CAPE Technologies

High Performance Dioxin/Furan Immunoassay Kit

DF1 Kit Insert (IN-DF1)

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A. Intended Use

The CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit is an Enzyme ImmunoAssay (EIA) for analysis of PolyChlorinated DibenzoDioxins and PolyChlorinated DibenzoFurans (PCDD/Fs) in prepared sample extracts. Samples can be prepared for analysis by EIA using a variety of simple and rapid methods (see Application Notes available from the CAPE Technologies web site http://www.cape-tech.com). Extracts of soil, sediment, food, water, fly ash, stack gas, tissue, or other samples which have been prepared by conventional extraction methods can also be exchanged to a water miscible solvent system for EIA analysis. Please read this kit insert and other related CAPE Technologies literature carefully to gain maximum understanding of the capabilities and limitations of the test. Refer to Technical Notes available from the web site for discussion of technical issues. **Important Note:** Samples analyzed by EIA must contain either no mass-labeled internal standards or immunoassay compatible internal standards. For discussion of issues related to internal standards, consult CAPE Technologies Technical Note TN-001.

B. Background Information

PCDD/Fs are a family of compounds with the same general structure. There are 75 dibenzodioxin congeners and 135 dibenzofuran congeners, containing from 1 to 8 chlorine atoms on the dibenzodioxin or dibenzofuran nucleus. Only 7 of the 75 PCDD congeners and 10 of the 135 PCDF congeners contain the 2,3,7,8 chlorination pattern thought to be required for dioxin-like toxicity. Only these 17 of the 210 total PCDD/F congeners contribute to the Toxic Equivalency (TEQ) of a sample, which is generally the ultimate analytical target. Based on a variety of toxicity data, these 17 congeners have been assigned Toxic Equivalency Factors (TEFs) of 1.0 to 0.0003 relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

The PCDD/F congener composition of samples can be highly variable. Because PCDD/Fs are formed unintentionally by a variety of chemical and combustion processes, samples usually contain a mixture of many different congeners. Samples from different sources often have very different mixtures of congeners that are consistent within the source. In most samples, the majority of the PCDD/F mass present does not contribute significantly to the total sample TEQ. Also, in most samples, only a few PCDD/F congeners are responsible for the majority of the TEQ. This immunoassay is designed to measure sample TEQ by responding to the toxic PCDD/F congeners in correlation with their TEFs. Variation in accuracy among samples may occur solely because of the variability of congener composition noted above. Best kit performance will be obtained when all samples are from a single group that shares as many properties as possible (common source of contamination, similar congener composition, similar sample matrix, etc.). To maximize accuracy, the congener composition of the target sample population should be known. Both screening and quantitative analysis are possible with this kit. Consult the appropriate CAPE Technologies Application Note for details. Please also consult CAPE Technologies Technical Note TN-004 for further discussion of quantitative use of the kit.

C. Test Principles

PCDD/Fs are typically extracted with organic solvents that are incompatible with the EIA. Before introduction of the sample into the EIA, a solvent exchange is required. PCDD/Fs have very low volatility and are retained during this solvent exchange in a small volume of a keeper solution (Triton X-100 detergent in polyethylene glycol [PEG]) after evaporation of the original solvent. Methanol is added to dilute this solution and the methanol-PEG-Triton mixture is added directly to the EIA tubes. It should be noted that the literature value for solubility of 2,3,7,8-TCDD in methanol is 10 ppm, which is 5000 times higher than the concentration of the highest standard recommended for this kit. Additionally, the solubility of PCDD/Fs in methanol is augmented significantly by the addition of PEG and Triton X-100. These factors assure the solubility of PCDD/Fs in the EIA system.

During the first EIA incubation, PCDD/Fs are specifically bound by the anti-dioxin antibodies, which have been immobilized on the EIA tube surface. After washing away the unbound material, the bound PCDD/Fs remain and a competitor-HorseRadish Peroxidase (HRP) conjugate is added. Bound PCDD/Fs occupy the dioxin binding sites of the antibodies in proportion to the PCDD/F content of the sample and prevent binding of the competitor-HRP conjugate. After a short incubation, unbound conjugate is removed and the test tubes are washed thoroughly. The amount of conjugate bound by the anti-dioxin antibody is inversely related to the amount of PCDD/Fs originally present in the sample.

Finally, a solution of chromogenic HRP substrate and hydrogen peroxide is added to the test tubes. Color development is directly proportional to enzyme concentration and inversely related to the PCDD/F concentration in the original sample. The test tubes are analyzed using a tube reader or spectrophotometer to measure the optical density (OD) at 450 nm. The OD values of unknown samples are compared to the OD values of standards to determine the level of PCDD/Fs in the samples.

D. Performance Characteristics

Sensitivity and Reproducibility

Standard curve data developed by CAPE Technologies for the High Performance Dioxin/Furan Immunoassay Kit are given in Table 1. Response values are expressed as a percentage of the negative control, which is 100 ppm Triton X-100 in 80:20 methanol:PEG. The detection limit of the kit is approximately 4 pg 2,3,7,8-TCDD per EIA tube. Results for samples that are compared to the standards shown in Table 1 must be related to the original sample concentration by back calculation using the proper dilution and volume factors. Matrix detection limits will vary according to matrix, sample size, and dilution factor. Consult the appropriate CAPE Technologies Application Note for further information. The data in Table 1 can be used to determine if kit performance is acceptable. If your results for the standards in Table 1 are not consistently within the percent of negative control ranges given, contact CAPE Technologies for assistance.

Table 1: Sensitivity and Reproducibility of the EIA Standard Curve. Data are accumulated responses for 2,3,7,8-TCDD standards over ten months. No sample matrix was present. A total of 41 tests were run in four different labs. The detection limit, which is approximated by the I_{85} or the concentration giving 85% of the negative control OD, was 3.9±1.4 pg/tube (mean±SD). The midpoint of the curve, defined as the I_{50} or the concentration giving 50% of the negative control OD, was 21.9±7.4 pg/tube (mean±SD).

Standard Number	1	2	3	4
ng/mL 2378-TCDD in standard (50 µL per EIA tube)	0.064	0.2	0.64	2
pg 2378-TCDD per EIA tube	3.2	10	32	100
mean percent of negative control (%NC)	87	66	41	29
standard deviation (SD)	6	7	7	6
range of mean±2SD	74-99	51-80	27-55	17-40

Specificity

The anti-dioxin antibody in this kit binds to different PCDD/F congeners with different affinities. The specificity of the test is predominantly for PCDD/Fs that contain 3 to 6 chlorines, with a strong preference for the 2,3,7,8 chlorinated congeners. Test specificity roughly parallels the TEF values of the individual PCDD/F congeners. Crossreactivity data given in the following table are reactivities relative to 2,3,7,8-TCDD.

Table 2: Specificity of the EIA. Response curves were prepared for each congener as noted. The percent crossreactivity = (((2,3,7,8-TCDD I_{50}) ÷ (congener I_{50})) x 100). Values are typically based on 2 to 4 independent curves, each containing at least 4 concentrations.

Compound Toxic Dioxin Congeners	Percent Crossreactivity
2,3,7,8-TCDD	100
1,2,3,7,8-PeCDD	105
1,2,3,4,7,8-HxCDD	1.6
1,2,3,6,7,8-HxCDD	7.9
1,2,3,7,8,9-HxCDD	39
1,2,3,4,6,7,8-HpCDD	0.7
OCDD	<0.001
Toxic Furan Congeners	
2,3,7,8-TCDF	20
1,2,3,7,8-PeCDF	4.6
2,3,4,7,8-PeCDF	17
1,2,3,4,7,8-HxCDF	0.4
1,2,3,6,7,8-HxCDF	1.0
1,2,3,7,8,9-HxCDF	3.3
2,3,4,6,7,8-HxCDF	4.9
1,2,3,4,6,7,8-HpCDF	0.02
1,2,3,4,7,8,9-HpCDF	0.9
OCDF	<0.001

Compound Other PCDD/F Congeners	Percent Crossreactivity
2,3-dichlorodibenzo-*p*-dioxin	0.13
2,7-dichlorodibenzo-*p*-dioxin	0.003
2,3-dichlorodibenzofuran	0.02
2,7-dichlorodibenzofuran	<0.002
2,3,7-trichlorodibenzo-*p*-dioxin	24
2,3,8-trichlorodibenzofuran	0.26
1,2,3,4-TCDD	<0.001
1,2,3,4-TCDF	<0.001
1,3,6,8-TCDD	0.05
1,3,6,8-TCDF	0.007
PolyChlorinated Biphenyls	
3,3',4,4' (PCB 77)	0.4
3,3',4,4',5 (PCB 126)	0.5
2,2',4,4',5 (PCB 153)	<0.1
3,3',4,4',5,5' (PCB 169)	<0.1
Aroclor 1254	<0.1

E. Precautions

- **Important: Please read the following precautions carefully.**
- **Follow precautions and instructions in this insert to achieve the best results.**

Safety:

- This kit should only be used by properly trained personnel in an appropriate laboratory environment.
- Treat PCDD/Fs, solutions that contain PCDD/Fs, and potentially contaminated samples as hazardous materials.
- Use gloves, proper protective clothing, and means to contain and handle hazardous material where appropriate.
- Obtain (if appropriate) permits pertaining to the handling, analysis and transport of dioxin-containing materials.
- **Stop solution is 1N hydrochloric acid. Handle carefully.**

Storage and Use of Kit:

- **Do not freeze test kit components or expose them to temperatures greater than 37°C (99°F).**
- **If desiccant in tube bag is not blue, do not use kit; contact CAPE Technologies.**

- **Do not expose substrate to direct sunlight.**
- If substrate is blue before adding to EIA tubes, do not use; contact CAPE Technologies.
- Store all test kit components at 2°C to 6°C (36 °F to 43°F) when not in use.
- Storage at ambient temperature (20°C to 25°C or 68°F to 77°F) on the day of use or overnight before the day of use is acceptable. Do not store at ambient temperature for extended periods.
- ***Allow all reagents to reach ambient temperature (20°C to 25°C or 68°F to 77°F) before beginning the test. This typically requires at least 60 minutes at ambient temperature to warm from recommended storage conditions. Warming will occur faster if bottles and tube bags are removed from the kit box.***
- Do not use test kit after the expiration date.
- Do not use components from one test kit with components from a different test kit.

Sample Preparation:

- ***Water immiscible solvents in sample extracts must be evaporated completely before diluting in methanol for the EIA.*** Residual solvents or excessive oil may cause precipitation when the sample is added to the EIA tubes. If this occurs, the test result may be invalid and the cause should be corrected before repeating the analysis. Quality assurance methods such as comparison of spiked and unspiked sample extracts are essential for determining the validity of such results. Contact CAPE Technologies for assistance with selection of appropriate quality assurance methods.
- ***Because this EIA recognizes many of the congeners in mass labeled internal standard mixtures designed for GC-MS methods, EIA samples must contain either immunoassay compatible internal standards or no internal standards.*** For specific recommendations regarding mass labeled internal standards, consult CAPE Technologies Technical Note TN-001.

EIA Protocol:

- ***When adding standards and samples to the EIA tubes, the methanol solutions must be dispensed directly into the liquid and not above the liquid surface or onto the side of the tube. Each EIA tube must be mixed briefly as soon as the sample or standard is added, until the tube contents appear uniform.***

Interpretation of Results:

- Consult the appropriate CAPE Technologies Application Note for proper interpretation of results.
- Distribution of PCDD/Fs in samples may vary greatly. The analyst is responsible for adequate frequency, distribution, and homogenization of samples.
- **Proper quality assurance is the responsibility of the analyst and is essential to analytical success. Please read Section N of this insert carefully before proceeding.**

F. Materials Provided

The contents of this kit are described in a separate **Materials List** (ML-DF1-12 or ML-DF1-60).

G. Materials Supplied by the User

- Sample preparation supplies and equipment (CAPE Technologies Sample Preparation Kits contain vials and other supplies for rapid preparation of soil, food, or other samples). You must consult the relevant Application Note for equipment, reagents, and supplies required for your application. CAPE Technologies Equipment Lists EL-001 and EL-002 provide helpful information on equipment and supplies needed. Visit the CAPE Technologies web site (www.cape-tech.com) or contact CAPE Technologies if additional technical assistance is required.
- The remainder of this list gives only those materials needed for a generic solvent exchange and the EIA portion of the analysis.

- HPLC or analytical grade methanol for solvent exchange
- Sample evaporation system for solvent exchange (nitrogen or other gas source)
- Glass tubes or vials for solvent exchange
- Glass vials (1-2 mL) with Teflon lined caps for storage of standards after opening (if using standards in ampoules)
- 1 variable volume glass capillary positive displacement pipettor for dispensing 50 to 100 µL of standards, samples, and keeper (Drummond 275 or equivalent)*
- 1 Eppendorf Repeater or Repeater Plus Pipettor and minimum of 6 tips (one 0.5 or 1 mL, four 10 or 12.5 mL, and one 50 mL), for pipetting 50 µL to 1.0 mL volumes (or equivalent repeating pipettor)*
- Portable photometer or other means for measuring OD of finished immunoassay tubes at 450 nm (alternatives include conventional spectrophotometers, other tube readers, or microplate readers)*
- Marking pen
- Watch or timer
- Reagent grade or bottled distilled water for tube washing
- Basin or other system for capture and disposal of wash water and other waste liquids.

* see Recommended List EL-001 from the CAPE Technologies web site for more information

H. Suggestions for Pipettor Use

- ***Please read these suggestions carefully before performing your first EIA.***
- Use empty tubes and extra tips to practice your pipettor technique before analyzing samples. For the glass capillary positive displacement pipettor, use methanol; for the Repeater pipettor, use water.
- Use a different tip for each reagent dispensed with the Eppendorf Repeater Plus Pipettor to avoid reagent cross-contamination, especially between conjugate and substrate. Label four 10 or 12.5 mL tips “Sample Diluent”, "Conjugate", "Substrate", and "Stop".
- Draw the desired reagent volume into the Repeater pipettor and dispense at least one portion of reagent back into the container to properly engage the ratchet mechanism. If this is not done, the first volume delivered may be inaccurate.
- When adding reagents to the EIA tubes using the Repeater pipettor, direct the liquid stream down the side of the tube slightly below the rim to avoid splashback.
- ***When using the glass capillary positive displacement pipettor for adding samples or standards to EIA tubes, the solution must be dispensed directly into the liquid in the tube, not above the liquid level or onto the side of the tube. The tubes must be individually mixed immediately after the addition of methanol-PEG-Triton solutions to distribute the analyte evenly and to avoid locally high or low concentrations at the antibody coated surface of the tube.***
- When using the glass capillary positive displacement pipettor for standards and samples, the following approach (as for a GC autosampler) has proven effective. After each pipetting operation, repeatedly rinse both inside and outside of the capillary by pipetting several methanol aliquots to a waste container. When changing capillaries, rinse the plunger to minimize carryover. ***If capillaries are not changed after each standard, any liquid on the outside of the capillary must be removed to avoid contamination of the standard with water.***

I. Preparation of PCDD/F Sample Extracts for EIA by Solvent Exchange

1. **Prepare keeper working solution from stock**: Locate the 8 mL vial labeled "PEG-Triton Keeper Stock" and stand upright for several minutes (or centrifuge briefly) to allow PEG-Triton mixture to drain away from lid. Make working solution of keeper by adding 6.0 mL of analytical grade methanol to the vial and mixing thoroughly. This solution of 100 ppm Triton X-100 in 80/20 methanol/PEG will be used for adding keeper to extracts prior to solvent exchange.

2. **Select evaporation protocol and keeper volume for solvent exchange**: If you have selected an Application Note, follow its instructions on sample preparation, including evaporation protocol and keeper volume. If not, you must first decide which evaporation protocol and keeper volume will be used. Three options are detailed below in Table 3. Your choice among these depends on the amount of sample available, whether you need replication in the EIA (not required except for periodic QA), and possible need for dilutions to accurately quantitate very high samples. For example, Option A is often specified for high pg/g soil screening because only a small fraction of the extract prepared is actually processed through the cleanup procedure. In contrast, high sensitivity applications, such as low pg/g food and feed screening, use Option B because it wastes the least possible sample. Option C is intermediate between A and B and allows for dilutions, but not replicates. Note that Option B can also be used for high pg/g soil screening, but at a cost of reduced flexibility.

   Table 3. Details of Protocols for Sample Evaporation and Reconstitution. All volumes are in µL.

Evaporation Protocol Option	A	B	C
Amount of sample available	not limited	limited	either
Number of EIA replicates possible	2	1	1
Extra volume available for sample dilutions	≈30	0	≈30
Working Keeper added to sample tube before evaporation	150	62.5	100
Residue left after evaporation (20% of initial keeper volume)	30	12.5	20
Methanol added to residue to dilute for EIA addition	120	50	80
Percentage of starting sample introduced to EIA tube	33%	80%	50%

3. **Select sample size for extraction and cleanup**: If you have selected an Application Note, follow its instructions on sample size for extraction and cleanup. If not, you must determine these factors before proceeding. The sample size is determined by both decision level and the evaporation protocol (Table 3). This kit is designed to make screening decisions at a level selected by the user. The best performance occurs when this decision level falls in the middle part of the EIA standard curve (Table 1), where precision and accuracy are highest. The following rule of thumb should be applied in unknown situations. The amount of sample (in g) to be introduced to the EIA tube after cleanup = 20 pg (the approx. standard curve midpoint in pg) ÷ decision level (in pg/g). For example, to make a decision at 100 pg/g in soil, an amount of prepared sample equivalent to 0.2 g of soil should be introduced to the EIA tube (20 pg ÷ 100 pg/g = 0.2 g). This value is then divided by the percentage in the last line of Table 3 to determine the amount of sample to be introduced into the cleanup. For example, the 100 pg/g decision level with Option A would require 0.6 g of soil extract (0.2 g/0.33 = 0.6 g or 33% of 0.6 g = 0.2 g), while Option B would require 0.25 g of soil extract (80% of 0.25 g = 0.2 g). Both would deliver the same 0.2 g of sample to each EIA tube, but A would use more sample in the cleanup. In such cases, the amount of sample extracted should be at least 5 g to minimize sample heterogeneity and only a small portion of the extract would be applied to the cleanup protocol. Analysis of samples at very low levels requires correspondingly larger sample sizes. For example, analysis of water at 30 ppq or 30 pg/L [≈0.03 pg/g] and using Table 3 Option B would require ((20 pg ÷ 0.03 pg/g) ÷ 0.8) = 833 g [0.83 L] of sample to be extracted. **Important Note:** This calculation assumes 100% recovery through the sample preparation procedure. This includes assumptions of 100% for both sample extraction efficiency and extract cleanup recovery. Some situations may require increased sample input to adjust for reduced analyte recovery.

4. **Perform solvent exchange**: Use a clean glass tube for evaporating each sample to be analyzed. Add the amount of methanol-PEG-Triton keeper solution determined above to each evaporation tube. Add sample in volatile solvent such as toluene, hexane, isooctane, or acetone. Solvents with boiling points

higher than toluene (111°C) should be avoided if possible. Evaporate solvent completely under a dry gas stream such as nitrogen. Compressed air or other gases can be used, but **it is critical that the gas stream be completely free of oil.** Recovery will not be adversely affected if the sample remains under the gas stream for a few minutes after complete evaporation of the solvent. **The original solvent must be completely gone- there should be no solvent odor.** Application of heat is required for removal of higher boiling solvents, but the temperature should be well below the boiling point of the solvent (e.g. no more than 40°C for hexane or 80°C for toluene). During solvent exchange the PEG and Triton X-100 function as a "keeper", similar to the conventional use of dodecane or tetradecane. It is essential that the gas flow rate be kept as low as possible to avoid aerosol formation (which can lead to loss of analyte). When the original solvent is completely evaporated, the PCDD/Fs stay in solution in the PEG-Triton and are easily diluted with methanol.

5. **Reconstitute sample**: Centrifuge the evaporation tubes for 2 minutes at 1-2000 x g to collect the residual PEG-Triton in the bottom of the tube. Add the volume of methanol to each tube that is indicated by Table 3 above or by your Application Note (equal to 80% of the original keeper volume used, replacing the evaporated methanol). Mix vigorously for 15 seconds to dissolve the keeper and sample completely. Sonication or longer mixing times are not necessary. Pipet the redissolved sample into the EIA tube which has been prepared, then mix immediately (as noted in Section J below). **Perform this sample dilution and EIA loading procedure in batches of 4 or fewer samples. This will minimize changes in sample concentration due to evaporation of the methanol.**

J. EIA Analysis of Standards and Prepared Samples

Prepare samples according to the directions above in "Preparation of PCDD/F Sample Extracts by Solvent Exchange" or in the appropriate Application Note. The following steps explain how to analyze your prepared samples using the CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit. For quick reference, a summary of this protocol is provided on a separate sheet (PS-DF1). The number of tubes per run should be limited by the amount of time it takes to add **Competitor-HRP Conjugate** in step 9 below, and is typically 20 or fewer. This is the largest batch size that can be done on one filling of the 10 mL tip of the Eppendorf Repeater Plus pipettor. Follow precautions in Section E above. **Do not expose Substrate to direct sunlight.** The kit bottles containing the Sample Diluent, Competitor-HRP Conjugate, HRP Substrate, and Stop Solution are overfilled approx. 10-20%. After dispensing each of these reagents to the EIA tubes, return the unused portion of each reagent to its original bottle so there will be enough for subsequent tests.

1. **Warm reagents**: Bring all reagents to ambient temperature as described in Section E (Precautions, Storage and Use of Kit). Before use, mix all reagents briefly by gently inverting several times.
2. **Prepare wash 1**: Locate the vial labeled "0.5 mL neat Triton X-100". Make a wash solution of 100 ppm (0.01% v/v) Triton in reagent grade or bottled distilled water by adding 10 µL of Triton X-100 to 100 mL of water and mixing thoroughly (this will typically take several minutes on a magnetic stirrer). This amount is sufficient for 20 tubes (20 tubes x 4 washes per tube x 1 mL/wash/tube = 80 mL nominal). This wash can be prepared in larger volumes and stored at room temperature.
3. **Prepare tubes**: Place the anti-Dioxin **antibody coated tubes** in the rack and label them. Put the standard tubes first, from low to high concentration, then the sample tubes.
4. **Prerinse tubes**: **Rinse** tubes once by filling each tube with reagent grade or bottled distilled **water.** Dump water out and tap inverted tubes on absorbent material to remove excess water.
5. **Add sample diluent**: Insert the 10 or 12.5 mL pipet tip labeled "sample diluent" into the Repeater pipettor and set volume to 500 µL. Dispense one **500 µL** aliquot from bottle of "**Sample Diluent**" into each tube.
6. **Add standards**: Using a glass capillary positive displacement pipettor, pipet **50 µL** of **standard** solution into each EIA standard tube. ***The solutions must be dispensed directly into the liquid and not above the liquid surface or onto the side of the tube.*** **Immediately after addition, mix each tube briefly until appearance is homogeneous**. The mixing should be vigorous enough to visibly swirl the liquid around the bottom of the tubes.

7. **Add samples**: Using a glass capillary positive displacement pipettor, pipet **50 µL** of **prepared sample** into each EIA sample tube. ***The solutions must be dispensed directly into the liquid and not above the liquid surface or onto the side of the tube.*** **Immediately after addition, mix each tube briefly until appearance is homogeneous**. Mix the rack of tubes by shaking for 10 seconds after adding the last sample. The mixing should be vigorous enough to visibly swirl the liquid around the bottom of the tubes. Incubate at room temperature for 2 to 24 hours. For longer incubation times, cover the rack of tubes or place in a closed plastic bag or other airtight container with limited headspace. The amount of time taken for addition of negative control, standard and sample has little effect on the results because of the long sample incubation. (It is preferred to incubate overnight at this point rather than 2 hours because of the slight improvement in sensitivity [up to two-fold] with the longer incubation). Also, results may be affected by proportionally higher variations in incubation time among samples, due to the sample addition process.

8. **Wash 1**: Dump or aspirate the EIA tube contents into a suitable waste container. Tap inverted tubes on absorbent material to remove excess liquid. Insert a 50 mL pipet tip into the Repeater pipettor and set volume to 1.0 mL. Dispense one **1 mL** aliquot of **100 ppm Triton X-100 in water** (made in step J2 above) into each tube. Dump or aspirate the EIA tube contents into a suitable waste container. **Repeat** this wash step three more times for a **total of 4 washes**. Be certain to shake or tap out as much wash solution as possible on each wash, especially the last one.

9. **Add conjugate**: Insert the 10 or 12.5 mL pipet tip labeled "conjugate" into the Repeater pipettor and set volume to 500 µL. Dispense one **500 µL** aliquot of "**Competitor-HRP Conjugate**" into each tube. Incubate tubes at room temperature for 15 minutes. Timing for this step is the most important of the EIA steps. Rapid and accurate addition of conjugate and consistent incubation times are necessary to maintain equal treatment within and among runs.

10. **Wash 2**: **Repeat** the **wash** procedure described in step 8 above *except use reagent grade or bottled distilled water with no detergent added*.

11. **Add substrate**: Insert the 10 or 12.5 mL pipet tip labeled "substrate" into the Repeater pipettor and set volume to 500 µL. Dispense one **500 µL** aliquot of "**HRP Substrate Solution**" into each tube. Incubate at room temperature for 30 minutes.

WARNING: Stop solution is 1N hydrochloric acid. Handle carefully.

12. **Add stop solution**: Insert the 10 or 12.5 mL pipet tip labeled "stop" into the Repeater pipettor and set volume to 500 µL. Dispense one **500 µL** aliquot of "**Stop Solution**" into each tube. The Stop Solution converts the developed color to yellow. If Stop Solution is not added, all tubes will eventually turn dark blue. **Read** the tubes as soon as possible after stopping; the yellow color is stable for only 30 minutes.

13. **Read OD values**: Wipe dry the outside of each EIA tube, then read and record the absorbance (optical density [OD]) of each tube at 450 nm. This can be done using a specialized tube reader, conventional spectrophotometer, or microplate reader. Consult the Recommended Equipment List (EL-001) for further information.

K. Interpretation of the Results

1. After each EIA run, calculate for each standard and sample the %NC value (OD as a percent of the negative control OD). The %NC values for standards should be compared to Table 1 to determine if the EIA has been performed properly. Refer to Table 3 and/or the appropriate Application Note for instructions on interpretation of sample results. Contact CAPE Technologies if additional assistance is required regarding interpretation of results.

2. The CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit is designed primarily for screening decisions. Quantitative interpretation of data may be possible in certain situations. Consult the appropriate Application Note and Technical Note TN-004 for discussion of this topic. Quantitative interpretation can be performed using **Calculation Module C** from the CAPE Technologies web site (**www.cape-tech.com**). This Microsoft Excel workbook file contains background information and operating instructions. The method utilizes a four parameter equation designed specifically for immunoassays and used in most commercial immunoassay software. Module C plots the actual data

and the calculated four parameter curve solution, then determines sample concentrations based on the calculated curve solution. It is necessary to use all the standards listed in Section D, Table 1 to produce an acceptable curve fit. Contact CAPE Technologies if additional assistance is required regarding interpretation of results.

L. Limitations of the Procedure

The CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit is designed for screening of samples according to their TEQ by responding to the toxic PCDD/F congeners in approximate correlation with their TEFs. Quantitative interpretation of data may be possible in certain situations. Consult the appropriate Application Note and Technical Note TN-004 for discussion of this topic. Confirmation of positive samples and a portion of the negative samples by GC-MS analysis is strongly recommended. Other quality assurance methods and samples should be used at all stages of sample preparation and analysis.

The distribution of PCDD/Fs can be extremely heterogeneous. Adequate sample number, distribution, and homogeneity are the responsibility of the analyst. Quality assurance samples to verify method performance are essential.

Samples that appear heterogeneous during the first incubation (step J7 above) are invalid due to phase separation. Adequate sample cleanup based on the protocol in the chosen Application Note must be assured by the analyst. Contact CAPE Technologies if this is a recurrent problem.

Samples analyzed by EIA must contain either immunoassay compatible internal standards or no internal standards. Consult Technical Note TN-001 and Calculation Module D for discussion and other assistance.

To ensure accurate and reliable results, every effort should be made to perform the CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit at temperatures between 20°C (68°F) and 25°C (77°F).

M. Storage

- Store all test kit components at 2°C to 6°C (36 °F to 43°F) when not in use.
- Do not expose test kit components to temperatures greater than 37°C (99°F). Storage at ambient temperature (20°C to 25°C or 68°F to 77°F) on the day of use is acceptable. Prolonged exposure (many days) or repeated exposure to ambient temperatures may cause a loss of reagent activity (especially conjugate), resulting in decreased OD values for all tubes.
- **Do not freeze test kit components. Kits which have been frozen must not be used.**
- **If desiccant in tube bag is not blue, do not use kit; contact CAPE Technologies.**
- Do not use test kit components after the expiration date printed on the kit box label.

N. Quality Assurance (READ CAREFULLY BEFORE BEGINNING)

- It is assumed that 40% of the tubes in each kit will be used for standards and QA (8 of 20 in DF1-12 and 40 of 100 in DF1-60). Replication, check samples, standard reference materials, method blanks, laboratory control samples, matrix blanks, and other QA samples and methods can and should be used with this kit, with the exception of conventional stable isotope labeled internal standards (as noted in the next paragraph). The Starter Packages DF1-ST-A and DF1-ST-B each may include two toluene check samples which can be used to determine if the solvent exchange portion of the sample preparation has been performed properly. Contact CAPE Technologies to obtain additional QA materials.
- Proper quality assurance is the responsibility of the analyst and is essential to analytical success. Your quality assurance plan should include the following items at a minimum: 1) evaporation controls to verify proper solvent exchange, 2) subsample spikes to verify extraction, 3) extract spikes to verify extract cleanup, 4) performance evaluation (PE) samples for method calibration and validation of analyst performance, 5) QA protocol and QA samples sufficient to statistically verify sample homogenization, and 6) GC-MS confirmation of some statistically meaningful fraction of both positive and negative results.

- ***The antibody used in this immunoassay recognizes PCDD/F congeners based on structure, not mass. Therefore, conventional stable isotope labeled internal standards are detected as native material. Typical levels of conventional stable isotope labeled internal standards can not be used with this EIA*.** Please consult Technical Note TN-001 for recommendations on the use of immunoassay compatible mass labeled internal standards. Contact CAPE Technologies if additional assistance is required regarding these issues. Immunoassay compatible internal standards are available from Wellington Laboratories (Guelph, Ontario, Canada).
- Samples which appear milky or opalescent during the first EIA incubation may contain more oil than can be tolerated by the test. These samples should be diluted or cleaned more, then analyzed again by EIA.
- Response values for 2,3,7,8-TCDD standards typically should be in the ranges given in Table 1. Optical Density (OD) values for the zero standard should be in the 0.75 to 1.80 range.
- If an obvious blue color does not develop in the negative control test tube within 10 minutes after adding the substrate solution, the test is invalid and must be repeated. If the problem persists, contact CAPE Technologies.

O. References

Most of the documents cited in this and other CAPE Technologies literature are available from the CAPE Technologies web site at "**www.cape-tech.com**". **Equipment Lists** provide detailed information about the equipment needed for both sample preparation and immunoassay analysis. **Application Notes** describe sample preparation methods for analysis of a variety of matrices at different TEQ levels. **Technical Notes** provide discussion and recommendations pertaining to important technical issues. **Technical References** are papers from technical journals, meetings, or other sources that provide extensive background information about selected topics relevant to immunochemical analysis of PCDD/Fs. Additional questions can be directed to CAPE Technologies using the contact information from the web site.

P. General Limited Warranty

CAPE Technologies, LLC ("CAPE Technologies") warrants the products manufactured by it against defects in materials and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the expiration date printed on the product. **CAPE Technologies MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of CAPE Technologies products appearing in CAPE Technologies' published catalogues and product literature may not be altered except by express written agreement signed by an officer of CAPE Technologies. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, CAPE Technologies' sole obligation shall be to repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies CAPE Technologies promptly of any such defect. The exclusive remedy provided herein shall not be deemed to have failed of its essential purpose so long as CAPE Technologies is willing and able to repair or replace any nonconforming CAPE Technologies product or part. **CAPE Technologies shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by any customer from the use of its products.**