

## Validation of a Time-Resolved Fluoroimmunoassay for Measuring Plasma Cortisol in Channel Catfish *Ictalurus punctatus*

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**Abstract.**—A time-resolved fluoroimmunoassay (TR-FIA) marketed for measuring cortisol in human sera was evaluated and validated for use in the quantification of plasma cortisol concentrations of channel catfish *Ictalurus punctatus*. Time-resolved fluoroimmunoassays provide non-isotopic alternatives to the use of radioimmunoassays (RIA). The evaluated TR-FIA satisfied strict criteria of precision (intra-assay coefficients of variation (CV) < 7%) and reproducibility (inter-assay CV  $\leq$  9%). Accuracy of the TR-FIA, calculated as the percent of exogenous cortisol recovered from spiked catfish plasma, averaged 99.5%. Assay sensitivity (minimum detection limit) in catfish plasma was 1.2 ng/mL, and the displacement curve for serially diluted channel catfish plasma paralleled the cortisol standard curve. Plasma cortisol concentrations of channel catfish in the presence and absence of a confinement stressor were used to characterize the immunoreactive cortisol measurable by TR-FIA, and compared favorably to RIA values for the same samples ( $r^2 = 0.95$ ,  $P < 0.001$ ).

Evaluations of stressors and stress responses of cultured fish can lead to improved management practices that minimize stress and thus improve production efficiency. An effective means of assessing the degree of stress in cultured fish is to measure changes in circulating levels of cortisol following administration of a stressor (Mazeaud et al. 1977; Donaldson 1981; Adams 1990; Barton and Iwama 1991). Accurate assessment of circulating cortisol levels is dependent upon a reliable and repeatable assay. Radioimmunoassays (RIA) are the most common method of quantifying plasma cortisol concentrations. Radioimmunoassays are highly sensitive, accurate and precise; however, the use of

radioisotopes diminishes the appeal of this methodology. Alternatively, enzyme-linked immunosorbant assays (ELISA) and other non-isotopic techniques have been used with some success to measure cortisol in fish plasma (Caldwell et al. 1990; Barry et al. 1993). More recently, the use of lanthanum chelates in time-resolved fluoroimmunoassays (TR-FIA) has provided a reliable non-isotopic alternative. The objective of this study was to evaluate and validate a commercially available cortisol TR-FIA for its use in measuring channel catfish *Ictalurus punctatus* plasma cortisol concentrations.

### Materials and Methods

Twenty fingerling channel catfish of approximately 100 g were acclimated for 7 d in a 535-L aquarium supplied with recirculating water held at 26 C. Following the acclimation period, a basket-confinement stressor was applied. Pre-stress blood samples were taken from ten fish and the remaining fish were confined in a plastic basket immersed so that the fish were in contact with each other and could not maintain their orientation. The basket was placed over an airstone to maintain adequate dissolved oxygen. After 2 h, the catfish were anesthetized with MS-222 (tricaine methanesulfonate) at a concentration of 0.2 g/L, and blood was collected from caudal vessels with heparinized syringes. Plasma was separated by centrifugation and stored at

-20 C for later analysis of cortisol concentrations.

Quantitative determination of cortisol in standards and channel catfish plasma was conducted using DELFIA® (dissociation enhanced lanthanide fluorescence immunoassay) cortisol reagents in a 96-well format (R060-101, PerkinElmer Wallac Inc., Gaithersburg, Maryland, USA). The DELFIA® cortisol assay is a solid phase TR-FIA in which europium (Eu)-labeled cortisol competes with sample cortisol for binding sites on cortisol specific, biotinylated mouse monoclonal antibodies. Streptavidin coated on the solid phase acts to bind the biotinylated antibody, thus separating antibody-bound cortisol from free cortisol.

Cortisol standards and assay solutions were prepared according to the manufacturers directions. The standard curve was generated from eight standards: 0, 2.7, 5.4, 10.9, 27.2, 72.5, 217.5, and 580.0 ng cortisol/mL. Non-specific binding was determined by omitting the binder (anti-cortisol antibody) in a zero standard sample. The Eu-cortisol tracer and cortisol antibody stock solution were combined (1:1) then diluted with Assay Buffer (10:1). Twenty-five  $\mu$ L of standards or plasma were diluted with the tracer-antibody-buffer solution and dispensed into streptavidin coated wells using a Hamilton Microlab 500 diluter-dispenser (Hamilton Company, Reno, Nevada, USA). Following a 1-h incubation at room temperature, the reaction wells were washed four times with the DELFIA® Wash Solution on an EL<sub>x</sub>50 Auto Strip Washer (Bio-Tek Instruments, Inc., Winooski, Vermont, USA). DELFIA® Enhancement Solution was then added, resulting in dissociation of the europium ions from the labeled cortisol into solution, and the formation of highly fluorescent chelates between the dissociated europium and components of the enhancement solution. Fluorescence was measured with a Victor<sup>2</sup> 1420 Multilabel Counter (PerkinElmer Wallac Inc., Gaithersburg, Maryland, USA).

Data acquisition, data reduction, and sta-

tistical analysis were performed using STA-TLIA® Immunoassay Workflow and Analysis Software (Brendan Scientific, Gross Point Farms, Michigan, USA). The standard curve was generated by plotting percent bound ( $B/B_0 \times 100$ ) against known cortisol concentrations using a five-parameter-logistic equation (Brendan Scientific Corporation 2000). Calculation of cortisol concentrations in the unknown samples, and the means and coefficients of variation (CV) for replicate samples were automatically calculated.

Precision and reproducibility of the TR-FIA, as estimated by intra- and inter-assay variation, were determined from separate samples of pooled ( $N = 3$ ) channel catfish plasma having low or elevated cortisol levels, and calculated as CV. Accuracy of the TR-FIA method was determined as the percent recovery of exogenous cortisol added at varying levels to catfish plasma. Cross-reactivity, at the 50% displacement level, was determined by the manufacturer for 20 different steroids. Assay sensitivity was calculated as the concentration of cortisol equal to  $B_0$  (fluorescence value of the zero standard) minus two standard deviations when interpolated from the standard curve. Cortisol levels in catfish plasma samples were also determined by RIA using the Chiron cortisol RIA kit (Chiron Diagnostics Corp., Norwood, Massachusetts, USA) which was previously validated for channel catfish (Davis et al. 1993). Results from the RIA and TR-FIA were correlated.

### Results and Discussion

A typical dose-response curve, covering the range of 2.7–580.0 ng/mL, is shown in Fig. 1. Cortisol concentrations of serially-diluted catfish plasma appeared parallel to the standard curve. The sensitivity of the assay, or minimum detection limit in catfish plasma, was 1.2 ng/mL. Accuracy of the TR-FIA, calculated as the percent of exogenous cortisol recovered from spiked plasma samples, averaged 99.5% (Table 1). Estimates of precision (intra-assay CV) and

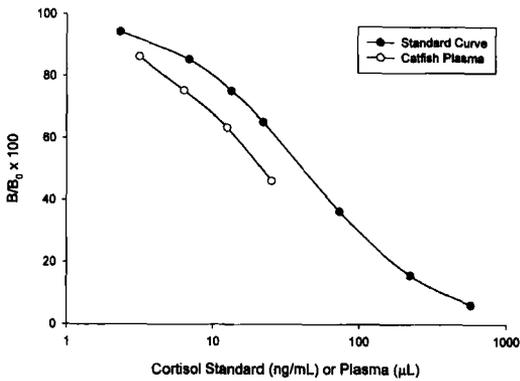


FIGURE 1. Displacement curves for cortisol standards and serially diluted channel catfish plasma. Each point is the mean of duplicate determinations.

reproducibility (inter-assay CV) for low (14.4 ng/mL) and elevated (46.5 ng/mL) cortisol levels are presented in Table 2. Assay results for 14 plasma samples by TR-FIA were compared to results obtained by conventional RIA. Resting plasma cortisol levels determined by TR-FIA averaged (mean  $\pm$  SE;  $N = 7$ )  $13.2 \pm 0.4$  ng/mL compared to  $9.1 \pm 1.2$  ng/mL as determined by RIA. Plasma cortisol levels determined by TR-FIA for fish subjected to net confinement averaged (mean  $\pm$  SE;  $N = 7$ )  $40.2 \pm 2.5$  ng/mL compared to  $40.3 \pm 2.4$  ng/mL as determined by RIA.

The major disadvantage of the RIA is the use of radioisotopes and the associated health hazards and waste disposal problems. While both the TR-FIA and the RIA depend on competition between labeled and unlabeled cortisol for a limited number of antibody binding sites, the TR-FIA utilizes the unique fluorescence property of europium as an alternative to the use of radioisotopes for labeling and detection. Eu-labeled compounds may be stable for over a year, and assay sensitivity is reported to be as good as or better than that of radioisotopes (Iwasawa et al. 1992).

The increasingly cumbersome and expensive regulatory controls over the use of radioactive materials makes it important to develop alternative methods to RIA for accurately determining hormone concentra-

TABLE 1. Recovery of exogenous cortisol added to channel catfish plasma.

Cortisol added (ng/mL)	Recovery <sup>a</sup>	
	(ng/mL)	(%)
13.6	15.1	110.0
36.3	34.7	95.6
108.8	103.3	94.9
290.0	282.2	97.3

<sup>a</sup>  $N = 4$ .

tions in small volumes. The TR-FIA evaluated here proved to be highly sensitive for measuring channel catfish plasma cortisol concentrations. The minimum detection level of the TR-FIA (1.2 ng/mL) is comparable to that of the RIA ( $\leq 2.5$  ng/mL). Because the TR-FIA also requires a small volume (25  $\mu$ L) of plasma be used in the assay, measurements can be taken from very small fish or repetitive samples can be taken from larger fish. Comparison of the plasma cortisol profiles of seven stressed and seven non-stressed catfish measured by TR-FIA and RIA, respectively, was favorable for the same fish ( $r^2 = 0.95$ ,  $P < 0.001$ ). Cost of the DELFIA<sup>®</sup> Cortisol TR-FIA kit, at about \$2.00 per sample, is somewhat more expensive than comparable RIA kits. Reasonable estimates are in the range of \$1.50 to \$1.80 per sample for RIAs. Cost of the time-resolved fluorometer may be as much as \$15,000 more than its gamma counter counterpart.

In conclusion, we have evaluated the use of a commercially available TR-FIA kit for measuring circulating levels of channel catfish cortisol. Plasma cortisol values ob-

TABLE 2. Intra- and interassay coefficients of variation (CV) in the TR-FIA for plasma pooled from three resting and three stressed channel catfish.

Sample	Assay results (ng/mL)	Intra-assay CV <sup>a</sup> (%)	Interassay CV <sup>b</sup> (%)
Resting	14.4	6.5	9.0
Stressed	46.5	5.3	5.1

<sup>a</sup>  $N = 4$ .

<sup>b</sup>  $N = 3$ .

tained with the TR-FIA correlated well to RIA values, and based on the criteria of inter- and intra-assay coefficients of variance, recovery of exogenous cortisol, and parallelism to the standard curve, our results indicate that the TR-FIA is a sensitive assay with acceptable accuracy, precision, and reproducibility for assessing plasma cortisol concentrations of channel catfish.

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