QUALITY CONTROL CRITERIA IMPLEMENTED
FOR MONITORING THE USE OF THE CALUX® BIOASSAY

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Introduction

The CALUX® (Chemically-Activated LUciferase eXpression) bioassay is a reporter gene based assay that has been used to detect the presence of dioxin-like compounds in biological[1] and environmental[2] samples. These studies indicated that the CALUX® assay can be used to detect the presence of dioxin like compounds and is predictive of gas chromatography high-resolution mass spectrometry (GC/HRMS) results. However, bioassays differ from chemical analysis methods, such as GC/HRMS, in that it is not possible to include internal standards for recovery determination. The use of isotopically labeled internal standards would contribute to the total TEQ determination for the sample, making it difficult or impossible to determine both recovery and sample TEQ in the same sample. The lack of internal standards limits the quality control criteria that can be used, it also means that the results should be considered semi-quantitative. We have been using the CALUX® bioassay in our laboratories as both a screening assay and as a semi-quantitative assay for estimating the dioxin TEQ contributions of dioxin-like polyhalogenated biphenyls (PHBs) and polyhalogenated dibenzodioxins/dibenzofurans (PHDD/PHDF). In this document we report the quality control criteria that have been developed in our laboratories to monitor the reproducibility and consistency of results from the CALUX® bioassay.

Materials and Methods

CALUX® Assay: Xenobiotic Detection Systems, Inc. has developed a cell line (mouse hepatoma H1L1) that was stably transfected with a vector that contains the gene for firefly luciferase under transactivational control of the aryl hydrocarbon receptor[3]. This cell line is used in a 96 well plates format to compare sample extracts to a standard curve of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as described previously[2].

Data analysis: Data for the dose response series were fit to a sigmoid curve described by the Hill Equation using least squares best fit modeling.

Results and Discussion

General characterization and validation of the CALUX® bioassay:

The sample clean up method used in our laboratories allows for the isolation of two fractions from a sample extract. The first fraction contains dioxin-like PHBs and the second fraction contains PHDD/PHDF. The method was optimized and characterized by submitting known mixtures of compounds to the clean up method and analyzing the components of the resulting

fractions using gas chromatography with electron capture detection. There are other compounds, such as polyaromatic hydrocarbons (PAH), that can activate the Ah receptor and produce a response in the CALUX® assay. Although PAH are important environmental contaminates we wanted to assure that they did not contribute to the TEQ determinations that we report. Over one hundred compounds, including pesticides, PAH and halogenated organic compounds were tested for activity in the CALUX® assay. Those compounds that were active were treated with our sample clean up method and the resulting extracts were analyzed for activity. Of the compounds tested, only one, chrysene, had significant activity following the clean up method, but it was less than 5% of the pre-clean up activity. This suggested that interference by compounds other than dioxin-like PHB and PHDD/PHDF should not be significant.

Relative Potency (REP) values were determined for 17 active chlorinated dibenzodioxin and dibenzofurans as well as for active dioxin-like polychlorinated biphenyls. The results were very similar to the WHO TEF values[4] for these compounds (results submitted as a separate abstract). These results indicated that based on efficacy of the tested compounds, the CALUX® assay would be more reliable for PHDD/PHDF TEQ determination when the response for the sample was less than 75% of the maximal response for TCDD and for PHB TEQ determination when the response for the sample was less than 50% of the maximal response for TCDD.

Validation of the CALUX® assay versus GC/HRMS analysis has been conducted for various sample types using a blinded study design. Coded samples were analyzed by both methods and the results were sent to an independent statistician for analysis. When completed, this validation study data will be used to define the different validation parameters (repeatability, reproducibility, accuracy, etc.). A portion of these validation studies have been published[1,2]. These studies indicate that there is a strong correlation between the two methods. The results for the CALUX® assay are generally higher for environmental samples, which results in some samples that could be considered false positives, but very few false negatives (less than 5%). The lower TEQ values provided by GC/HRMS could be due to the GC/HRMS result only including chlorinated compounds while the CALUX® assay will also detect brominated and mixed halogenated compounds. This has not been confirmed and further analysis is being conducted to test this hypothesis.

Cross-laboratory validation studies have been initiated to test the reproducibility of results between laboratories. Coded samples are being distributed to the participating laboratories and results will be reported to the independent statistician.

Analytical quality control concepts based on Good Laboratory Practices (GLP) have been incorporated into the development, characterization and subsequent use of the CALUX® assay. Standard Operating Procedures (SOP) have been developed, all equipment used in the storage, preparation and analysis of samples are regularly monitored and data is stored in a manner that assures its integrity and validity.

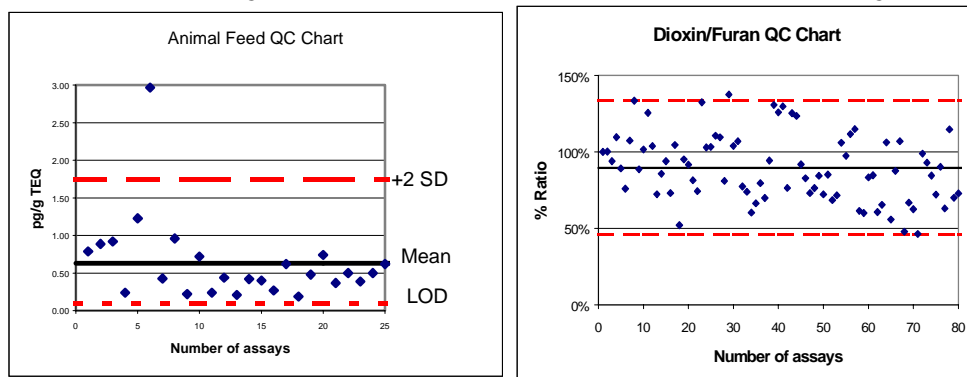*Quality control criteria for use of the CALUX® assay as a screening assay*:
Monitoring of solvents used in sample preparation is conducted on a regular basis. Each lot of solvent is tested by evaporating a 10 ml aliquot of the solvent and resuspending the residue in four microliters of DMSO. The DMSO solution is suspended in cell culture medium and exposed to the cells. A response greater than 5% of the maximal response for TCDD is considered unacceptable. 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 CALUX® activity from any of the solvents or column matrices used in sample preparation.

Reference samples were prepared from appropriate materials that were finely ground (solids) or homogenized (liquids) and analyzed by GC/HRMS for dioxin TEQ. The material was then spiked with an appropriate amount of an equimolar mixture of the 17 active chlorinated dibenzodioxins and dibenzofurans to provide a final dioxin TEQ concentration equivalent to the action level. The reference material was shaken or stirred for three days, aliquotted and an aliquot was analyzed by GC/HRMS. A reference sample is included in each sample batch and is prepared and analyzed using the same method as the unknown samples.

Quality Control (QC) charts are maintained for all reference samples as well as for a standard solution of PCB 126 and a mixture of PHDD/PHDF that are analyzed on each plate (each of these standard solutions produces a response near the middle of the dose response curve). These charts are generally reported as a three-month average (figure 1), however the data for these samples can be monitored over longer time periods to insure against longer term variation in the assay. In QC charts, the results for the standard mixtures are reported as a ratio relative to the 15.6 ppt point of the 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 differ 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.

Figure 1. QC charts for animal feed reference material and Dioxin/Furan standard mixture. Solid line is the 3-month average and dashed lines are two standard deviations from the average.

Quality control criteria for use of the CALUX® assay as a semi-quantitative estimate of dioxin TEQ:
In addition to the quality controls used in the screening assay, the TCDD standard curve is modeled to a sigmoid curve described by the four variable 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 limitations listed above (i.e. PCB fraction response must be less than 50% of 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 (described below) are reanalyzed using appropriate dilutions.

Recovery determination 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 (spiked at approximately 10 times the expected concentration for the sample). Using the radioactively labeled spike the recovery is the percent of the recovered spike versus the amount added to the sample as determined by scintillation counting. For unlabeled spike the recovery is determined by subtracting the TEQ for the sample from the TEQ for the spiked sample and dividing the result by the TEQ for the spike.

Limits of detection are determined based on the y-intercept from the Hill Equation and the standard deviation of the DMSO blanks from the plate. The limits of detection for the plate in relative light units is defined as the y-intercept plus 2.5 times the standard deviation of the DMSO blanks. The limits of detection for the plate in pg of 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.

Conclusions

Parts of the quality control criteria outlined in this document are based on methods for GC/HRMS such as US EPA SW846 method 8290 and 40 CFR part 136 method 1613[5]. However, because the CALUX® bioassay cannot include an internal standard, modifications were necessary. These modifications have been made based on experience using the CALUX® assay in our laboratories. Characterization of the CALUX® assay has been very important to the development of these QC criteria and we expect that the QC criteria will be refined further as additional sample matrices are analyzed.

Acknowledgements

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References

1. Van Overmeire, I., Goeyens, L., Beernaert, H., Srebrnik, S., De Poorter, G., Baeyens, W., Clark, G., Chu, M., Chu, A., Chu, D., Morris, R., and Brown, D. (2000) Organohal. Comp. 45, 196-199.
2. Brown, D., Kishimoto, Y., Ikeno, O., Chu, M., Nomura, J., Murakami, T., Murata, H., (2000) Organohal. Comp. 45, 200-203.
3. Garrison, P.M., Tullis, K., Aarts, J.M.M.J.G., Brouwer, A., Giesy, J.P., and Denison, M.S., (1996) Fundam. Appl. Toxicol. 30, 194-203.
4. Van den Berg, M., Birnbaum, L., Bosveld, A., Brunström, B., Cook, P., Feeley, M., Giesy, J., Hanberg, A., Hasegawa, R., Kennedy, S., Kubiak, T., Larsen, J., Van Leeuwen, R., Djien Liem, A., Nolt, C., Peterson, R., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F. and Zacharewski, T. (1998) Env. Health Perspec. 106, 775-792.
5. EPA method 1613, US Environmental Protection Agency, 40 CFR Part 136.