

## IMMUNOASSAY FOR CADMIUM IN AMBIENT WATER SAMPLES

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## **UNIT TITLE: Immunoassay for Cadmium in Ambient Water Samples**

### **UNIT INTRODUCTION:**

The cadmium immunoassay uses a key reagent, a monoclonal antibody 2A81G5 that binds tightly to ionic cadmium [Cd(II)] complexed to ethylenediaminetetraacetic acid (EDTA) but not to metal-free EDTA (Blake *et al.* 1996). The water sample is mixed with a buffer containing millimolar concentrations of EDTA, to insure that any ionic cadmium in the sample forms a stable EDTA complex. The protocol described herein utilizes a competitive ELISA format; the sample containing the Cd(II)-EDTA complex is mixed with the monoclonal antibody, then the mixture is allowed to bind to a microwell plate containing immobilized Cd(II)-EDTA covalently conjugated to a carrier protein. The presence of Cd(II)-EDTA in the sample inhibits the ability of the monoclonal antibody to bind to the immobilized Cd(II)-EDTA-protein conjugate. After the unbound 2A81G5 antibody has been washed from the microwell plate, an enzyme-labeled anti-species antibody is added. After an additional wash to remove unbound anti-species antibody, the assay is completed by the addition of a colorimetric substrate for the labeling enzyme. The greater the amount of cadmium in the sample, the lighter the color will be. Conversely, if the sample has little or no ionic cadmium, a greater amount of color will be produced because all of the 2A81G5 antibody will be available for binding to the immobilized Cd(II)-EDTA conjugate in the microwell plate. The limit of detection in the immunoassay is 7 ppb Cd(II). Each ambient water sample is diluted 10% for pH adjustment and subsequently 3-fold during the analysis procedure; the limit of detection in the original environmental sample is therefore approximately 30 ppb.

Ca(II), Na(I), and K(I), cations commonly encountered in ambient water samples, do not interfere with the cadmium immunoassay at concentrations approaching their limit of solubility. The assay will reliably measure Cd(II) in the presence of >50,000 ppb Zn(II), Ni(II), Fe(III), and

Pb(II). Mg(II) causes positive interference at concentrations >24,000 ppb and In(III) and Mn(II) cause positive interference in the immunoassay at concentrations higher than 5,000 ppb. Hg(II) causes a false positive in the assay, but only at concentrations higher than 2,000 ppb. The ability of the assay to give false positive in the presence of mercury concentrations above 2,000 ppb indicates that those environmental samples which give a positive response in the assay should be reanalyzed by an independent method to distinguish between samples contaminated with ionic cadmium from those containing ionic mercury.

### **QUALITY CONTROL**

When considering QA indicators of confidence, the user should make use of both generic and core indicators (Coakley *et al.* 1996). Generic indicators of confidence are those which are common to all analytical methods and include blanks, matrix spikes, calibration standards, procedures for sample preparation, replicate analyses, and confirmation analyses. Core performance indicators focus upon errors that are associated with the specific immunoassay method; these may include temperature, antibody specificity, non-analyte interferences, dilutions, pH and stability of reagents, and reaction time.

Minimum quality control requirements include the analysis of field reagent blanks, field fortified blanks, and field fortified sample matrices. When available, certified standard reference materials should be used to verify method performance.

#### **Initial demonstration of assay capabilities**

Prepare four replicate field-fortified blanks by spiking 100 and 1000 ppb of atomic adsorption grade cadmium into metal-free water. Assay the fortified blanks according to the protocol described below and calculate the mean recovery and standard deviation of the recoveries. The mean recovery value, expressed as a percentage of the spiked concentration, must be >50% and

the % RSD should be <30%. If these criteria are met, performance is considered acceptable and sample analysis may begin. If these criteria are not met, the procedure must be repeated until satisfactory performance has been demonstrated.

### **Field reagent blanks**

Blanks should be included with field sample-collection activities, and must be included with samples being sent for confirmatory analysis. Before processing any sample, at least one field reagent blank (FRB) must be analyzed to demonstrate that all interferences introduced by equipment and reagents are under control. A FRB must be also be analyzed each time reagents are changed. If the FRB produces a positive response, the source of contamination must be determined and the interference eliminated before proceeding with the analysis.

### **Field fortified blanks**

The analyst must analyze at least one field fortified blank (FFB) with every 20 samples, or one FFB with every sample set (i.e., all samples tested in one 24-hour period), whichever represents the greater number of samples. Water samples fortified with 200-500 ppb of cadmium are recommended. Percent recovery must be calculated; if the recovery falls outside the control limits of the analytical method (i.e., 50% to 150%) the method is judged out of control and the source of the problem must be identified and resolved before continuing the analysis.

### **Field fortified sample matrix**

The analyst must perform analyses of the field fortified sample matrix (i.e., add known concentrations of cadmium to a minimum of 10% of the actual samples). The spike levels should be equivalent or greater than the background concentrations in the samples selected for fortification. The mean percent recovery (R) is calculated for each analyte after correcting for the total mean measured concentration (A) in the fortified sample for the background concentration (B) measured in the unfortified sample:

$$R = 100(A-B)/C$$

where C is the fortified concentration (the known concentration added). These values should be compared to the control limits of the FFB. If the method accuracy falls outside the 50% to 150% range, the accuracy problem encountered with the FFB may be judged to be matrix-related. The results for the unfortified samples must be labeled suspect and the data user informed that the result is suspect because of matrix effects.

## **MATERIALS FOR BASIC PROTOCOL**

Ambient water samples for analysis

Water used for making all reagent solutions should be purified by filtration through a

Nanopure II water purification system (Barnstead/Thermolyne, Inc.) or any equivalent device that will provide 16-18 mega ohm water)

Phosphate-buffered saline: 137 mM NaCl, 3 mM KCl, 10 mM sodium phosphate buffer, pH 7.2

HEPES-buffered saline (10x): 1.37 M NaCl, 30 mM KCL, 100 mM HEPES, pH 7.2

HEPES-buffered saline (1x): 137 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.2

Cd(II)-EDTA-bovine serum albumin conjugate: 0.5 µg/ml in phosphate-buffered saline

NOTE: this conjugate may be prepared as described in (Blake *et al.* 1996) using 1-(4-isothiocyanobenzyl)ethylenediamine-N,N,N'.N'-tetraacetic acid purchased from Dojindo Laboratories (Kumamoto, Japan)

Washing solution: Phosphate-buffered saline containing 0.05% Tween 20

Bovine serum albumin (BSA): fatty acid ultrafree from Boehringer-Mannheim Biochemicals

NOTE: This product has a lower heavy metal content than many other commercial products

2A81G5 monoclonal antibody: 0.15 µg IgG1/ml diluted into 1x HEPES-buffered saline

containing 0.75% BSA and 115 mM EDTA, pH 7.2 (available via Materials Transfer Agreement with Tulane University School of Medicine)

Goat anti-mouse-HRP conjugate, Fc specific (Sigma Chemical Co., or equivalent): 1:1500 dilution in phosphate-buffered saline containing 1% BSA

Cadmium standards (see reagents and standards)

Trisma base (Sigma Chemical Co. or equivalent)

1 N NaOH: for pH adjustment

Chromogenic substrate: TMB Microwell Substrate (Kirkegaard-Perry)

Stop solution: 1N HCl

ELISA microwell plates: Available from Costar, Inc.

Micropipettor capable of delivering 20-200  $\mu$ l (eg. Rainin p200)

Micropipettor capable of delivering 200-1000  $\mu$ l (eg. Rainin p1000)

Octapipette capable of delivering 50  $\mu$ l

Metal-free pipettor tips: for p200 and p1000 pipettors (Oxford Labware or equivalent)

1.5 ml disposable polypropylene tubes

Whatman 45 filter paper

0.45  $\mu$ m syringe filters: Gelman (Ann Arbor, MI) NOTE: Gelman filters have been tested by our laboratory for heavy metal contamination and do not appear to contribute significant quantities of heavy metals to the sample

25-50 ml disposable plastic syringes

50 ml acid-washed polypropylene centrifuge tubes

pH meter that can read accurately to the nearest 0.1 pH unit

Microtiter plate washer (Denley Instruments or an equivalent device)

Microplate reader with a 450 nm filter and software for calculating standard curve: Vmax

Microplate Reader from Molecular Devices, or equivalent device

CAUTION: Cadmium is acutely toxic to kidneys and lung, depending upon route of ingestion; chronic low doses of cadmium have been linked to increased risk of cancer.

### **BASIC PROTOCOL STEPS**

#### **Preparation of microwell plates**

1. Dissolve the Cd(II)-EDTA-BSA conjugate in phosphate-buffered saline at a concentration of 0.5  $\mu\text{g/ml}$  and add 50  $\mu\text{l}$  each well of a 96-well microtiter plate. Incubate at room temperature for 1 h or overnight at 4°C. The plates may also be covered and stored at 4°C for up to 3 months before use.
2. On the day of the assay, wash each well of the plate 3 times with phosphate-buffered saline containing 0.05% Tween.
3. Add 50  $\mu\text{l}$  of 3% BSA in phosphate-buffered saline to each well of the plate to block any excess protein binding sites. Incubate 30-60 minutes at room temperature, then wash each well 3 times with phosphate-buffered saline containing 0.05% Tween.

#### **Preparation of Cadmium Standards**

4. Adjust the pH of the cadmium standard (1000 ppm in 2% nitric acid) to pH 7.2 with dry Trisma base.
5. Dilute the pH-adjusted cadmium standard (1000 ppm) into 1-5 ml of HEPES-buffered saline (1x) to final dilutions of 0, 30, 90, 150, 240, 500, and 1500 ppb. Because each standard will be diluted 1:3 in the final assay mixture, these standard concentrations will yield the following final Cd(II) concentrations in the assay: 0, 10, 30, 50, 80, 167, and 500 ppb.

#### **Preparation of Environmental Water Samples**

6. Water samples should be collected in acid-washed gallon jugs and stored at 4°C until tested (usually within 24 h.).

7. The water should be filtered through Whatman 45 paper to remove coarse particulates, then passed through 0.45  $\mu\text{m}$  syringe filters and stored in the acid-washed 50 ml centrifuge tubes.
8. Adjust the pH of the water samples 7.2 by the addition of a 10% volume of HEPES-buffered saline (10x).
9. Check the pH of the diluted water samples and adjust to 7.2 with 1 N NaOH or HCL. Record the original volume of the water sample and the final volume after pH adjustment.
10. Samples should be subsequently diluted into HEPES-buffered saline (1x) if necessary to bring them into the linear range of the assay.

#### **Analyze samples by ELISA**

11. Mix 50  $\mu\text{l}$  of each standard cadmium sample with 100  $\mu\text{l}$  of the 2A81G5 monoclonal antibody.
12. Mix 50  $\mu\text{l}$  of each pH-adjusted ambient water sample with 100  $\mu\text{l}$  of the 2A81G5 antibody solution.
13. Add duplicate 50  $\mu\text{l}$  aliquots of each mixture to wells of the coated, blocked microtiter plates and incubate for 1 h at 25 °C. Include duplicate wells on the microwell plate for an internal control which contains no 2A81G5 monoclonal antibody.
14. Wash each well of the plate 3 times with washing solution.
15. Add 50  $\mu\text{l}$  of a 1:1500 dilution of goat anti-mouse-HRP conjugate and incubate an additional hour at 25°C. Wash each well of the plate as in step 14.
16. Add 50  $\mu\text{l}$  of TMB microwell substrate and incubate for 5 min. at 25°C. Stop the reaction by the addition 50  $\mu\text{l}$  of 1 N HCl.
16. Within 15 minutes after stopping the reaction, read the optical density of each well at 450 nm using the Vmax Microplate Reader.
17. Determine the concentration of cadmium in each sample by comparing the results to the

standard curve; dilute any samples with cadmium concentration above 1500 ppb with HEPES-buffered saline (1x) and reanalyze.

### Calculate Results

18. The data from the standard curve may to analyzed by non-linear regression (Enzfitter, Biosoft) to estimate the parameters of an empirical fitting equation:

$$OD = OD_{max} - Cap * [Cd(II)] / (IC_{50} + [Cd(II)]) \quad [1]$$

where  $OD_{max}$  is optical density signal in the absence of soluble Cd(II), OD is the optical density in the presence of a known quantity of soluble Cd(II), Cap is the maximal decrease in the OD at saturating Cd(II) concentrations, and  $IC_{50}$  is the cadmium concentration that produces a 50% inhibition in the signal.

19. The concentrations of cadmium in the environmental samples (X) may then be calculated as weighted averages for 2 to 4 measurements at appropriate dilutions as:

$$X = IC_{50} / (Cap / (OD_{max} - OD) - 1) * Id \quad [2]$$

where Id is a dilution of the sample. The effect of small changes in parameters that contain random errors on the precision of the calculated concentrations of cadmium was approximated by a full differential of equation [2], which was rearranged to produce estimations of relative errors for X:

$$\delta X = Cap / (OD - Bckg) * (\delta IC_{50} + \delta Cap + \delta (OD_{max} - OD) + \delta Id) \quad [3]$$

where  $\delta X = \Delta X / X$ ;  $Bckg = (OD_{max} - Cap)$ ;  $\delta IC_{50} = \Delta IC_{50} / IC_{50}$ ;  $\delta Cap = \Delta Cap / Cap$ ;

$\delta (OD_{max} - OD) = (\Delta OD_{max} + \Delta OD) / (OD_{max} - OD)$ ; and  $\delta Id$  is the relative error in the dilution.  $\Delta OD_{max}$ ,  $\Delta Cap$ , and  $\Delta IC_{50}$  were obtained as standard errors by non-linear regression of the cadmium standard curves using equation [1].  $\Delta OD$  was estimated by analyzing replicate measurements on the ELISA plates, and the  $\delta Id$  was calculated by using values of errors that were supplied by the manufacturers of the pipettors and the volumes used in dilutions for

individual measurements. The statistical weight for each measurement was calculated as:

$$W = 1/(\delta X * X)^2 \quad [4]$$

This approach of estimation of standard errors is different from the one proposed by Fare *et al.* 1996, because it does not neglect random errors in parameters of a fitting equation.

## **REAGENTS AND STANDARDS**

### **Cadmium**

The cadmium standard (1000 ppm in 2% nitric acid) may be purchased from Perkin-Elmer Corporation. A standard from Spex (10,000 ppm in 5% nitric acid) gives equivalent results. Once the standards have been diluted into the HEPES-buffered saline, they should be used within 24 h.

## **COMMENTARY**

### **Metal ion ELISAs**

Environmental and occupational pollution from heavy metals is steadily increasing and the general population may be exposed to heavy metals by ingestion of contaminated water and food (Faroom *et al.* 1994). Heavy metals require long-term monitoring because of their persistence in the environment and their ability to be mobilized by changes weather patterns and hydrology. Heavy metal contamination in ambient water samples is presently assessed by either atomic absorption spectroscopy or inductively coupled plasma emission spectroscopy. However, measurement of specific heavy metals by ELISA could have significant advantages; immunoassay are quick, inexpensive, simple to perform and sufficiently portable to be used at the site where the sample is taken. In this unit, an assay for cadmium in ambient water samples has been developed using a monoclonal antibody that recognizes an cadmium-EDTA complex, but not metal-free EDTA (Blake *et al.* 1996).

The cadmium immunoassay employs a competitive format similar to that previously

described by our laboratory for immunoassay of indium (Chakrabarti *et al.* 1994). A protein conjugate containing a covalently bound Cd(II)-EDTA complex is adsorbed to the wells of microtiter plates. The monoclonal antibody is subsequently mixed with cadmium in the presence of excess metal-free EDTA. This molar excess of EDTA insures that any metal cations in the immunoassay will be present as a metal-EDTA complex, the form of the metal recognized by the monoclonal antibody. The incubation mixture is added to the microwell plate, where the soluble Cd(II)-EDTA complex and the immobilized Cd(II)-EDTA-protein conjugate compete for antibody binding sites; any antibody bound to the soluble Cd(II)-EDTA complex will be removed during the subsequent wash of the microwell plate. An enzyme-labeled anti-species antibody is then added, and after an additional wash step, color is developed. As in other competitive assays, color formation is inversely proportional to the amount of analyte in the incubation mixture. A typical cadmium standard curve is shown in Figure 1.

## **DEFINITIONS**

**2A81G5 monoclonal antibody.** A protein produced in response to injection of a metal-chelate complex into BALB/c white mice. The affinity of this antibody for various metal-chelate complexes has been extensively characterized (Blake *et al.* 1996).

**Cd(II)-EDTA-BSA.** Bovine serum albumin-thioureido-L-benzyl-ethylenediaminetetraacetic acid-cadmium conjugate that is immobilized onto microwell plates. This conjugate may be prepared and characterized as described in (Blake *et al.* 1996).

**Cross reactivity.** The ability of the antibody to bind to metal-chelate complexes other than cadmium-EDTA.

**ELISA.** (enzyme-linked immunosorbent assay) A method that uses an immobilized reagent (in this unit, a metal-chelate-protein conjugate) to facilitate separation of the bound and unbound antibody.

**False negative.** A negative response for a sample at the stated action level of the target analyte. The maximum permissible false negative rate is 5% as measured by analyzing split samples using both ELISA and a reference method.

**False positive.** A positive response for a sample that does not contain the analyte at the action level.

**Field duplicates.** Two separate samples collected at the same time and place under identical conditions and treated exactly the same throughout field and laboratory procedures. Analyses of field duplicates provide a measure of the precision associated with sample collection, preservation, and storage, as well as with field procedures.

**Field reagent blank.** An aliquot of reagent-grade water that is subjected to exactly the same treatments as a field sample, including exposure to all glassware, plasticware, equipment, and reagents that are used with field samples. The field reagent blank (FRB) is used to determine if method analytes or other interferences are present in the field environment, the reagents, or the apparatus.

**Field fortified blank.** An aliquot of reagent-grade water to which known quantities of cadmium are added in the field. The field fortified blank (FFB) is analyzed exactly like a sample, and its purpose is to determine whether the methodology is under good control and whether the field instrument is capable of making accurate and precise measurements.

**Field fortified sample matrix.** A aliquot of the environmental sample to which known quantities of cadmium are added in the field. The field fortified sample matrix (FFM) is analyzed exactly like a sample and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentration of the analyte in the sample matrix must be determined on a separate portion of the sample and the measured values in the field fortified sample matrix corrected for background concentrations.

## **SAFETY**

High doses of cadmium are acutely toxic to kidney and lung, depending upon route of ingestion; chronic low doses have been linked to increased risk of cancer. The toxicity and carcinogenicity of the TMB microwell substrate and other reagents used in this protocol have not been precisely defined; therefore these compounds should be treated as a potential health hazard and exposure should be minimized. The field laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data safety sheets should also be made available to all field personnel involved in the analyses. Additional references to laboratory safety are available.

## **CRITICAL PARAMETERS AND TROUBLE SHOOTING**

### **Preparation of water samples for analysis**

A critical source of error in this protocol is improper pH adjustment of the sample. The assay response is very dependent upon the pH of the incubation mixture; response is optimal between pH 7.0 and 7.2, but is strongly inhibited when the pH of the incubation mixture falls below 7.0 or rises above 7.3. In a competitive immunoassay, any factor which depresses color formation will be read as a positive response; the strict pH dependence displayed by the 2A81G5 monoclonal antibody requires that the pH of the incubation mixture be carefully controlled to avoid false positives when assessing environmental samples. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.2 was chosen as the buffer salt for these experiments because of its  $pK_a$  and its negligible metal-binding capacity (Good *et al.* 1966).

## **ELISA**

Allow all reagents to come to 20-25°C before use in the assay. Do not lower the concentration of bovine serum albumin in the 2A81G5 monoclonal antibody formulation, because these concentrations of BSA mask some of the cross-reactivity to Hg(II). Do not transfer the antibody

to glass containers. To prevent cross-contamination, use clean pipette tips for each sample and avoid contact between reagent droplets on the test tubes and pipette tips. Avoid foam formation during mixing steps. Refer to the User's Guide for Environmental Immunochemical Methods (Gee & Van Emon, 1994) for general instructions regarding ELISA protocols.

### **METHOD PERFORMANCE**

At present, the cadmium immunoassay is a prototype, and performance in field trials has not been established. In experiments with environmental water samples spiked with 40-600 ppb of cadmium, the results from the immunoassay correlated well with the values obtained from atomic absorption spectroscopy (AAS) as shown in Figure 2. Linear regression analysis of the data in Fig. 2 yielded a slope of 0.951, an intercept of  $23.07 \pm 9.5$  ppb, and a correlation coefficient of 0.931. The immunoassay correctly identified minimally, moderately, and heavily contaminated water samples. There was some positive bias in the immunoassay as indicated by the non-zero intercept of the graph in Fig. 2; however, such a positive bias is acceptable in an assay designed to be used as primarily as a field portable screening test. The limit of detection in this immunoassay (defined as 2 standard deviations above the minimum detectable level) is 7 ppb.

### **Cross-Reactivity**

A summary of the effect of other metal ions on the cadmium immunoassay is presented in Table 1. The ability of a metal-EDTA complex to interfere in the competitive immunoassay was well correlated with its affinity for the 2A81G5 monoclonal antibody. The only metal which showed an ability to interfere with the immunoassay at concentrations likely to be present in field samples was Hg(II). The immunoassay was not affected by Hg(II) at concentrations lower than 1000 ppb; however, concentrations of 2000 ppb or higher caused significant positive interference in the immunoassay. The ability of the assay to give false positive in the presence of

mercury concentrations above 2000 ppb indicates that those environmental samples which give a positive response in the assay should be reanalyzed by an independent method to distinguish between samples contaminated with ionic cadmium from those containing larger quantities of ionic mercury.

### **TIME CONSIDERATIONS**

The average time required to process a batch of 20 samples is 4-6 hours, if the microwell plates have been previously coated with the Cd(II)-EDTA-BSA conjugate. Calibrators and sample preparation can be initiated while the plates are being blocked with BSA. Unless all microwell plates are of the same series (i.e., coated and stored as one batch) it is recommended that calibration standards, the FRB, FFB, and FFM be included on each plate.

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**Table 1**

Affinity of a Metal-EDTA Complex for the 2A81G5 Antibody  
Correlates with Its Inhibitory Effect in Competitive Immunoassay

Metal-EDTA Complex	Equilibrium Dissociation Constant (M) <sup>a</sup>	Inhibitory Concentration in Immunoassay (ppb) <sup>b</sup>
Pb(II)	$7.4 \times 10^{-5}$	>207,000
Fe(III)	$5.4 \times 10^{-5}$	> 56,000
Mg(II)	$2.2 \times 10^{-4}$	> 24,000
Zn(II)	$2.2 \times 10^{-6}$	65,000
Ni(II)	$2.1 \times 10^{-6}$	59,000
In(II)	$6.2 \times 10^{-7}$	11,400
Mn(II)	$4.1 \times 10^{-7}$	5,500
Hg(II)	$2.6 \times 10^{-8}$	2,000

<sup>a</sup> Binding affinity of antibody for metal-EDTA complexes taken from Blake *et al.* 1996

<sup>b</sup> Concentration of metal-EDTA complex which inhibits color formation in the competitive immunoassay by >50%

## FIGURE LEGENDS

**Figure 1. Representative standard curve for cadmium.**

**Figure 2. Comparison of ELISA and atomic absorption results in the analysis of environmental water samples.** ●, ▼, ▲, and ◆; samples prepared in water collected on different dates from Bayou Trepagnier, located approximately 22 miles west of Metropolitan New Orleans adjacent to the Bonnet Carre' Spillway. Bayou Trepagnier was chosen to test the cadmium immunoassay because its water chemistry is typical of polluted bayous found in southern Louisiana. Linear regression analysis of the data generated a line with a slope of 0.951, an intercept of  $23.07 \pm 9.5$  ppb and a correlation coefficient of 0.931.

Figure 1.

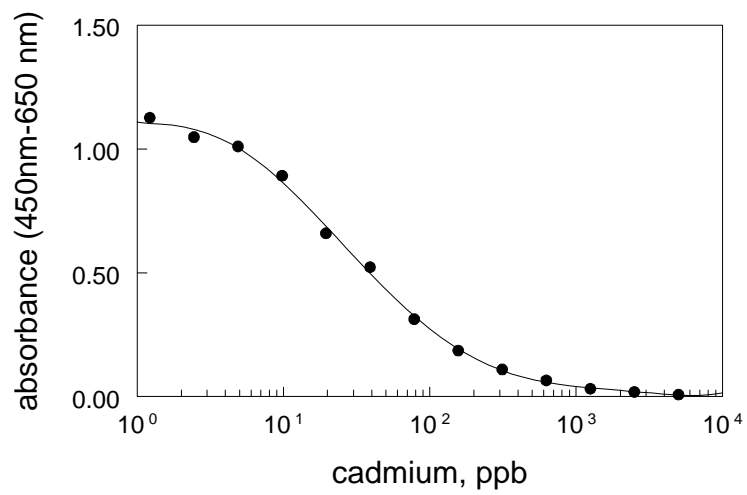


Figure 2.

