HIGHLY SENSITIVE CALUX ASSAY FOR SCREENING DIOXINS IN RETAIL FISH

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Introduction

Fishery products have been identified as the main source of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyl (dl-PCBs), collectively referred as to dioxins, in the Japanese diet. It is therefore highly important to develop screening methods for dioxins in retail fish in Japan. Reporter gene assays, such as the chemical-activated luciferase gene expression (CALUX) assay, are currently considered the best screening method for dioxins. A couple of studies have reported on the utility of the CALUX assay as a possible alternative for screening dioxins in fish samples [1-3]. However, since dioxin concentrations in fish samples are typically very low, a more sensitive CALUX assay is useful. The CALUX assay uses a genetically modified cell line that can detect dioxins on the basis of their activation of the aryl hydrocarbon receptor (AhR), which in turn increases the expression of the luciferase reporter gene. The expression of the luciferase gene under the control of the mouse mammary tumor virus promoter is regulated by dioxin responsive elements (DREs, the DNA recognition site of dioxin-bound AhR). Recently, a more sensitive CALUX assay using a new cell line (H1L7.5) has been developed [4]. The H1L7.5 cell line was constructed with a new AhR-inducible reporter plasmid containing an increased numbers of DREs (20 DREs). However, the H1L7.5 cell CALUX bioassay has not yet been used for screening dioxins in fish samples. We have developed a similar amplified cell line (pGL7.3 cell) by stable transfection of mouse hepatoma (hepa1c1c7) cells with AhR-inducible reporter plasmid containing 12 DREs (pGudLuc7.3) [4], and have found that the resulting CALUX assay using this cell line is highly sensitive [5]. Here, we report on the applicability of the new CALUX assay for screening dioxins in retail fish samples.

Materials and methods

Samples: Fish samples were purchased from supermarkets in Tokyo, Japan. The samples (muscular sections) were homogenized using a food cutter and stored at –20ºC until analysis.

CALUX assay: A 10 g sample of homogenized fish was mixed with 15 ml of acetone and mechanically shaken for 5 min. The sample was then extracted with 10 ml of dichloromethane/*n*-hexane (1:2, v/v) by shaking for 2 min. The extract was passed through a layered column with (from the bottom) 1.0 g of celite and 7.3 g of anhydrous sodium sulfate. Each sample was extracted three times, and then passed through the column. After elution with an additional 10 ml of *n*-hexane, the column elute was evaporated to dryness and weighed to estimate the lipid content of the sample. After dissolution in 20 ml of *n*-hexane, then 16 g of 45% (w/w) sulfuric acid-silica gel was added, the supernatant was loaded onto a layered sulfuric acid silica gel column with (from the bottom) 3.4 g of anhydrous sodium sulfate, 0.7 g of 33% (w/w) sulfuric acid-silica gel, and 4 g of 45% (w/w) sulfuric acid-silica gel. The sample was eluted with 20 ml of *n*-hexane, then further loaded onto an XCARB column. After sample addition, the XCARB column was washed with *n*-hexane (10 ml); then the first fraction, containing dl-PCBs, was eluted with *n*-hexane/toluene/ethyl acetate (8:1:1, v/v) (15 ml), and the second fraction, containing PCDD/Fs, was eluted with toluene (20 ml). The elutes were concentrated and resuspended in *n*-hexane (4 ml). A portion of the cleaned-up extract (maximum 1.5 ml of *n*-hexane) was solvent exchanged into 300 µl of MEM culture medium supplemented with 10% (v/v) FBS and 500 µg/ml G418 (the final DMSO concentration in the culture medium was 1%). In addition, 2,3,7,8-TCDD standard solutions (3 µl of DMSO) added to 1 ml of *n*-hexane were prepared using vacuum centrifugation and diluted with the culture medium (300 µl). The pGL7.3 cells (7.5×10^4/well) were cultured in the culture medium in 96-well plates at a temperature of 37ºC, in the presence of 5% CO_2. After incubation for 20-24 h, the cells were exposed in triplicate (95 µl/well)

for samples. The induced luciferase activity was determined with the luciferase assay system using a Centro LB 960 Microplate Luminometer (Berthold Technologies) or an EnSpire® Multimode Plate Readers (PerkinElmer) and expressed in terms of relative light units (RLUs). Average RLUs were calculated from individual RLUs obtained from triplicate wells for samples and standards. All RLU values were corrected by subtraction of the RLUs from the blanks (standard wells containing no 2,3,7,8-TCDD). Fitting of standard curves and calculation of dioxin contents in fish samples were performed using a four-variable Hill equation.

HRGC/HRMS analysis: Extraction, cleanup, and analysis of dioxins were performed in accordance with a previously reported protocol using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) [6]. The limits of detection (LODs) for PCDD/Fs were 0.01–0.05 pg/g. The LODs for dl-PCBs were 0.1 pg/g for non-*ortho* PCBs and 1 pg/g for mono-*ortho* PCBs. The toxic equivalent (TEQ) concentrations were calculated using WHO toxic equivalency factors (2005). In calculating the total TEQ concentration in a sample, it was assumed that all isomer concentrations lower than the LODs were equal to zero.

Results and discussion

Standard curve of the CALUX assay: Figure 1 shows the standard curve of the pGL7.3 CALUX bioassay, derived from five individual assays performed on separate days. The curves were highly reproducible, as indicated by the low SDs. The mean concentration, which resulted in 50% of the maximal response for 2,3,7,8-TCDD (EC50), was approximately 7.6 pg/ml. The minimum concentration of 2,3,7,8-TCDD detectable by the CALUX assay (defined as 3 SDs above the mean RLU value for the zero standards) was approximately 0.24 pg 2,3,7,8-TCDD/ml (approximately 0.19 pg 2,3,7,8-TCDD/assay). To determine the limit of quantification (LOQ), 8 concentrations of 2,3,7,8-TCDD (0.49 to 31 pg/ml) were assayed in triplicate on 5 different days. From 0.24 to 31 pg 2,3,7,8-TCDD/ml, the residual errors were small (-3.3 to 3.2%), and the coefficients of variation (CVs) over the same range were also low (0.8 to 6.0%). Therefore, the assay's quantification range was determined to be from 0.49 to 31 pg/ml for the PCDD/Fs and dl-PCBs fractions. The 0.49 pg/ml quantification limit corresponded to 0.039 pg 2,3,7,8-TCDD/g, when a 10 g fish sample was tested. The quantification limit was 2 times lower than a conventional CALUX assay using the H1L6.1 cell line [2].

Effect of fish matrix on the CALUX assay: To examine the matrix effect for the CALUX assay under the sample preparation procedure, two-fold serial dilutions of the cleaned-up extracts of the fish samples were tested in the CALUX assay. Three different fish extracts were diluted serially with *n*-hexane and assayed. As shown in Figure 2, the values observed for the PCDD/Fs and dl-PCBs fractions in these samples tended to increase with dilution. This indicates that the dilution process might eliminate the matrix effects in the CALUX assay. For this reason, serial dilutions of fish extracts (dilution factors of 1, 2, 4, and 8) were measured in the CALUX assay, and the maximum concentration was used to reduce the rate of false-negative results.

A recovery test using the fish samples was also carried out to examine the effect of the matrix on the CALUX assay. Fish samples were spiked with known concentrations of PCDD/Fs and PCB 126, extracted, cleaned-up, and assayed with the serial dilution procedure. Acceptable recoveries (74 to 95%) were obtained over the tested range for two varieties of fish analyzed (Table 1).

Reproducibility of the CALUX assay: The reproducibility of the CALUX assay using the sample preparation procedure described above was tested by analyzing replicate samples from the same fish. The samples were extracted, cleaned-up, and assayed in five separate analyses on different days. The CVs for two varieties of fish samples were 17 to 21% for PCDD/Fs and 12 to 23% for dl-PCBs (Table 2). The CALUX assay therefore has acceptable precision for dioxin analysis. However, the ratio of the minimum to maximum concentrations of dl-PCBs in mackerel was nearly 1:2. To reduce the impact of variation, the dioxin concentration determinations reported for the CALUX assay were the mean of two independent sample preparations analyzed in duplicate.

Comparison of the CALUX assay with HRGC/HRMS analysis: The TEQ concentrations of PCDD/Fs and dl-PCBs in 32 retail fish samples were determined by both the CALUX assay and HRGC/HRMS analysis (Figure 3). The correlation coefficients between the two methods were 0.94 for the PCDD/Fs and 0.96 for the dl-PCBs. The high correlation between the results obtained by the two methods suggested that the CALUX assay would be a useful screening method for predicting TEQ concentrations in fish.

In conclusion, the present study evaluated a new CALUX assay using the pGL7.3 cell line for determination of dioxin concentrations in retail fish. The assay would be a useful dioxin TEQ screening method prior to HRGC/HRMS analysis.

Acknowledgements
This work was supported by a Health Sciences Research Grant from the Ministry of Health, Labour, and Welfare of Japan and a grant from the National Institute of Environmental Health Sciences of the United States (ES04699).

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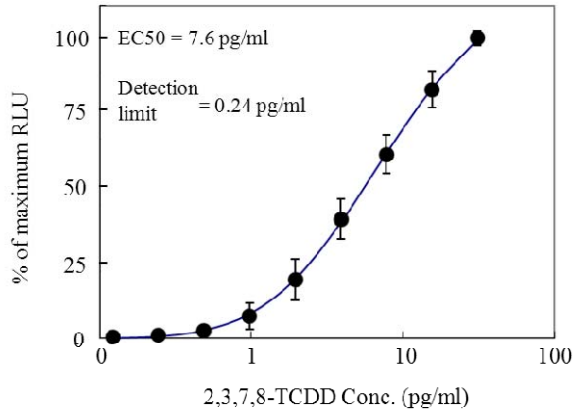

Figure 1. Standard curve for the CALUX assay.

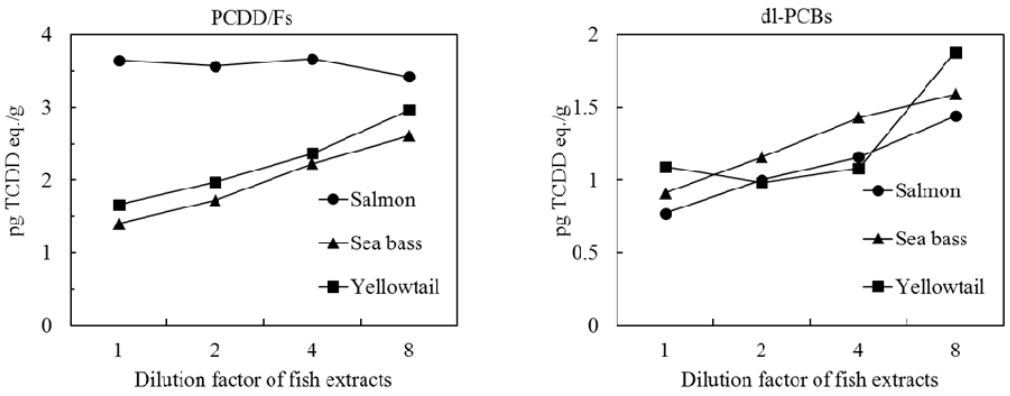

Figure 2. Effect of the dilution factor on the determination of dioxins in cleaned-up extracts from natively contaminated fish.

Table 1 Recovery of dioxins from fish samples [a]

		Spiked Conc. (TCDD eq./g)	Recovery (%) Mean ± SD
Salmon	PCDD/Fs	0.58	95 ± 6
		2.5	84 ± 12
	PCB 126	0.80	95 ± 13
		2.3	86 ± 2
Tuna	PCDD/Fs	0.57	80 ± 5
		2.6	74 ± 9
	PCB 126	0.86	76 ± 13
		2.4	87 ± 12

[a] Fish samples spiked with known quantities of PCDD/Fs or PCB 126 were extracted, cleaned up, and measured by the CALUX assay (n = 3).

Table 2 Reproducibility of the CALUX assay combined with the sample preparation procedure [a]

		TCDD eq./g						
		1st	2nd	3rd	4th	5th	Mean ± SD	CV(%)
Horse mackerel	PCDD/Fs	0.44	0.52	0.37	0.33	0.32	0.40 ± 0.084	21
	dl-PCBs	0.23	0.27	0.24	0.23	0.30	0.25 ± 0.031	12
Mackerel	PCDD/Fs	0.95	1.32	0.94	1.07	0.85	1.03 ± 0.18	17
	dl-PCBs	0.91	0.90	0.77	0.48	0.86	0.78 ± 0.18	23

[a] The fish contaminated in the natural environment were extracted, cleaned up, and assayed by the CALUX assay in five separate runs on different days.

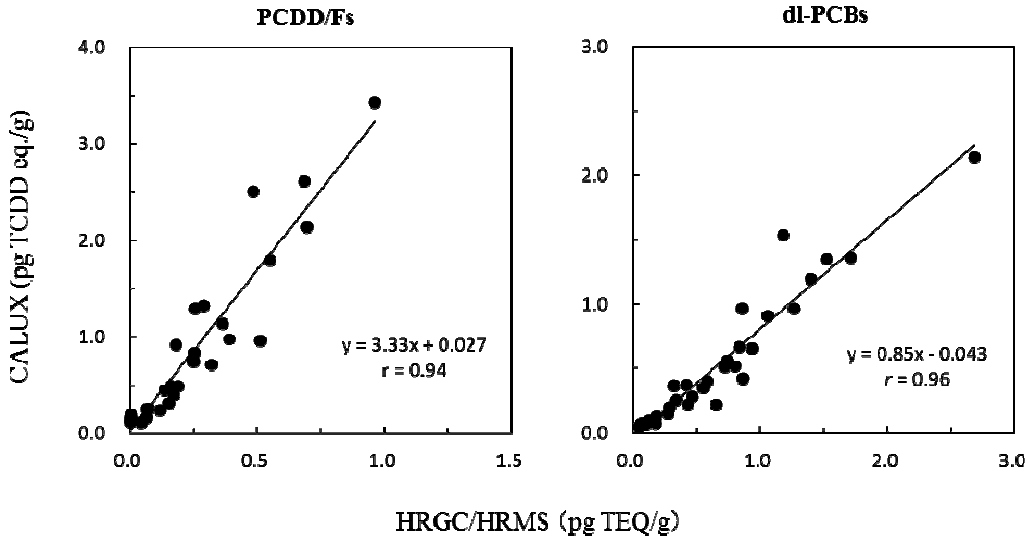

Figure 3. Comparison of the CALUX assay with HRGC/HRMS measurements of fish samples