8%	800.00
Company-2 Asia	Soil	Soil-6	7306.87	1021.26	14%	2600.00
Company-2 Asia	Soil	Soil-7	20539.55	4661.64	23%	1200.00
Company-3 USA	Soil	17100 A, B	207.51	28.56	14%	63.90
Company-3 USA	Soil	17101 A, B	12869.85	1057.71	8%	4852.00
Company-3 USA	Soil	17102 A, B	306.05	19.80	6%	84.20
Company-3 USA	Soil	17103 A, B	539543.83	42835.93	8%	94330.00
Company-3 USA	Soil	17105 A, B	441485.41	79369.82	18%	63113.00
Company-3 USA	Soil	17106 A, B	637162.72	29247.40	5%	44411.00
Company-3 USA	Soil	17107 A, B	394642.48	19514.21	5%	55581.00
Company-3 USA	Soil	17108 A, B	2078549.92	80574.11	4%	333638.00
Company-3 USA	Soil	17109 A, B	25574.14	154.39	1%	8547.00
Company-3 USA	Soil	17118 A, B	272858.59	2423.72	1%	98560.00
Company-3 USA	Soil	17120 A, B	7469.31	299.10	4%	1398.00
Company-3 USA	Soil	17126 A, B	790.04	85.95	11%	207.00
Company-3 USA	Soil	17128 A, B, C, D, E, F	191.58	10.11	5%	65.00
Company-3 USA	Soil	17130 A, B	260510.86	2495.27	1%	10891.00
Company-3 USA	Soil	17131 A, B	885605.65	83917.76	9%	115539.00
Company-3 USA	Soil	17132 A, B	823636.59	93127.53	11%	115451.00
Company-3 USA	Soil	17134 A, B	392784.46	468.45	0%	116792.00
Company-3 USA	Soil	17138 A, B	6703.40	172.76	3%	2337.00
Company-3 USA	Soil	17149 A, B	459.40	23.40	5%	89.50
Company-1 Asia	Soil	Soil Sample 1	416.00	32.13	8%	150.00
Company-1 Asia	Soil	Soil Sample 2	5.65	0.01	0%	1.20
Company-1 Asia	Soil	Sample No. 2 soil/1185-2	27.87	3.13	11%	7.90
Company-1 Asia	Soil	Sample No. 3 Soil/ 1185-3	14.41	2.17	15%	7.90
Company-1 Asia	Soil	Sample No. 4, Soil./ 1185-4	39.90	6.60	17%	21.00
Company-1 Asia	Soil	Sample No. 6, Soil./ 1185-6	16.98	2.85	17%	2.40
Company-1 Asia	Soil	Sample No. 7, Soil./ 1185-7	7.67	1.19	16%	6.00
Company-1 Asia	Soil	Sample No. 8, Soil./ 1185-8	9.98	1.47	15%	6.00
Company-1 Asia	Soil	Soil Sample 1.	9.05	0.78	9%	0.21
Company-1 Asia	Soil	Soil Sample 3.	15.22	2.36	15%	0.31
Company-1 Asia	Soil	Soil Sample 4.	21.82	0.06	0%	3.30
Company-1 Asia	Soil	Soil Sample 5	4699.31	171.10	4%	4000.00
Company-1 Asia	Soil	Soil Sample 6	6725.04	525.67	8%	5900.00
Company-1 Asia	Soil	Soil Sample 14.	24.04	0.43	2%	3.80
Company-4 Europe	Soil	A	160.00	0.01	0.00%	17.67
Company-4 Europe	Soil	C	600.00	0.07	0.01%	180.00
Company-4 Europe	Soil	D	1061.00	0.09	0.01%	323.33
Company-4 Europe	Soil	B	154.00	0.04	0.02%	19.67
Company-4 Europe	Soil	C	570.00	0.11	0.02%	496.67
Company-5 USA	Soil	XDSG	16.28	0.62	4%	8.79
Company-5 USA	Soil	XDSG30	37.24	1.39	4%	38.83

TABLE 10

COMPARISON OF METHOD 4435 AND GC/MS RESULTS FOR SOIL EXTRACTS

Company	Sample Matrix	Sample ID	TEQ-ppt mean (pg/g)	std error	Percent std error	CALUX (pgTEQ/10g)	GCMS (pgTEQ/10g)
Company-1 Asia	Soil Extract	S-74	115.02	16.01	14%	120.00	23.00
Company-1 Asia	Soil Extract	S-121	74.50	10.37	14%	76.00	6.80
Company-1 Asia	Soil Extract	S-16	12.23	3.40	28%	12.00	0.92
Company-1 Asia	Soil Extract	S-61	522.67	73.34	14%	540.00	110.00
Company-1 Asia	Soil Extract	S-119	55.27	7.56	14%	55.00	5.40
Company-1 Asia	Soil Extract	S-54	104.50	17.76	17%	110.00	25.00
Company-1 Asia	Soil Extract	S-120	176.43	30.43	17%	180.00	15.00
Company-1 Asia	Soil Extract	S-132	847.00	157.42	19%	860.00	150.00
Company-1 Asia	Soil Extract	S-43	1.21	0.17	14%	1.00	0.07
Company-1 Asia	Soil Extract	S-17	2.70	0.52	19%	3.00	0.11
Company-1 Asia	Soil Extract	S-49	89.67	10.74	12%	92.00	12.00
Company-1 Asia	Soil Extract	S-53	345.28	22.20	6%	360.00	87.00
Company-1 Asia	Soil Extract	S-18	8.90	0.61	7%	9.00	0.39
Company-1 Asia	Soil Extract	S-55	82.48	8.22	10%	84.00	8.20
Company-1 Asia	Soil Extract	S-28	313.50	37.88	12%	310.00	42.00
Company-1 Asia	Soil Extract	S-42	98.96	10.41	11%	103.00	12.00
Company-1 Asia	Soil Extract	S-127	682.20	67.87	10%	680.00	110.00
Company-1 Asia	Soil Extract	S-19	5.97	0.73	12%	6.00	0.26
Company-1 Asia	Soil Extract	S-7	93.94	12.16	13%	160.00	17.00
Company-1 Asia	Soil Extract	S-52	102.10	10.46	10%	105.00	18.00
Company-1 Asia	Soil Extract	S-75	316.10	18.96	6%	320.00	21.00

TABLE 11

COMPARISON OF METHOD 4435 AND GC/MS RESULTS FOR ASH SAMPLES

Company	Sample Matrix	Sample ID	TEQ-ppt mean (pg/g)	std error	Percent std error	GC/MS (pg/g)
Company-2 Asia	Ash	Ash-1	7.34	1.89	26%	2.80
Company-2 Asia	Ash	Ash-2	105.70	27.01	26%	60.00
Company-2 Asia	Ash	Ash-3	584.55	61.40	11%	400.00
Company-2 Asia	Ash	Ash-5	52795.74	9307.38	18%	27000.00
Company-1 Asia	Ash	Sample No.9, Ash,1185-9	402.60	14.84	4%	360.00
Company-1 Asia	Ash	Sample No.10, Ash/ 1185-10	684.47	38.78	6%	470.00
Company-1 Asia	Ash	Ash Sample 7	142.83	14.06	10%	89.00
Company-1 Asia	Ash	Ash Sample 8	260.60	33.29	13%	71.00
Company-1 Asia	Ash	Ash Sample 9	19870.12	983.14	5%	7000.00
Company-1 Asia	Ash	Ash Sample 11	1598.46	73.54	5%	1100.00
Company-4 Europe	Ash	B	36.00	0.00	0%	32.00
Company-4 Europe	Ash	C	523.00	0.02	0%	390.00
Company-4 Europe	Ash	B	63240.00	9.11	0%	57500.00
Company-4 Europe	Ash	C	545.00	0.04	0%	285.33

TABLE 12

COMPARISON OF METHOD 4435 AND GC/MS RESULTS FOR ASH EXTRACTS

Company	Sample Matrix	Sample ID	TEQ-ppt mean (pg/g)	std error	Percent std error	CALUX (ngTEQ/10g)	GCMS (ngTEQ/10g)
Company-1 Asia	Ash Extract	A-20	12721.95	1048.59	8%	12.00	2.80
Company-1 Asia	Ash Extract	A-17	28261.60	4132.32	15%	18.00	2.20
Company-1 Asia	Ash Extract	A-23	84.69	5.55	7%	0.10	0.012
Company-1 Asia	Ash Extract	A-40	193299.28	20703.51	11%	210.00	19.00
Company-1 Asia	Ash Extract	A-18	1442.06	84.06	6%	1.00	0.22
Company-1 Asia	Ash Extract	A-66	5862.67	736.10	13%	6.10	1.20
Company-1 Asia	Ash Extract	A-57	16871.73	1857.26	11%	17.00	1.50
Company-1 Asia	Ash Extract	A-43	7896.12	334.81	4%	8.60	1.20
Company-1 Asia	Ash Extract	A-42	4525.55	457.10	10%	4.60	0.85
Company-1 Asia	Ash Extract	A-50	48462.10	5962.94	12%	34.00	5.00
Company-1 Asia	Ash Extract	A-56	4646.37	333.91	7%	2.90	0.68
Company-1 Asia	Ash Extract	A-2	2443.32	188.29	8%	3.00	0.39
Company-1 Asia	Ash Extract	A'-2	2192.71	143.06	7%	2.20	0.23
Company-1 Asia	Ash Extract	A'-1	1844.48	187.46	10%	1.10	0.23
Company-1 Asia	Ash Extract	A'-5	1273.59	114.65	9%	1.30	0.28
Company-1 Asia	Ash Extract	A-13	128781.60	13106.95	10%	130.00	10.00
Company-1 Asia	Ash Extract	A-19	22838.28	1799.11	8%	16.00	2.70
Company-1 Asia	Ash Extract	A-9	60.94	5.96	10%	0.10	0.0038
Company-1 Asia	Ash Extract	A-59	6812.57	902.82	13%	20.90	2.00
Company-1 Asia	Ash Extract	A-60	92283.85	12021.15	13%	280.00	20.00
Company-1 Asia	Ash Extract	A-28	77824.56	4318.94	6%	102.00	6.20
Company-1 Asia	Ash Extract	A-41	103465.03	15583.39	15%	71.00	8.10
Company-1 Asia	Ash Extract	A-34	327501.35	27583.75	8%	200.00	26.00
Company-1 Asia	Ash Extract	A-61	20.66	2.61	13%	0.012	0.0035
Company-1 Asia	Ash Extract	A-27	56.60	6.85	12%	0.034	0.0061
Company-1 Asia	Ash Extract	A-45	60374.36	8614.39	14%	60.00	3.10
Company-1 Asia	Ash Extract	A-14	164.08	17.35	11%	0.10	0.019
Company-1 Asia	Ash Extract	A-16	47848.74	6204.24	13%	32.00	3.30
Company-1 Asia	Ash Extract	A-44	3985.64	239.54	6%	4.00	0.53
Company-1 Asia	Ash Extract	A-75	512.53	24.61	5%	0.30	0.074
Company-1 Asia	Ash Extract	A-65	458.24	19.23	4%	0.28	0.035
Company-1 Asia	Ash Extract	A-15	2630.39	200.54	8%	2.70	0.36
Company-1 Asia	Ash Extract	A-58	5399.43	168.09	3%	6.00	1.40
Company-1 Asia	Ash Extract	A-48	201.53	11.01	5%	0.12	0.012
Company-1 Asia	Ash Extract	A-8	14904.29	1335.97	9%	15.00	1.10
Company-1 Asia	Ash Extract	A-74	781.28	61.79	8%	0.74	0.13
Company-1 Asia	Ash Extract	A-31	975.80	47.11	5%	0.60	0.11

TABLE 13

COMPARISON OF METHOD 4435 AND GC/MS RESULTS FOR EXHAUST EXTRACTS

Company	Sample Matrix	Sample ID	TEQ-ppt mean(pg/sample)	std error	Percent std error	CALUX (ngTEQ/M3)	GCMS (ngTEQ/M3)
Company-1 Asia	Exhaust Extracts	G1	6581.26	622.42	9%	16.00	3.3
Company-1 Asia	Exhaust Extracts	G7	75.53	4.38	6%	0.29	0.036
Company-1 Asia	Exhaust Extracts	G8	21436.70	829.11	4%	68.50	9.20
Company-1 Asia	Exhaust Extracts	G9	548320.73	29207.76	5%	2900.00	210.00
Company-1 Asia	Exhaust Extracts	G10	605.72	42.02	7%	1.50	0.30
Company-1 Asia	Exhaust Extracts	G17	457.44	13.64	3%	3.70	0.28
Company-1 Asia	Exhaust Extracts	G18	46.06	1.64	4%	0.15	0.012
Company-1 Asia	Exhaust Extracts	G19	5875.83	390.38	7%	17.00	2.30
Company-1 Asia	Exhaust Extracts	G20	8164.75	269.70	3%	24.00	3.50
Company-1 Asia	Exhaust Extracts	G24	1728.17	115.02	7%	6.10	0.75
Company-1 Asia	Exhaust Extracts	G25	2009.68	120.16	6%	7.80	1.10
Company-1 Asia	Exhaust Extracts	G36	1981.81	132.78	7%	6.20	0.65
Company-1 Asia	Exhaust Extracts	G37	19927.99	1227.69	6%	100.00	8.30
Company-1 Asia	Exhaust Extracts	G38	34.94	3.79	11%	0.115	0.0089
Company-1 Asia	Exhaust Extracts	G40	533.58	15.08	3%	1.90	0.38
Company-1 Asia	Exhaust Extracts	G41	8002.56	539.02	7%	30.00	5.30
Company-1 Asia	Exhaust Extracts	G42	2410.17	178.49	7%	7.60	0.90
Company-1 Asia	Exhaust Extracts	G43	2958.76	252.68	9%	10.80	1.40
Company-1 Asia	Exhaust Extracts	G48	1477.56	20.27	1%	3.60	0.66
Company-1 Asia	Exhaust Extracts	G50	212.27	12.76	6%	1.50	0.19
Company-1 Asia	Exhaust Extracts	G51	2465.49	141.12	6%	7.60	0.83
Company-1 Asia	Exhaust Extracts	G58	173086.48	9011.13	5%	800.00	44.00
Company-1 Asia	Exhaust Extracts	G62	34.30	10.53	31%	0.10	0.017
Company-1 Asia	Exhaust Extracts	G66	191.14	26.82	14%	0.75	0.11
Company-1 Asia	Exhaust Extracts	G67	1186.26	9.92	0.84%	9.00	1.90
Company-1 Asia	Exhaust Extracts	G70	36803.65	1739.43	5%	300.00	38.00
Company-1 Asia	Exhaust Extracts	G77	4548.33	389.91	9%	29.60	4.60
Company-1 Asia	Exhaust Extracts	G80	4457.20	350.10	8%	16.00	4.40
Company-1 Asia	Exhaust Extracts	G91	21.57	2.46	11%	0.30	0.0032
Company-1 Asia	Exhaust Extracts	G96	2381.29	240.40	10%	7.70	0.90
Company-1 Asia	Exhaust Extracts	G97	3941.68	302.96	8%	20.00	3.50
Company-1 Asia	Exhaust Extracts	G98	219387.37	16146.81	7%	900.00	69.00
Company-1 Asia	Exhaust Extracts	G99	2886.31	176.12	6%	16.00	2.40
Company-1 Asia	Exhaust Extracts	G100	1361.66	111.82	8%	7.10	0.97
Company-1 Asia	Exhaust Extracts	G105	66.99	4.26	6%	0.23	0.023
Company-1 Asia	Exhaust Extracts	G107	19.87	1.52	8%	0.032	0.00045
Company-1 Asia	Exhaust Extracts	G109	34.32	3.21	9%	0.13	0.013
Company-1 Asia	Exhaust Extracts	G113	33.91	4.52	13%	0.117	0.014
Company-1 Asia	Exhaust Extracts	G119	255.60	34.76	14%	1.60	0.30
Company-1 Asia	Exhaust Extracts	G120	329.13	17.24	5%	0.87	0.21
Company-1 Asia	Exhaust Extracts	G121	746.96	55.48	7%	2.80	0.80
Company-1 Asia	Exhaust Extracts	G129	1478.34	158.49	11%	8.60	2.40
Company-1 Asia	Exhaust Extracts	G131	804.38	42.69	5%	4.20	0.95
Company-1 Asia	Exhaust Extracts	G132	1263.64	66.85	5%	4.20	1.10
Company-1 Asia	Exhaust Extracts	G133	1359.57	144.41	11%	4.80	0.88
Company-1 Asia	Exhaust Extracts	G134	2162.18	99.76	5%	7.00	2.40
Company-1 Asia	Exhaust Extracts	G135	1563.29	74.61	5%	7.30	2.80
Company-1 Asia	Exhaust Extracts	G136	1170.80	36.58	3%	6.70	1.60

TABLE 14

COMPARISON OF METHOD 4435 AND GC/MS RESULTS FOR SOLUTION SAMPLES

Company	Sample Matrix	Sample ID	TEQ-ppt mean (pg/ul)	std error	Percent std error	GC/MS (pg/ul)
Company-4 Europe	solution	F	448.34	N/A	N/A	229.67
Company-4 Europe	solution	H	8.39	N/A	N/A	3.34
Company-4 Europe	solution	Standard Solution L	381.00	35.04	9%	160.33

FIGURE 1

SCHEMATIC REPRESENTATION OF THE BIOCHEMICAL PROCESSES INVOLVED IN DIOXIN TOXICITY AND TEQ ESTIMATIONS BY METHOD 4435

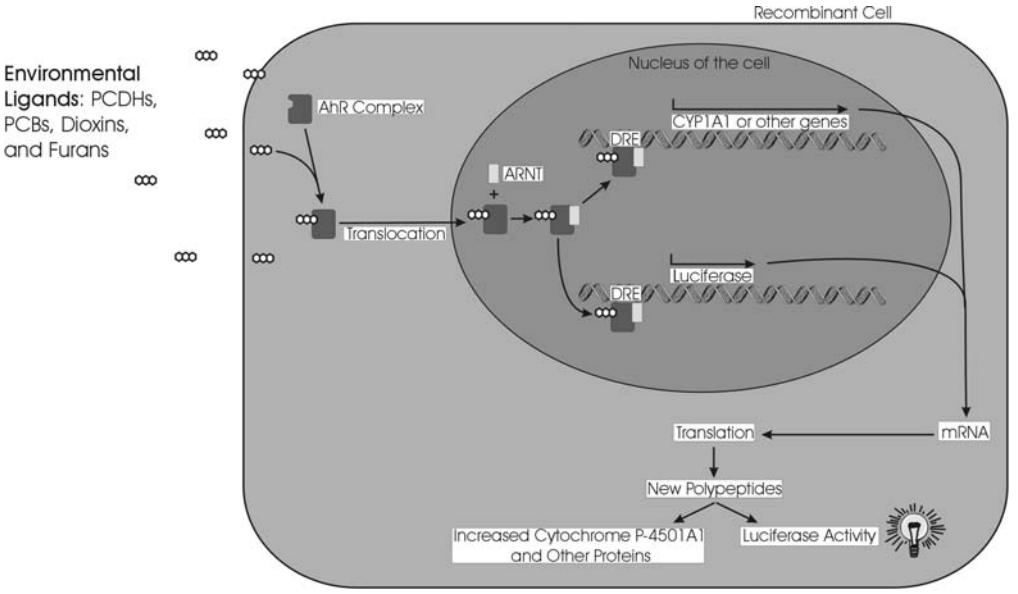
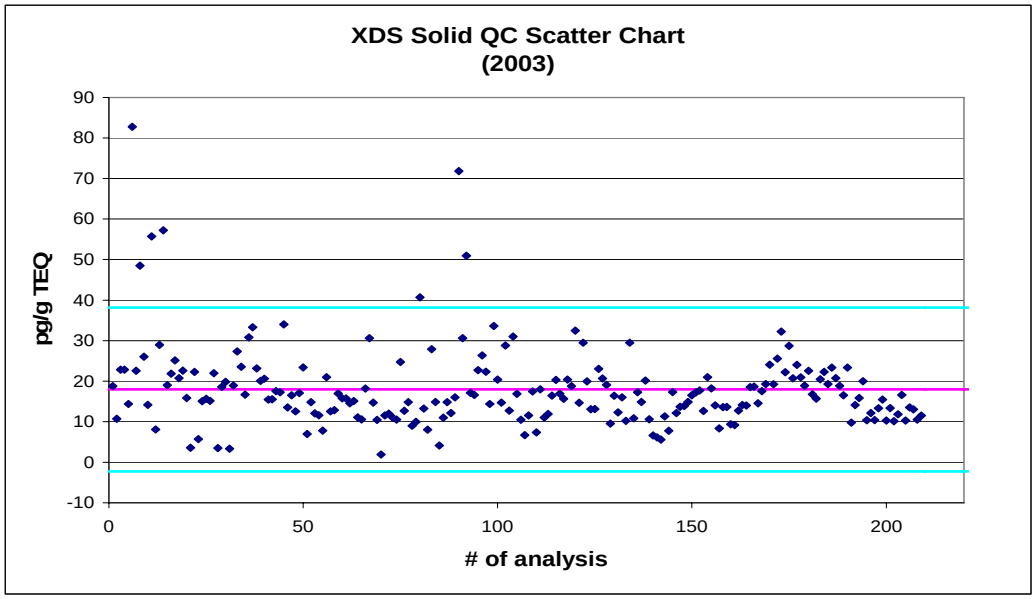


FIGURE 2

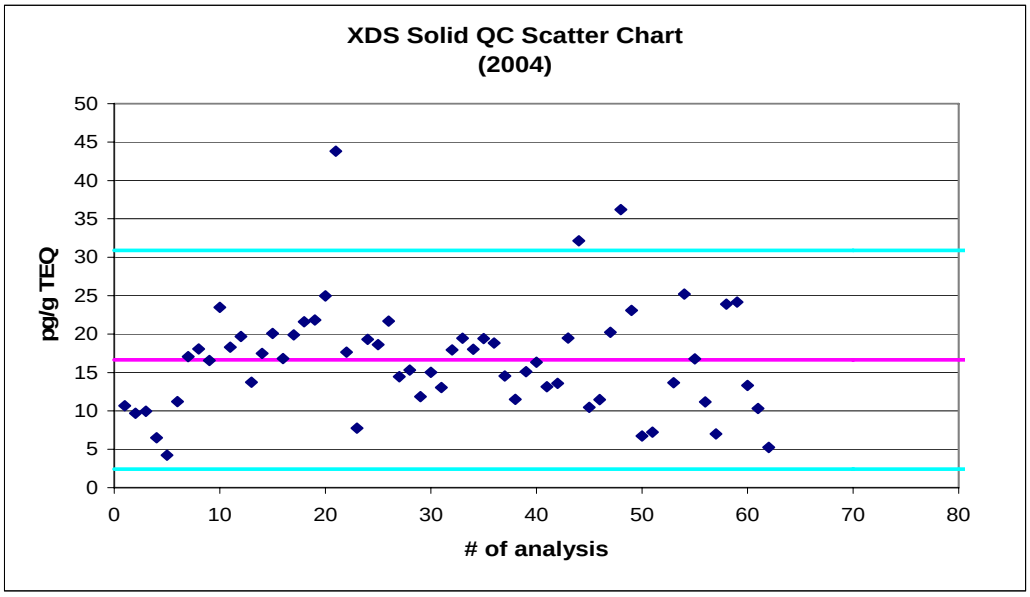
LAKE SEDIMENT REFERENCE SAMPLE QC CHART FOR 2003



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 3

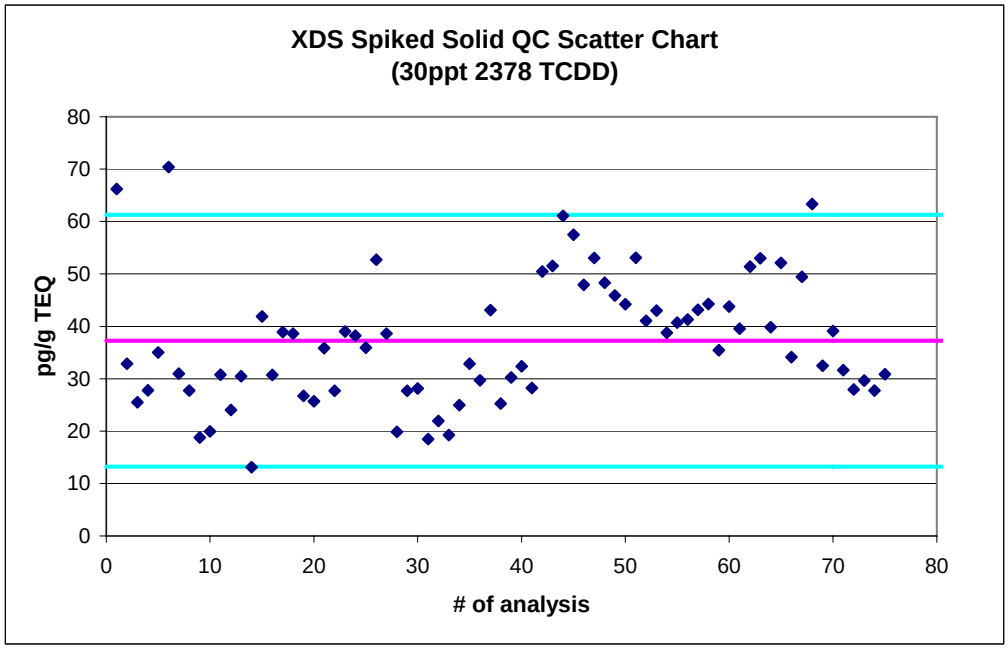
LAKE SEDIMENT REFERENCE SAMPLE QC CHART FOR 2004



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 4

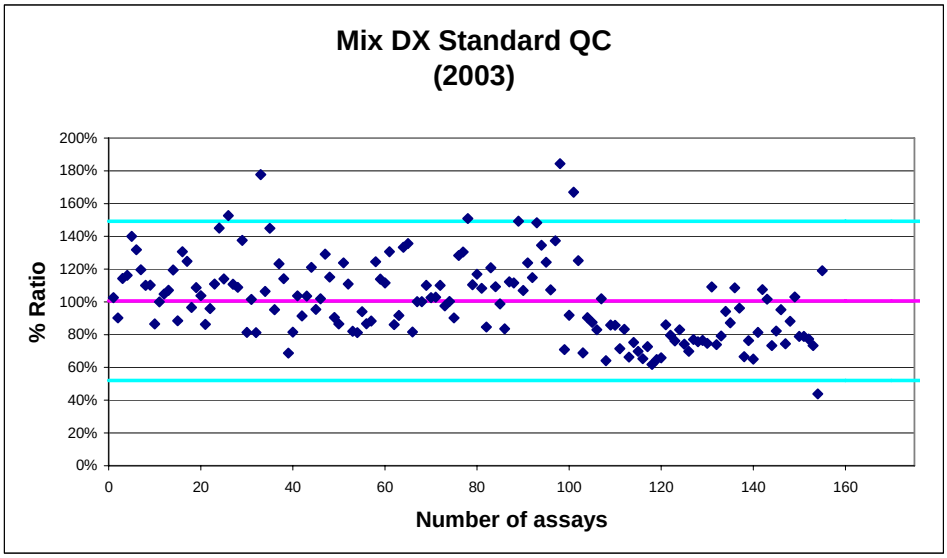
LAKE SEDIMENT REFERENCE SAMPLE QC CHART SPIKED WITH 30 pg/g 2,3,7,8-TCDD



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 5

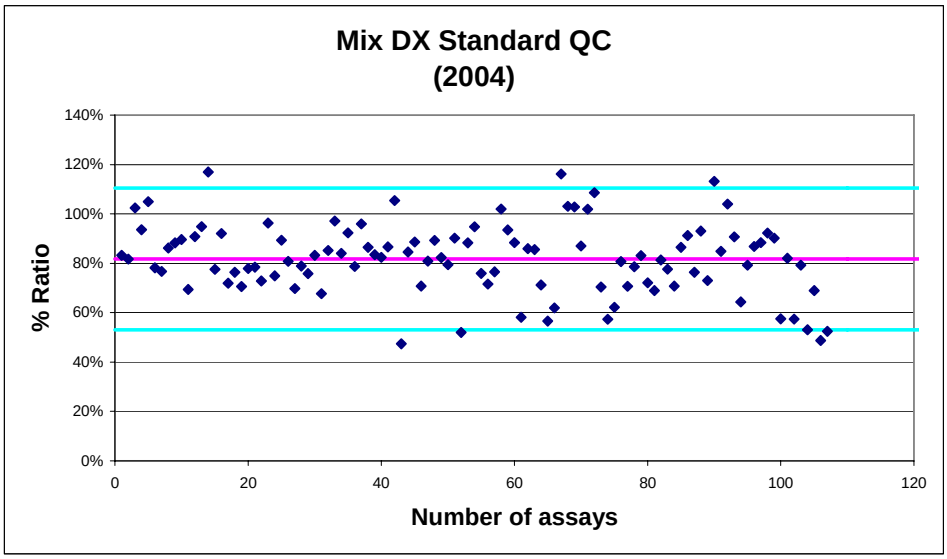
QC CHARTS FOR DIOXIN/FURAN STANDARD MIXTURE FOR 2003



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 6

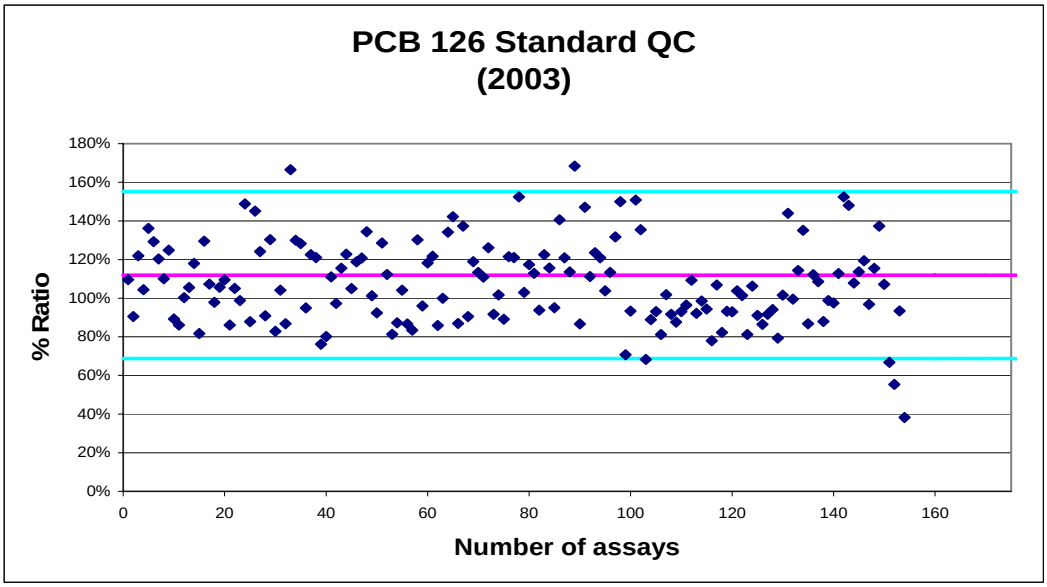
QC CHARTS FOR DIOXIN/FURAN STANDARD MIXTURE FOR 2004



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 7

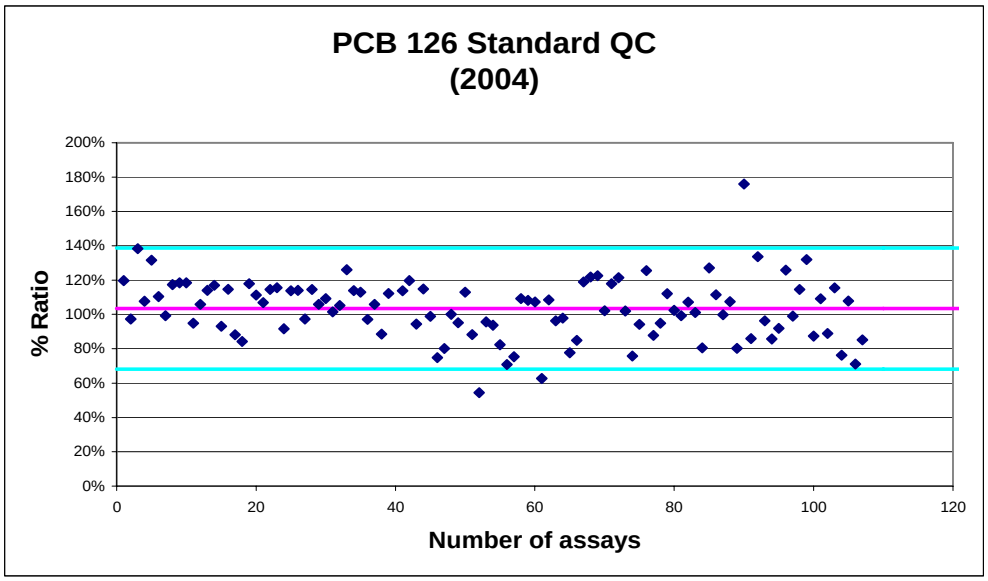
QC CHARTS FOR PCB 126 STANDARD FOR 2003



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 8

QC CHARTS FOR PCB 126 STANDARD FOR 2004



The middle line is the mean and the two outer lines are 2 standard deviations from the mean. The dots represent the individual assays.

FIGURE 9

2,3,7,8-TCDD STANDARD CURVES WITH ERROR BARS FOR 2003

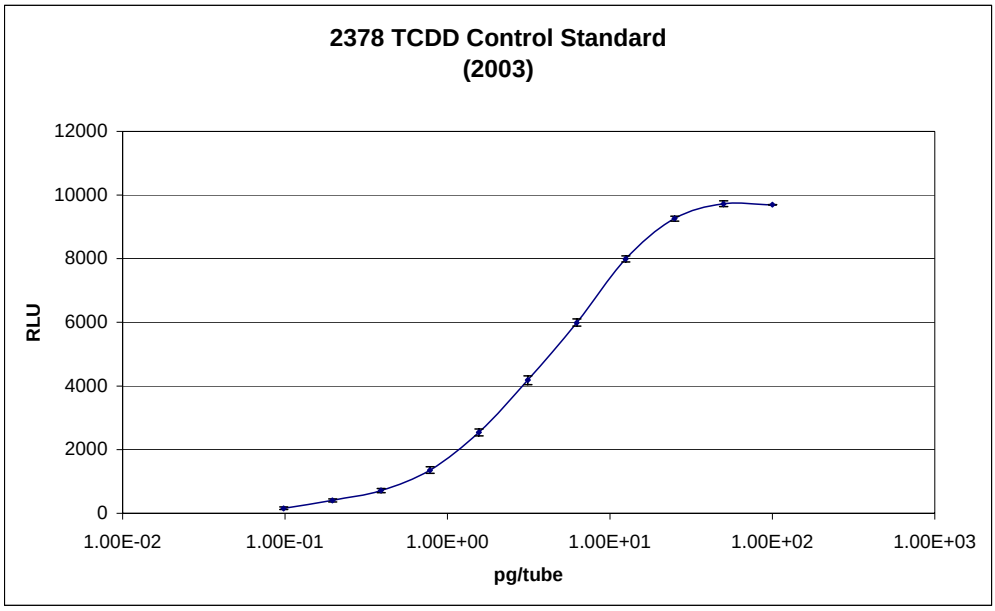


FIGURE 10

2,3,7,8-TCDD STANDARD CURVES WITH ERROR BARS FOR 2004

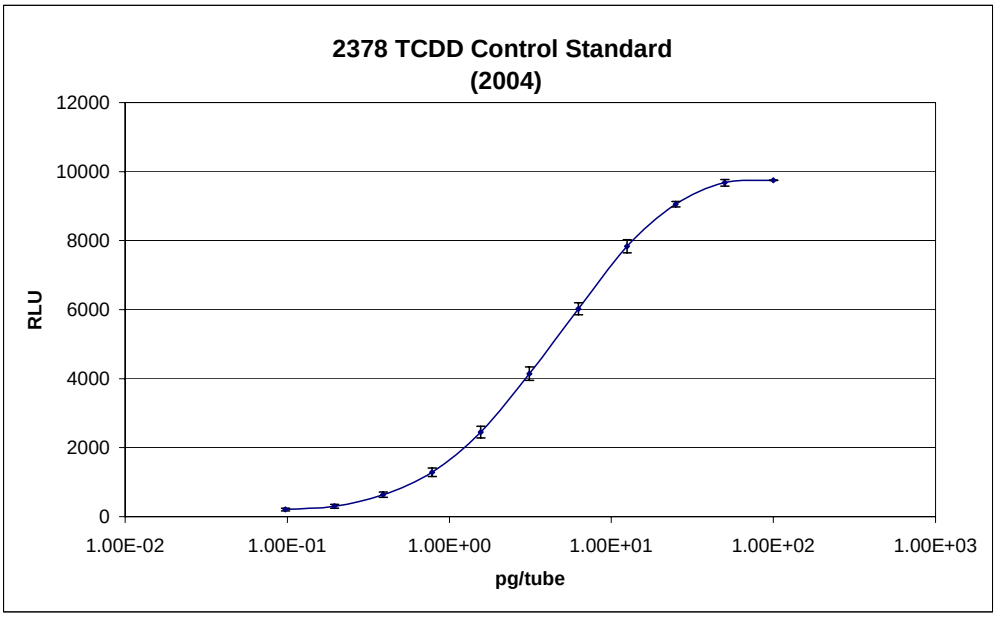


FIGURE 11

TEMPLATE FOR 96 WELL PLATE

	2	3	4	5	6	7	8	9 20 hour exposure	10	11
A										
B	2,3,7,8 TCDD 1.00E+02 TCDD 13853	2,3,7,8 TCDD 1.56E+00 TCDD 2066	Negative Control DMSO control 1	XDS-1 1:10 Soil 980	XDS-3 1:10 Soil 7573	XDS QC-1 1:10 Matrix QC sample 341	XDS-1 1:100 Soil 269	XDS-3 1:100 Soil 4679	XDS-1 1:500 Soil 237	XDS-3 1:500 Soil 2136
C	2,3,7,8 TCDD 5.00E+01 TCDD 13047	2,3,7,8 TCDD 7.81E-01 TCDD 898	Negative Control No DMSO control 1	XDS-1 1:10 Soil 1040	XDS-3 1:10 Soil 7293	XDS QC-2 1:10 Martix spike sample 3750	XDS-1 1:100 Soil 263	XDS-3 1:100 Soil 3846	XDS-1 1:500 Soil 251	XDS-3 1:500 Soil 1998
D	2,3,7,8 TCDD 2.50E+01 TCDD 11673	2,3,7,8 TCDD 3.91E-01 TCDD 436	Postive Control High-level PCB-QC control 8081	XDS-1 1:10 Soil 1363	XDS-3 1:10 Soil 8254	XDS QC-3 1:10 Soil QC sample 4208	XDS-1 1:100 Soil 375	XDS-3 1:100 Soil 4106	XDS-1 1:500 Soil 345	XDS-3 1:500 Soil 2123
E	2,3,7,8 TCDD 1.25E+01 TCDD 9382	2,3,7,8 TCDD 1.95E-01 TCDD 112	Postive Control High-level DX-QC control 8010	XDS-2 1:10 Soil 3863	XDS-4 1:10 Soil 5829	XDS-5 1:10 Method Blank 452	XDS-2 1:100 Soil 1588	XDS-4 1:100 Soil 2954	XDS-2 1:500 Soil 532	XDS-4 1:500 Soil 1834
F	2,3,7,8 TCDD 6.25E+00 TCDD 6480	2,3,7,8 TCDD 9.77E-02 TCDD 43	Negative Control DMSO control 1	XDS-2 1:10 Soil 4174	XDS-4 1:10 Soil 6419	XDS-6 1:10 Method Blank 398	XDS-2 1:100 Soil 1533	XDS-4 1:100 Soil 3026	XDS-2 1:500 Soil 863	XDS-4 1:500 Soil 1642
G	2,3,7,8 TCDD 3.13E+00 TCDD 3819	Negative Control DMSO control 1	Negative Control DMSO control 1	XDS-2 1:10 Soil 4422	XDS-4 1:10 Soil 5719	XDS-7 1:10 Method Blank 583	XDS-2 1:100 Soil 1824	XDS-4 1:100 Soil 3212	XDS-2 1:500 Soil 825	XDS-4 1:500 Soil 1358
H										

Includes 2,3,7,8-TCDD standard curve, 4 DMSO controls, 1 No DMSO control, 2 positive control QC points; and samples (including THE MANUFACTURER I.D.#; sample dilution; matrix (or client I.D.#); and RLU result)

FIGURE 12

RAW DATA FROM THE LUMINOMETER, CORRECTED BY BACKGROUND DMSO SUBTRACTION

Raw Data

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		14620	2833	1110	1747	8340	1108	1036	5446	1004	2903	
C		13814	1665	936	1807	8060	4517	1030	4613	1018	2765	
D		12440	1203	8848	2131	9021	4975	1142	4873	1112	2890	
E		10149	879	8777	4630	6596	1219	2355	3721	1300	2601	
F		7247	810	849	4941	7186	1166	2300	3793	1630	2409	
G		4586	726	726	5189	6486	1350	2591	3979	1592	2125	
H												

Raw Data - Blank

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		13853	2066	1	980	7573	341	269	4679	237	2136	
C		13047	898	1	1040	7293	3750	263	3846	251	1998	
D		11673	436	8081	1363	8254	4208	375	4106	345	2123	
E		9382	112	8010	3863	5829	452	1588	2954	532	1834	
F		6480	43	1	4174	6419	398	1533	3026	863	1642	
G		3819	1	1	4422	5719	583	1824	3212	825	1358	
H												

Average background 767

Refer to template (Figure 11) for well descriptions.

FIGURE 13

BACKGROUND CORRECTED CALCULATIONS USING THE RLU DATA (FIGURE 12) TO PRODUCE THE RESULTS FOR THE REPORT (FIGURE 14), USING THE 4 PARAMETER HILL EQUATION

THE MODEL:
RLU = (v*(d^n))/(d^n + k^n)

'd' is the natural logarithm of TCDD concentration
"v" is the limiting value of the RLU response as TCDD concentration increases
"k" is the dose at which the response is 50% of maximum
"n" is a parameter that determines sigmoidal shape of curve
"b" is the interecept parameter

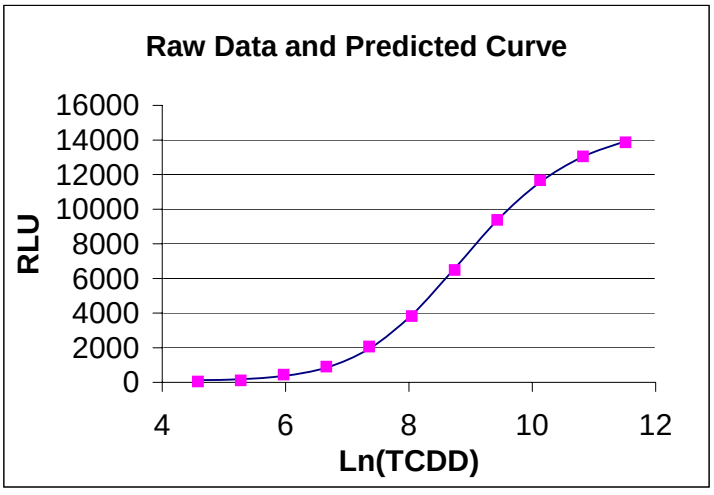
Initial Values (replaced with final estimates by 'Solver')

k	8.9922483
n	9.8105504
v	15042.549
b	100.78584

To fit standard curve, paste TCDD and RLU data into the framed columns below. Then select 'Solver; from the Tools menu. Be sure that the 'Target Cell' is set to **D43**, the 'Equal To' option is set to **min**, and the 'By Changing Cells' option is set to **B8:B11**

TCDD	Ln(TCDD)	RLU
100000.00	11.51	13853.18
50000.00	10.82	13046.78
25000.00	10.13	11672.91
12500.00	9.43	9381.71
6250.00	8.74	6480.18
3125.00	8.05	3818.58
1562.50	7.35	2066.24
781.25	6.66	897.58
390.63	5.97	435.78
195.31	5.27	111.84
97.66	4.58	42.98

Pred	Res	Res^2
13919.75	66.58	4432.26
13036.99	-9.78	95.73
11568.32	-104.59	10939.57
9357.55	-24.16	583.75
6580.49	100.31	10061.56
3887.57	68.99	4759.79
1936.94	-129.30	16719.11
853.06	-44.52	1981.92
365.49	-70.28	4939.97
180.61	68.76	4728.28
120.90	77.93	6072.35
		6.53E+04



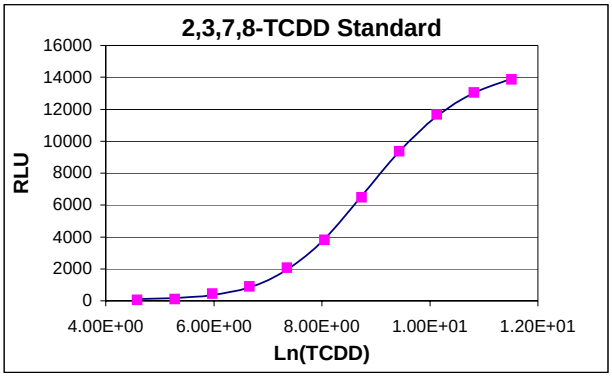
To obtain predicted Ln(TCDD) concentrations from observed RLU data, paste observed RLU values into the framed cells below. These are your predicted concentrations based on the standard curve

Observed Response	Predicted Ln(TCDD)	pg
980.11	6.77386	0.874681
1039.78	6.822278	0.918074
1363.44	7.048033	1.150593
3862.51	8.039923	3.102374
4174.31	8.128638	3.390179
4421.78	8.196694	3.628932
7572.58	8.980196	7.944188
7293.31	8.912427	7.423655
8253.98	9.147964	9.395293
5828.58	8.557404	5.205153
6418.84	8.701257	6.010462
5718.64	8.530309	5.06601
341.11	5.908263	0.368066
3749.98	8.006966	3.001795
4208.18	8.138066	3.422292
451.84	6.145653	0.466684
398.44	6.040917	0.420278
582.71	6.353161	0.574305
268.71	5.693441	0.296914
263.24	5.674053	0.291212
374.64	5.988836	0.39895
1588.18	7.178723	1.311232
1533.11	7.148216	1.271835
1824.38	7.300454	1.480972
4679.31	8.265665	3.888056
3845.64	8.035017	3.087192
4105.58	8.109386	3.325534
2953.78	7.754997	2.333203
3026.31	7.779599	2.391317
3211.64	7.84077	2.542161
237.11	5.572525	0.263098
250.98	5.628357	0.278205
344.51	5.916868	0.371247
532.44	6.280019	0.533799
862.51	6.669828	0.78826
825.18	6.633984	0.760506
2136.31	7.443251	1.708295
1998.31	7.382232	1.607173
2122.91	7.437461	1.698432
1833.51	7.304898	1.487569
1641.58	7.207478	1.349485
1357.78	7.044507	1.146544
0.00	#NUM!	#NUM!
0.00	#NUM!	#NUM!
0.00	#NUM!	#NUM!
0.00	#NUM!	#NUM!
0.00	#NUM!	#NUM!

FIGURE 14

REPORT PRODUCED FROM THE RAW DATA AND CALCULATIONS (FIGURES 12 AND 13)

pg/tube	RLU
1.00E+02	13853
5.00E+01	13047
2.50E+01	11673
1.25E+01	9382
6.25E+00	6480
3.13E+00	3819
1.56E+00	2066
7.81E-01	898
3.91E-01	436
1.95E-01	112
9.77E-02	43



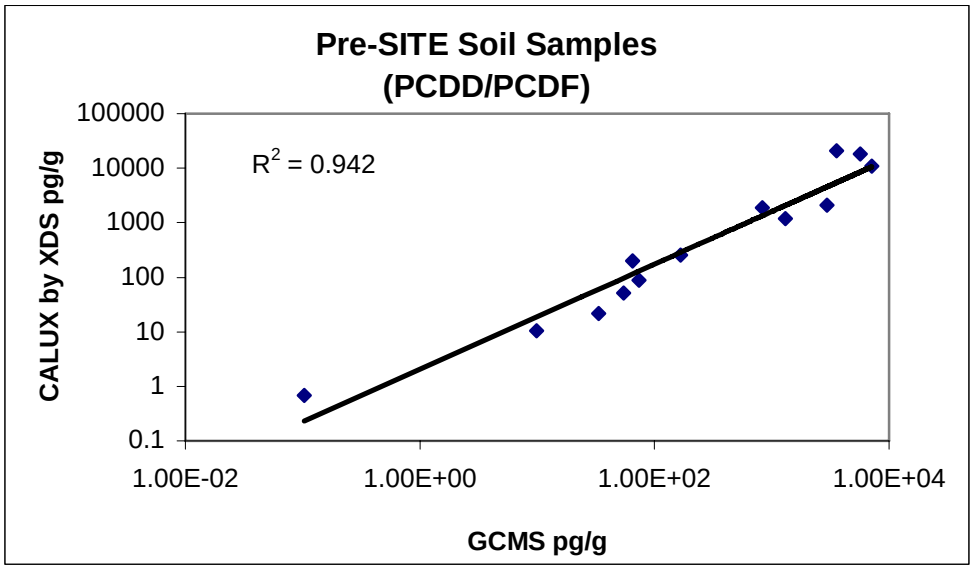
	PCB QC	6.25pg	Ratio
High Level	8081	6480	0.80
Low Level			
	DX QC	**6.25pg**	**Ratio**
High Level	8010	6480	0.81
Low Level			
	DMSO	**mean**	
	726	853	
	1110		
	849		
	726		

Sample	identity	fraction	RLU	TEQ, pg/tube	ppt/sample	corrected	mean	std error	% error
XDS-1	Soil	1:10	980	=calculation!J19-AVERAGE(E$34:E$36)	=E19/(2/10)	=F19/(67/100)	3.69	0.64	17%
XDS-1	Soil	1:10	1040	0.43	2.15	3.22			
XDS-1	Soil	1:10	1363	0.66	3.32	4.95			
XDS-2	Soil	1:10	3863	2.62	6.54	9.76	10.77	0.57	5%
XDS-2	Soil	1:10	4174	2.90	7.26	10.83			
XDS-2	Soil	1:10	4422	3.14	7.85	11.72			
XDS-3	Soil	1:10	7573	7.46	18.64	27.82	28.98	2.20	8%
XDS-3	Soil	1:10	7293	6.94	17.34	25.88			
XDS-3	Soil	1:10	8254	8.91	22.27	33.24			
XDS-4	Soil	1:10	5829	4.72	11.80	17.60	18.43	1.10	6%
XDS-4	Soil	1:10	6419	5.52	13.81	20.61			
XDS-4	Soil	1:10	5719	4.58	11.45	17.09			
XDS QC-1	Matrix QC sample	1:10	341	-0.12					
XDS QC-2	Martix spike sample	1:10	3750	2.51					
XDS QC-3	Soil QC sample	1:10	4208	2.94	29.35	**43.81**			
XDS-5	Method Blank	1:10	452	0.47					
XDS-6	Method Blank	1:10	398	0.42					
XDS-7	Method Blank	1:10	583	0.57					
XDS-1	Soil	1:100	269	0.30	14.85	22.16	24.55	2.61	11%
XDS-1	Soil	1:100	263	0.29	14.56	21.73			
XDS-1	Soil	1:100	375	0.40	19.95	29.77			
XDS-2	Soil	1:100	1588	1.31	32.78	48.93	50.55	2.39	5%
XDS-2	Soil	1:100	1533	1.27	31.80	47.46			
XDS-2	Soil	1:100	1824	1.48	37.02	55.26			
XDS-3	Soil	1:100	4679	3.89	97.20	145.08	128.12	8.86	7%
XDS-3	Soil	1:100	3846	3.09	77.18	115.19			
XDS-3	Soil	1:100	4106	3.33	83.14	124.09			
XDS-4	Soil	1:100	2954	2.33	58.33	87.06	90.38	2.32	3%
XDS-4	Soil	1:100	3026	2.39	59.78	89.23			
XDS-4	Soil	1:100	3212	2.54	63.55	94.86			
XDS-1	Soil	1:500	237	0.26	65.77	98.17	113.50	12.62	11%
XDS-1	Soil	1:500	251	0.28	69.55	103.81			
XDS-1	Soil	1:500	345	0.37	92.81	138.53			
XDS-2	Soil	1:500	532	0.53	66.72	99.59	129.51	15.04	12%
XDS-2	Soil	1:500	863	0.79	98.53	147.06			
XDS-2	Soil	1:500	825	0.76	95.06	141.89			
XDS-3	Soil	1:500	2136	1.71	213.54	318.71	311.81	6.01	2%
XDS-3	Soil	1:500	1998	1.61	200.90	299.85			
XDS-3	Soil	1:500	2123	1.70	212.30	316.87			
XDS-4	Soil	1:500	1834	1.49	185.95	277.53	247.74	18.48	7%
XDS-4	Soil	1:500	1642	1.35	168.69	251.77			
XDS-4	Soil	1:500	1358	1.15	143.32	213.91			

The formula under “TEQ pg/tube” is the TEQ calculation using the data from the calculation (Figure 13). The formula under “pg/g/sample” takes into account the weight (or volume) of the sample and the dilution the sample was analyzed at. The formula under “corrected” takes into account the percent recovery. The corrected values are then averaged for a “mean” and the standard error and percent error are calculated to determine precision.

FIGURE 15

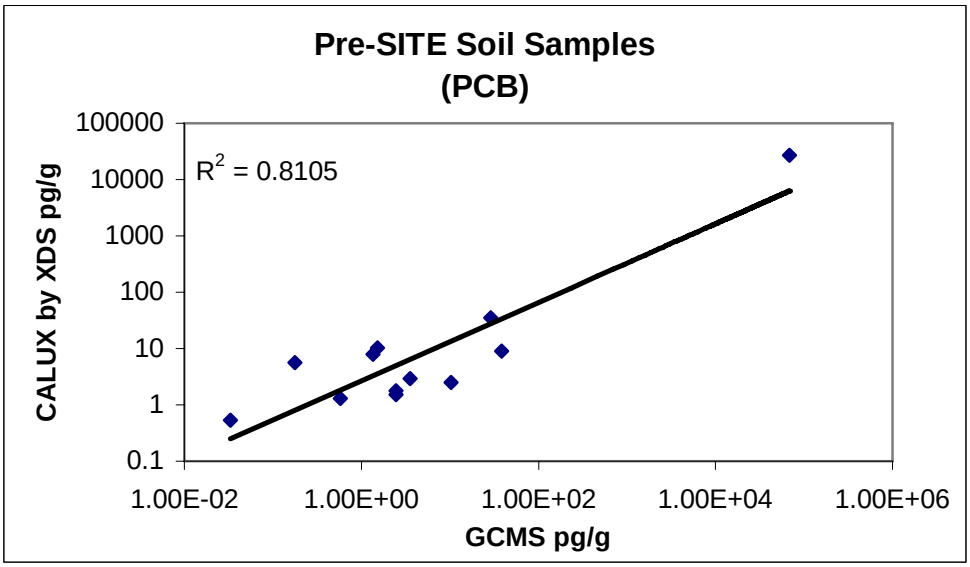
PRE-FIELD TEST COMPARISON FOR DIOXIN / FURAN



Represents the dioxin / furan TEQ comparison of Method 4435 to GC/MS for soil samples for pre-field testing during the EPA SITE field study. These samples were processed in March of 2004 using the screening technique with a surrogate recovery. These TEQs were calculated using the WHO 1997 REP values.

FIGURE 16

PRE-FIELD TEST COMPARISON FOR PCBs



Represents the PCB TEQ comparison of the Method 4435 to GC/MS for soil samples for pre-field testing during the EPA SITE field study. These samples were processed in March of 2004 using the screening technique with a surrogate recovery. These TEQs were calculated using the WHO 1997 REP values.

FIGURE 17

PRE-FIELD TEST COMPARISON FOR INCUBATOR TO AIRTIGHT BOX

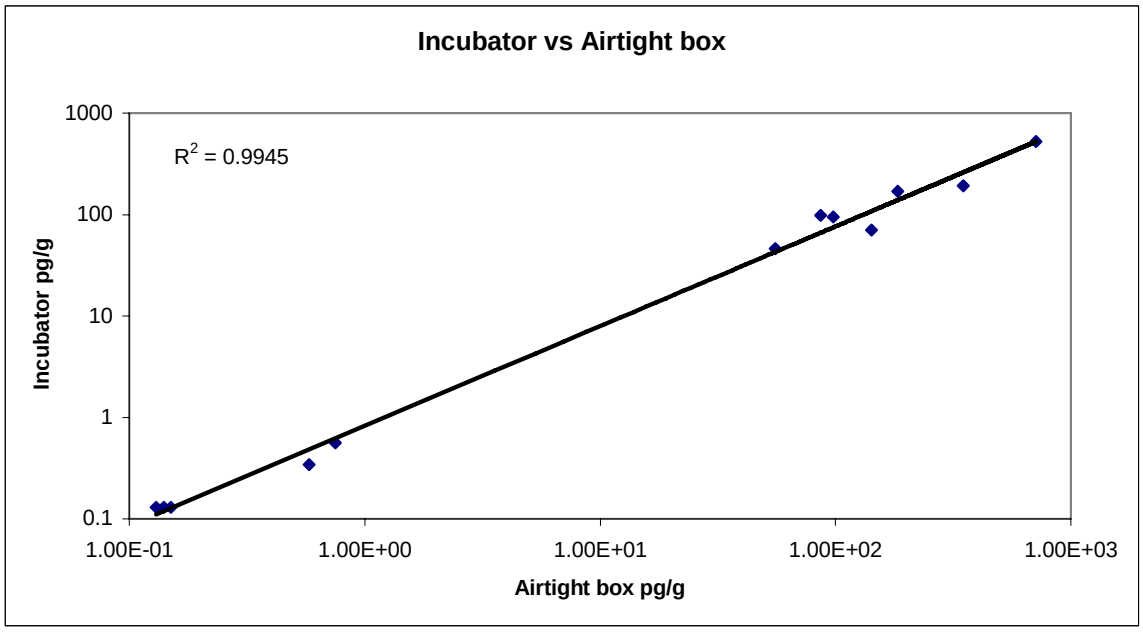


FIGURE 18

SOIL SAMPLE DATA COMPARISON

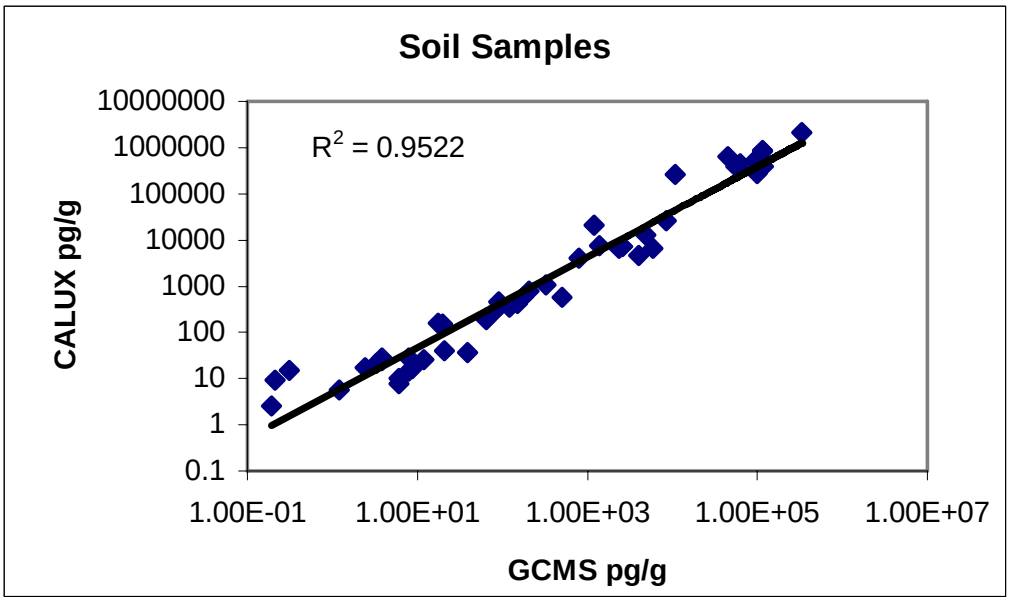


FIGURE 19

ASH SAMPLE DATA COMPARISON

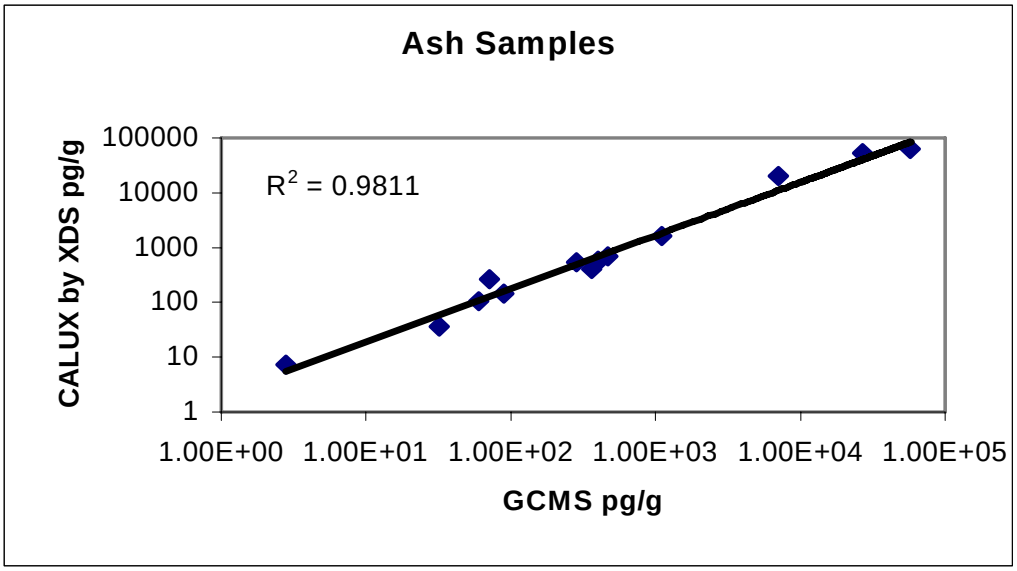


FIGURE 20

EXHAUST EXTRACT SAMPLE DATA COMPARISON

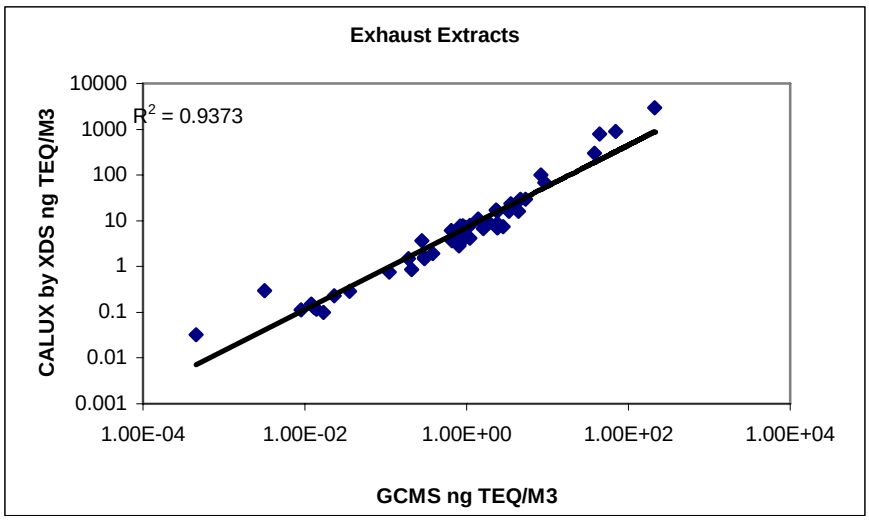


FIGURE 21

SOIL EXTRACT SAMPLE DATA COMPARISON

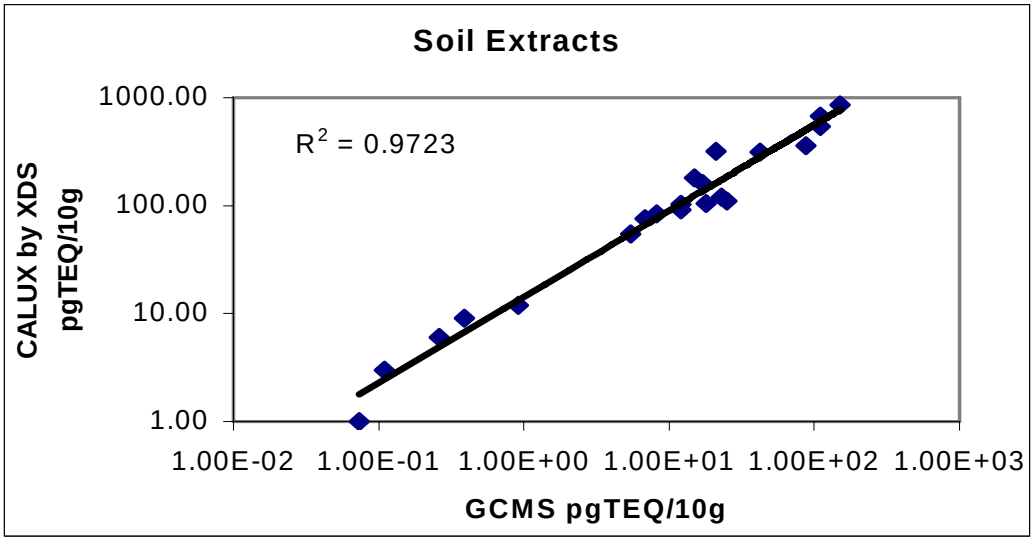


FIGURE 22

ASH EXTRACT SAMPLE DATA COMPARISON

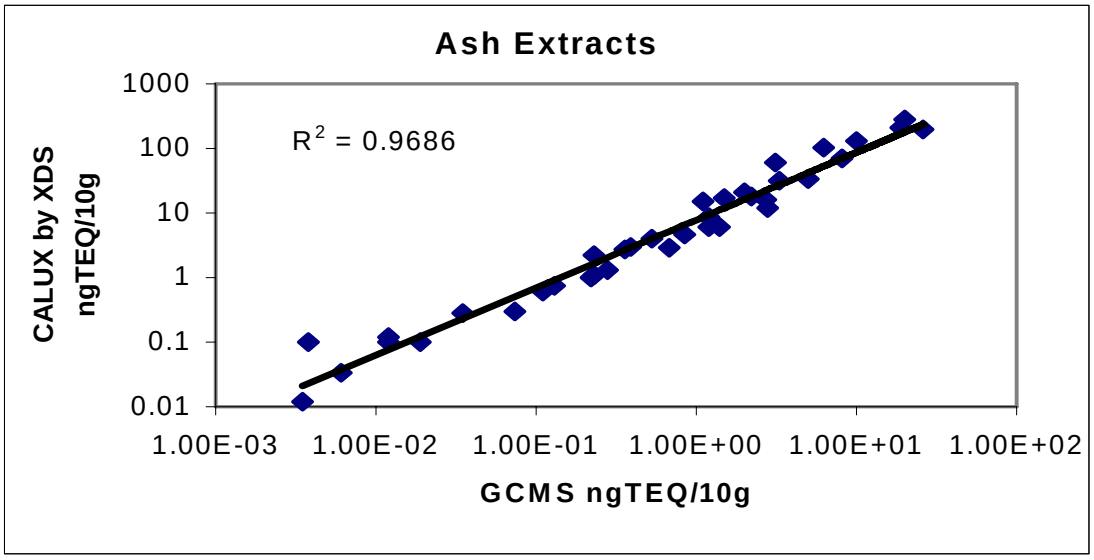


FIGURE 23

STANDARD SOLUTION SAMPLES DATA COMPARISON

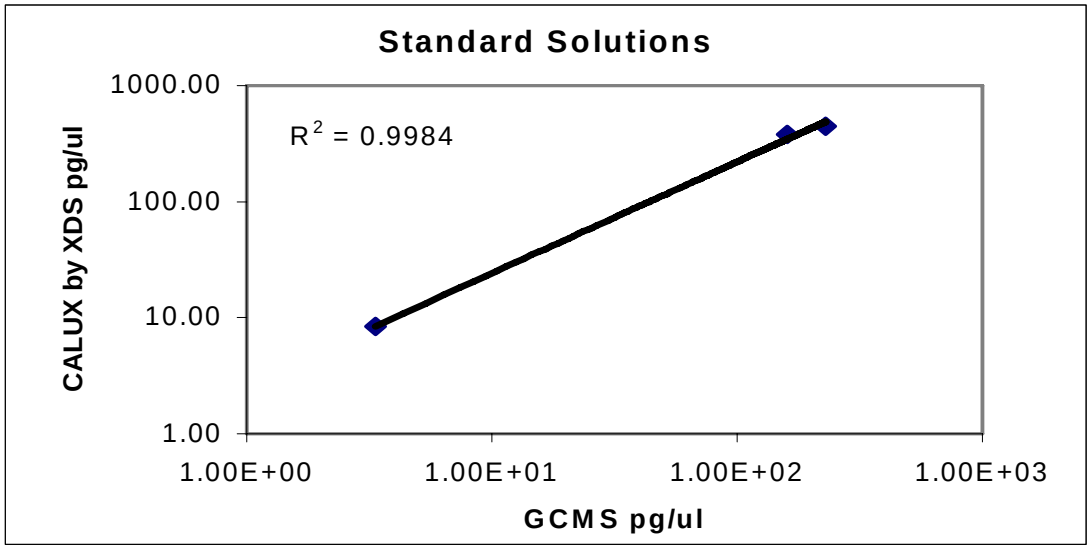


FIGURE 24

ALL SAMPLE MATRICES DATA COMPARISON