# CALUX™ RESULTS CORRELATE WITH GC/MS DATA FROM KAZAKSTAN BREAST MILK SAMPLES AND SUPPORT NEW TEF VALUES

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**Introduction**

In 1994, in the first comprehensive investigation of persistent organochlorine contaminants in a country of the former Soviet Union, we measured congener-specific levels of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), as well as 19 organochlorine pesticides (OC) in breast milk samples collected using the WHO protocol from first-time mothers ("primiparae") living in southern Kazakhstan *(1-3).* High levels (up to 80 pg/g fat) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were found in breast milk samples from villages in a cotton-growing region in southern Kazakhstan, with TCDD contributing 70-80% of the I-TEQ (*1-2*). A follow-up study in 1997 measured levels of PCDDs/PCDFs in samples from the region's cotton-growing State Farms *(4).* As part of our continuing characterization of this TCDD contamination, we investigated the effectiveness of the CALUX™ assay to screen and identify breast milk samples with high contaminant levels. In this case, the I-TEQ arises from only two congeners, TCDD and the pentachlorodibenzo-p-dioxin. We report the results of correlation studies on CALUX™ and GC/MS data using these samples..

**Materials and Methods**

***Study design.*** Twenty-one breast milk samples and their organic extracts were used in the correlation studies. The samples were collected in 1997 using the WHO/EURO *(5),* protocol for breast milk sample collection, in which donors were healthy primiparae with healthy infants 2-8 weeks of age. The procedures followed in these exposure assessment studies (design, exposure assessment questionnaire, informed consent, PCDD/PCDF target analytes, analytical methods, and statistical analysis) are described in detail elsewhere *(1-2*).

Split samples of breast milk were analyzed by HRGCMS and by the CALUX™ assay. Results from the two methods were compared using statistical tests.

***Analytical methods.*** Breast milk samples (100 mL) were collected from donors in chemically clean sample jars with teflon-lined caps, frozen immediately, and stored at -20°C until analysis. Samples were thawed, shaken, and weighed, and a 5 gram aliquot was removed for the CALUX™ assay ("milk aliquot"). The remainder was extracted in ethanol:hexane:diethyl ether (2:1:1), and a 10% aliquot was set aside for CALUX™ assay ("extract aliquot"). The remainder of the extract was evaporated to dryness and the residue analyzed by HRGCMS

***HRGCMS.*** Extracts of breast milk samples were spiked with 15 $^{13}C_{12}$-labeled PCDD/PCDF standards and analyzed for congener-specific PCDD/PCDFs in a U.S. Food and Drug

Administration (USFDA) laboratory using quadrupole ion storage tandem MS *(7).* Lipids were determined gravimetrically, and residue levels were expressed as pg/g milk lipid. I-TEQs for PCDDs/PCDFs were calculated using both old and new TEF values *(8,9).*

**CALUX™ *Assay.*** Xenobiotic Detection Systems, Inc. (XDS), has a patented genetically engineered cell line which contains the firefly luciferase gene under trans-activational control of the aryl hydrocarbon receptor (10). The cell line can be used for the detection and relative quantification of PCDDs, PCDFs, and coplanar PCBs when used with our patent pending sample processing procedure (11). The assay using this cell line is called the Chemical-Activated Luciferase expression, or CALUX™ assay**.**

Using the CALUX™ assay, breast milk samples ("milk aliquots") and the set of organic extracts ("milk extracts"), stored at –20° C, were analyzed for total TEQ activity. The "milk aliquots" (5 ml) were transferred to hexane-rinsed glass vials with a PTFE-lined cap and were shaken and extracted three times with a acetone/hexane mixture. The three extracts were pooled and evaporated to dryness under nitrogen, and the remaining residue was weighed to determine organic extractables ("milk lipid"). The residues of "milk aliquots" and "milk extracts" were re-suspended in hexane and cleaned up for the bioassay using our proprietary patent pending clean-up procedure (11).

The sample extracts in DMSO were suspended in cell culture medium, just prior to dosing on monolayers of H1.L1.6 mouse hepatoma cells that were grown in 96-well culture plates. In addition to the dilution of samples, a standard curve of TCDD-concentrations was assayed (128.8, 64.4, 32.2, 16.1, 8.0, 4.0, 2.0, 1.0, 0.5, and 0.1 ppt of TCDD). The plates were incubated for optimal induction of luciferase activity in a humidified $CO_2$ incubator. After the incubation, the media was removed and the cells were microscopically observed for viability. The luciferase activity was quantified using the substrate kit of Promega.

***Statistical Analysis.*** Analytical data were stored in EXCEL 5.0 (Microsoft, Redmond, WA). All statistical analyses were conducted in STATA 5.0 (Stata Corp, College Station, TX). Spearman Rank Correlation was used to assess the similarity of results from the HRGCMS and CALUX™ analytical methods. In addition, qualities of the distributions of the I-TEQ levels from both methods were compared using the Wilcoxon Signed Rank Test.

**Results and Discussion**

From the GC/MS analysis of milk samples, the ITEQs (pg/g fat) were calculated using either the old or new TEFs (PeCDD TEFs of 0.5 vs 1.0, respectively). The CALUX™ assay was run on the provided milk (n = 13) or milk extract samples (n=21). For the 8 extract samples with no milk samples, CALUX™ results were predicted from regression analysis of CALUX™ data from the 13 milk and milk extract samples. Thus, a CALUX™ data set was assembled on milk samples of 21 values, 13 measured and 8 extrapolated.

***Correlation Analysis.*** The relationship between the GC/MS and CALUX™ results was examined by an extensive correlation analysis. The linear relationship between GC/MS and CALUX™ results was stronger when new, rather than old, TEF values were used, and when all PCDD/PCDF congeners were included in the calculation o f the TEQ (Table 1).

**Table 1: Comparison of GC/MS and CALUX**

| | | CALUX | | |
|---|---|---|---|---|
| | | Milk (original n=13) | Milk 13 + 8 predicted (n=21) | |
| GC/MS | ITEQ new TEF (n=21) | 0.7418 | 0.7819 | |
| | ITEQ old TEF (n=21) | 0.6823 | 0.7379 | |
| | TCDD+PeCDD new TEF (n=21) | 0.7363 | 0.7532 | |
| | TCDD+PeCDD old TEF (n=21) | 0.6648 | 0.6857 | |
| | TCDD (n=21) | 0.5942 | 0.6329 | |

***Sensitivity, Specificity, Predictive Value of the* CALUX™ *assay.*** Based upon the distributional characteristics of CALUX results for the 21 milk samples (13 measured and 8 predicted), four cutoff points were selected for analysis of specificity, sensitivity, and predictive value of the CALUX™ assay: 25, 38.12 (CALUX™ median ITEQ), 45, and 60 pg/g fat. Results are summarized in Table 2. The median cutoff gives the best balance of specificity and sensitivity.

**Table 2: Sensitivity, Specificity, Predictive Value of CALUX Assay**

| | Cutoff Point | | | |
|---|---|---|---|---|
| **Estimate of Measure** | 25 pg/g fat | 38.12 pg/g fat (CALUX median) | 45 pg/g fat | 60 pg/g fat |
| Sensitivity | 0.94 | 0.78 | 0.50 | 0.33 |
| Specificity | 0.20 | 0.75 | 0.92 | 1.00 |
| Predictive Value (positive test result) | 0.79 | 0.70 | 0.80 | 1.00 |
| Predictive Value (negative test result) | 0.50 | 0.82 | 0.75 | 0.79 |

**Definitions:**
Sensitivity: P(CALUX>=cutoff | GCMS>=cutoff)
Specificity: P(CALUX<cutoff | GCMS<cutoff)
Predictive value (positive test result): P(GCMS>=cutoff | CALUX>=cutoff)
Predictive value (negative test result): P(GCMS<cutoff | CALUX<cutoff)

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