

AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) METHOD FOR THE DETERMINATION OF RESIDUES OF SULFONYLUREAS IN WATER AND SOIL

Johann C. Strahan and Christine L. Rankin

INTRODUCTION/SUMMARY

Scope

The herbicidal properties of the sulfonylurea class of compounds is well known. Their low toxicity combined with their low application rates makes these compounds especially attractive from an environmental and human health standpoint¹.

This AMR describes the enzyme linked immunosorbent assay (ELISA) used for the determination of residues of sulfonylureas (SUs) in water and soil. Details specific to a particular SU method and the validation data for each of the SU methods may be found in the Appendices.

The limit of quantitation (LOQ) of a water sample is typically 0.5 ng/mL. The LOQ of a soil sample is typically 1.0 ng/g. Note that the LOQ is dependent on both the matrix effects and sensitivity of the particular SU assay. The LOQs reported here for water and for soil are intended to encompass an unknown world of samples and to be LOQs that will provide reliable and reproducible results for all of the SU assays. Mean recoveries of SUs spiked into water or soil are within 100 ± 30%.

Principle of Method

- (1) Polyclonal or monoclonal antibodies (Ab) and buffer are added to sample containing an unknown amount of SU and incubated. The antibodies bind to any SU molecules present in the sample.
- (2) Aliquots of the solution are added to wells on a 96-well microtiter plate, which have been coated with an SU derivative-ovalbumin conjugate. Any excess Ab **not**

¹Sulfonylurea Herbicides E. M. Beyer, M. J. Duffy, J. V. Hay & D. D. Schiueter, Herbicides Chemistry, Degradation, and Mode of Action, Vol 3 Marcel Dekker, Inc. 1988.

bound to SU in the sample will bind to SU immobilized on the microwell plate. The plate is then washed to remove any Ab not bound to the plate. The amount of Ab bound to the microwell is an inverse measure of the amount of SU in the sample.

- (3) To detect the Ab bound to each microwell, an anti-rabbit or an anti-mouse antibody conjugated to an enzyme, alkaline phosphatase (Ab-E), is added to each well and incubated. This Ab-E will bind to any anti-SU antibodies bound to the microwell. The microwell plate is then washed to remove any unbound Ab-E.
- (4) An alkaline phosphatase substrate, *para* nitrophenyl-phosphate, is added to the microwells. The enzyme-substrate reaction produces a yellow color which is inversely proportional to the concentration of SU in the sample. A microtiter plate reader quantitates the absorbance in each well at 405 nm. Computer software is used to construct a standard curve from standards run on the plate and to calculate SU concentrations for each unknown on the plate.

MATERIALS AND METHODS

Equipment

Disposable plasticware is recommended for all possible uses to minimize carry-over. Polypropylene is preferred for plasticware, but high-density polyethylene is also acceptable. Comparable equipment may be substituted for the equipment specified below.

- (1) ELISA Plate Washer - Titertek® Plus M96V Microplate Washer with Validation Station (Catalog No. 79-960-00). ICN Biomedicals, Inc., 3300 Hyland Avenue, Costa Mesa, CA 92626. Tel. (800) 854-0530.
- (2) ELISA Plate Reader - VMax® Kinetic Microplate Reader (Catalog No. 0200-0100). Molecular Devices Corporation, Menlo Oaks Corporation Center, 4700 Bohannon Drive, Menlo Park, CA 94025. Tel. (800) 635-5577.
- (3) Computer and Software - IBM or IBM Compatible Computer with 4 MB RAM and 386 or 486 Microprocessor. Package should include Windows 3.1 (with mouse), DOS 5.0, VGA Graphics Adapter and Monitor, a keyboard

and a printer. SOFTMAX Software for Windows (Catalog No. 0200-0120). Molecular Devices Corporation, Menlo Oaks Corporation Center, 4700 Bohannon Drive, Menlo Park, CA 94025. Tel. (800) 635-5577.

- or -

Computer and Software - Macintosh Classic II with 4 MB RAM, keyboard and mouse. Package should also include a printer. SOFTMAX Software for Apple Macintosh Computers (Catalog No. 0200-0109). Molecular Devices Corporation, Menlo Oaks Corporation Center, 4700 Bohannon Drive, Menlo Park, CA 94025. Tel. (800) 635-5577.

- (4) Centrifuge - Sorvall® RT 6000D. DuPont Biotechnology Systems Customer Service, P.O. Box 80024, Wilmington, DE 19880. Tel. (800) 551-2121.
- (5) Analytical Balance - Mettler Toledo AG104 (Catalog No. 11274-452). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (6) Top Loading Balance - Mettler Toledo PM600 (Catalog No. 11275-260). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (7) Infrared Moisture Determination Balance - A&D Moisture Analyzer, Model No. AD-4712. A&D Engineering, Inc., 1555 McCandless Drive, Milpitas, CA 95035.
- (8) Vortex Mixer - Vortex Genie 2 Mixer (Catalog No. 58815-178). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (9) Stirrer - VWR Brand Model 360 Stirrer (Catalog No. 58935-351). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (10) Platform Mixer - Nutator Mixer, Model 11105 Clay Adams (Catalog No. 15172-203). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (11) Plate Shaker - Titertek 4 Plate Shaker (Catalog No. ~~77-478~~ 00). ICN Biomedicals, Inc. 3300 Hyland Avenue, Costa Mesa, CA 92626. Tel. (800) 854-0530.
- (12) pH Meter - Beckman phi 34 (Catalog No. BK123141). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.

- (13) Refrigerator/Freezer - General Purpose. Should not have self-defrost option on freezer. This prevents the repeated thawing and re-freezing of small aliquots of the Coating Antigen Master Stock Solution.
- (14) Oven - Drying oven/incubator (used to dry plates after coating, ~35 °C). Oven dimensions: 12 x 12 x 12 inches (interior measurements)
- (15) EDP Motorized Digital Pipettor - 10.0 mL adjustable pipette (Catalog No. EP-10ML). Rapid Charge Stand (Catalog No. 6101-049). Portable Power Pack (Catalog No. 6100-080). Tips (Catalog No. RC-10ML). Rainin Instrument Co., Mack Road, Woburn, MA 01801. Tel. (617) 935-3050.
- (16) Microman Positive Displacement Pipettes - 1 to 25 μ L pipette (Catalog No. M-25). Tips (Catalog No. CP-25). 20 to 50 μ L pipette (Catalog No. M-50). Tips (Catalog No. CP-50). 50 to 250 μ L pipette (Catalog No. M-250). Tips (Catalog No. CP-250). Rainin Instrument Co., Mack Road, Woburn, MA 01801. Tel. (617) 935-3050.
- (17) Multichannel Pipettor - Digital 12 Channel Finnpipette, 50-300 μ L (Catalog No. 4172-317). Tips (Catalog No. 9401 230). Rack (Catalog No. 9420-120). Elkay LabSystems, 800 Boston Turnpike, P. O. Box 5247, Shrewsbury, MA 01545. Tel. (800) LAB-PROD.
- (18) Biohit Pipettor - 50 to 1000 μ L pipette (Biohit No. 710 022, Catalog No. 53495-205). Tips (Biohit No. 780 020, Catalog No. 53495-254). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (19) Disposable Centrifuge Tubes - Blue Max 15 & 50 mL (Catalog Nos. 21008-918 and 21008-951). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (20) Polypropylene Cube 2ubes™ (Catalog No. CT-205). DBM Scientific Corp., 13149 Ingres Ave., Granada Hills, CA. Tel. (818) 360-3610.
- (21) Cube 2ube™ Rack (Catalog No. CT-750) or Multi-Use rack (Catalog No. ACT-850). DBM Scientific Corp., 13149 Ingres Ave., Granada Hills, CA. Tel. (818) 360-3610.
- (22) ELISA Plates - Nunc Immuno 96F Maxisorp with certificate (Nunc No. 439454, Catalog No. 62409-832). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.

- (23) ELISA Plate Lids (Nunc No. 264122, Catalog No. 62409-126). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (24) Plate Sealing Film (Nunc No. 236366, Catalog No. 62409-122). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (25) Filters - Millex-HV 25 mm diameter, 0.45 μ m pore size (Catalog No. SLHV025NB). Millipore Products Division, Bedford, MA 01730. Tel. (800) 225-1380.
- (26) Syringes - Luer lock, disposable, without needle, 10cc (Catalog No. BD309604). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (27) Reagent Reservoirs - disposable, polypropylene (Costar No. 4870, Catalog No. 53504-035). VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.
- (28) Other lab supplies - VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000.

Timers	VWR Brand 100	62344-756
Stir Bars	Color Coded-Octagon	58948-074
Pipettes	Graduated, Plastic, 5 mL	14670-205
Bottles	Polypropylene, 500 mL	16129-040
	Polypropylene, 1000 mL	16129-061
Beakers	Polypropylene, 100 mL	13915-522
	Polypropylene, 250 mL	13915-566
	Polypropylene, 400 mL	13915-588
	Polypropylene, 600 mL	13915-599
Supports	Test Tube Rack, Vinyl	60941-029
	Centrifuge Tube Rack	60916-101
Kimwipes		
Lint-free towels		

Reagents

If the vendor and catalog number of a chemical are not specified, reagent grade chemicals from any reputable vendor may be used. If the vendor and catalog number are specified, then substitution with material from another vendor may.

require method validation studies to assure quality performance.

(1) **Water** - Water quality is critical to reagent stability and assay performance. Use Milli-Q™ or a comparable system. This water is deionized, carbon-filtered and micro-organism-filtered at a pore size of 0.2 µm. It is important to maintain the system so as to keep the quality of the water high. Unless otherwise specified, "water" in this method refers exclusively to Milli-Q™ water or water of equivalent quality, i.e. HPLC-grade water.

(2) **PBS** - Phosphate Buffered Saline Solution (pH 7.4 at 25 °C)
Add one envelope PBS to 1 liter water and mix well. Do not use a stir bar. Typically made up in polypropylene bottles. Store at nominal 2 to 8°C (maximum desirable range 1 to 10°C) for a maximum of one month.

PBS - Phosphate Buffered Saline (Catalog No. 1000-3).
Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.
Tel. (800) 325-3010. STORE AT ROOM TEMPERATURE.

(3) **10X PBS/1% BSA (10X)**

Add one envelope of Sigma PBS and 1.0 g of Bovine Serum Albumin (BSA), Fraction V, or BSA giving equivalent performance in the assay, to 100 mL of water. Add stir bar and mix for at least two hours (this stir bar should only be used to mix the 10X). Store 10 mL aliquots in 15-mL polypropylene tubes at 2 to 8°C for a maximum of one month. Equilibrate at room temperature with stirring before use to dissolve crystals which may have formed.

BSA - Bovine Serum Albumin, Fraction V (Catalog # JTA464-2). VWR Scientific, P.O. Box 626, Bridgeport, NJ 08014. Tel. (800) 932-5000. STORE AT 2-8°C.

(4) **PBS/0.5% BSA (1X)**

Add 5.0 g of Bovine Serum Albumin, Fraction V (BSA) to 1000 mL of prepared PBS. Add stir bar and mix well (this stir bar should only be used to mix the 1X). Store at 2 to 8°C for a maximum of one month.

(5) **Wash Buffer (PBS-Tween)**

Add four envelopes of Sigma PBS and 2 mL of Tween 20 to 4 L water. Add a stir bar and mix for approximately 20

minutes (this stir bar should only be used to mix the wash buffer). Make fresh daily.

TWEEN 20 - Polyoxyethylenesorbitan Monolaurate (Catalog No. P1379). Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178. Tel. (800) 325-3010. STORE AT ROOM TEMP.

(6) Coating Antigen Master Stock Solution

Ovalbumin conjugates, stored frozen at -20°C or -70°C . Details for each SU can be found in the Method Performance Specification Summaries of the Appendices.

(7) Coating Antigen Intermediate Stock Solution

Prepare a dilution of an aliquot of Coating Antigen Master Stock Solution in PBS to give a concentration such that 1 mL of this dilution + 99 mL of PBS provides the desired coating antigen concentration for coating five plates.

For example, 1 mL of a $10\ \mu\text{g}/\text{mL}$ intermediate stock solution + 99 mL PBS provides a coating antigen solution of $0.1\ \mu\text{g}/\text{mL}$.

To make 20 plates, add 4 mL of coating antigen intermediate stock solution to 396 mL PBS. Stir and use immediately. Note that a separate stir bar should be reserved for each SU coating antigen intermediate stock solution prepared in order to prevent problems with contamination.

(8) Anti-SU Antilody Tablets

Hydrate the tablet(s) in 10 mL of 10X PBS/1%BSA and rock gently until tablet(s) is completely dissolved. Do not use a stir bar. This reagent combines the antibody with the protein buffer for a one-step addition. Add 0.1 mL to each 0.9 mL of standard or sample. Note that the titer of the antibody in the 10X PBS/1%BSA is 10x desired titer in assay. Store tablets, with a desiccant pack, at $2-8^{\circ}\text{C}$ for up to one year (longer if no change in performance is observed).

(9) Second Antibody-Enzyme Conjugate Stock

Use an affinity-purified anti-(rabbit (polyclonal) or mouse (monoclonal) IgG (H+L))-alkaline phosphatase conjugate. Reconstitute each vial of lyophilized powder with 1 mL of water 24 hours before use to ensure full solubilization. Rock gently for a minimum of 1 hour. Do not vortex or shake. Follow directions on insert sheet. Store hydrated

stock at 2 to 8°C according to vendor's recommendation. Store lyophilized powder at 2 to 8°C for up to 1 year or for the shelf-life specified by the vendor. Alternate vendors may be qualified.

2nd Ab-Enzyme Conjugate - Anti-Rabbit IgG (H+L), polyclonal, alkaline phosphatase conjugate (Catalog No. 111-055-003). Jackson Immunoresearch Laboratories, Inc. P.O. Box 9, 872 West Baltimore Pike, West Grove, PA 19390. Tel. (800) 367-5296. STORE AT 2-8°C.

(10) Second Antibody-Enzyme Conjugate Reagent (2 plates)

Add 40 µL of Second Antibody-Enzyme Conjugate Stock to 40 mL of PBS/0.5% BSA. Mix gently in a 50 mL polypropylene tube. Do not use a stir bar. This gives a titer of 1:1000. Note that the titer may be adjusted in order to optimize the substrate development time. New lots or new vendors' materials may also require re-optimizing titer to obtain the desired substrate development time. Perform crossover studies between lots or vendors to ensure adequate performance in terms of substrate development time, precision, accuracy and recovery.

(11) Substrate (2 plates)

Add 8 mL of DEA buffer concentrate and two 20 mg pNPP tablets to 32 mL HPLC grade water. Rock gently until tablets completely dissolve. Do not use a stir bar.

DEA Buffer 5X Concentrate - Catalog No. 77544. New England Nuclear, 549 Albany Street, Boston, MA 02115. STORE AT 2-8°C.

pNPP - *para* nitrophenyl phosphate tablets, 20-mg foil wrapped (Catalog No. N-2765). Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178. Tel. (800) 325-3020. STORE AT -20°C.

(12) SU Analytical Standard (powder)

Analytical standards are nominally 99% pure and are available from E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Wilmington, DE 19880-0402.

(13) SU Master Stock Solution (1.0 mg/mL)

Weigh out 10.0 mg of an SU. Dissolve in 10-mL, HPLC-grade acetonitrile. Store preferably in glass vial with solvent-resistant cap or high-density polyethylene bottle. Seal and store in secondary container in freezer at

nominal -20°C. Expiration of this solution is six months; if no evaporative loss is observed, or longer if concentration is still determined to be 1.0 mg/mL (recheck at six-month intervals). Note that a 0.1 mg/mL Master Stock Solution may be prepared in lieu of the 1.0 mg/mL Stock if problems with solubility arise.

(14) SU Intermediate Stock Solutions

These are generally diluted further for control sample fortifications. The 1 ng/mL Intermediate Stock Solution (C) is serially diluted to prepare the standard curve.

Spiking Stock Concentration	Volume Spiking Stock (mL)	Volume PBS (mL)	Intermediate Stock Solution (ng/mL)
1 mg/mL	0.1	9.9	10
A	0.1	9.9	0.1
B	0.1	9.9	0.001

(15) Standard Curve Preparation

Serially dilute the 1 ng/mL Intermediate Stock Solution (C) to the following concentrations: 0.200 ng/mL, 0.100 ng/mL, 0.050 ng/mL and 0.025 ng/mL.

ANALYTICAL METHOD

Microtiter Plate Preparation

An antigen or analog of the antigen covalently conjugated to ovalbumin is used to prepare the antigen-coated 96 well, polystyrene microtiter plates. The adsorptive characteristics of the ovalbumin on polystyrene and the protein-adsorptive capacity imparted during the manufacture of the plates provides the stability of the protein adsorption to the solid phase, which is required through the several washing steps of the ELISA assay. Note that all microtiter plates from all vendors are not the same, and selection of a different manufacturer requires testing in the assay format. Plates from different vendors or from different grades from the same vendor vary in total protein absorptive capacity and in the precision of the amount of protein adsorbed per well. Note

Immuno Plate, MaxiSorp F96 microtiter plates (or equivalent) are coated with the coating antigen (200 μ L/well), dispensed from a disposable reagent reservoir with a 12-channel pipetter. Plates are covered and incubated at room temperature for a minimum of two hours or they may incubate overnight at 2 to 8°C. The plates are then washed two cycles of three (2 x 3), 300 μ L washes each. Plates are air-dried or dried in a 37°C oven for 30 minutes. Cover each plate and store in groups of five in a plastic "ziploc"-type bag with desiccant packs. Store at 2 to 8°C for up to three months (longer if performance stability is demonstrated).

Water Sample Preparation

Filter all samples containing visible particulate matter. Millipore-HV filters (0.45- μ m pore size, 25-mm diameter) are recommended. These filters contain Durapore membranes, which are made from polyvinylidene difluoride (an inert, high purity, hydrophilic polymer) and do not retain SUs. A minimum dilution of 1:10 (one part sample plus nine parts PBS) is recommended. Since natural waters may contain a wide variety of minerals, organic substances, acids, bases and suspended solids like colloidal silica, sediment, algae, microorganisms, etc., one must be alert to the possibility of a significant matrix effect on the assay response versus the aqueous standard curve. The 1:10 recommended dilution of the sample in the assay is intended to reduce the matrix effect. If control water samples are available, it is necessary to validate the method by performing spiking/recovery studies at appropriate concentrations and dilution factors to encompass the concentrations to be measured in the unknown samples. If no control samples are available, spiking/recovery studies and dilution/recovery studies should be performed on selected samples in order to validate the quantifiable range in that matrix.

Soil Sample Preparation

Step 1

Thaw approximately 35 grams of each soil sample to be analyzed overnight at 4 to 8°C in a 50-mL polypropylene centrifuge tube. This provides enough soil for the assay and for repeat analyses, if necessary. If the soil is very wet, centrifuge the following morning at 4000 RPM (3100 G) and

0°C for 30 minutes to remove excess water. Decant supernatant and proceed to next step.

Step 2

Transfer 5.0 g of each sample to a moisture balance to determine % moisture. This will be used to correct the soil wet weight in the final calculations (see Table II). Transfer 10.0 g of each soil to 50 mL tubes for extraction. Carefully transfer sample LD. At this time also set-up fortified soil controls.

Step 3

Add 20.0 mL extraction buffer to each of the 10.0-g soil samples (1:2 dilution). For fortified soils, add the spike and then the extraction milieu to 20.0 mL total volume. Vortex and then tumble for one-hour extraction. Note that the specific extraction buffers for the SUs are identified in the Method Performance Specification Summaries of the Appendices.

Step 4

Remove samples from tumbler and centrifuge at ~ 4000 RPM, 0°C for 30 minutes. Decant supernatant and filter through a Millipore-HV, 0.45- μ m pore size, 25-mm diameter filter into a 50-mL polypropylene tube, transferring label information onto that tube. Refer to Table I for any solvent specific extraction procedures before proceeding to Step 5.

Step 5

Prepare a dilution of the filtered soil extract that results in a final dilution of 1:20. The soil extracts may be held overnight at 2 to 8°C.

Assay Procedure

Step 1: Reagents

Take reagents, plates, standards and samples out of the refrigerator and allow them to warm up to room temperature before starting the assay. Inspect the 10X PBS/1% BSA tube to

make sure there are no precipitates. If it is necessary to warm the tube in a 37°C water bath to solubilize any precipitates, allow the contents to equilibrate to room temperature before adding the antibody tablet(s).

Step 2: Anti-SU Antibody Tablets

Visually inspect the integrity of the tablets before use. Do not use broken tablets. Dissolve tablet(s) in the appropriate volume of 10X PBS/1% BSA (see the Method Performance Specification Summaries in the Appendices for specific information on antibody titer). Rock tube gently until tablet(s) completely dissolve. This reagent combines the antibody with the protein buffer for one-step addition. This reagent is good for three working days if maintained at 2 to 8°C. Normally, it is made fresh and used up each day.

Step 3: Controls

Fortified (spiked) controls are prepared by spiking soil or water with appropriate concentrations across the quantifiable range using the Spiking Stock Solutions (see Reagents (14)). The controls are then processed exactly like the samples. The usual practice is to run at least one fortified control on each microtiter plate. In each working day, include controls to cover the assay range. It may also be necessary to run a dilution control for those samples out of range requiring a further dilution.

Note that the measured ng/mL in the assay must be multiplied by the dilution factor to determine ng/mL in the water samples. Calculations for ng/g in soil samples are shown in Table II.

Step 4: Assay Set-Up

Set up an appropriate number of cube 2ubes™ for each plate. See Figure 1 for a sample plate design. The use of cube 2ubes™ allows for the use of a multichannel pipette so that as many as 12 samples can be treated simultaneously. Standards are not diluted. Dispense 0.9 mL each. Controls and samples have all been pre-diluted. Dispense 0.9 mL each of control or sample. Add antibody (0.1 mL) to each of the tubes simultaneously using a reagent reservoir and a multichannel pipette and mix by aspirate/dispense X 5. Incubate at room temperature (see the Method Performance Specification

Summaries in the Appendices for specific information on incubation times). To allow for completion of each step in the assay and to keep the time the same for each plate, a 15-minute delay is suggested between plates.

Alternatively, 12 x 75 polypropylene culture tubes may be used if preferred to cube 2ubes™. If the assay is set up this way, the multichannel pipette cannot be used for antibody addition or plate loading. The EDP (or comparable) pipette, set for a multi-dispense mode, is used for each individual tube. Vortex to mix.

Step 5: Addition of Reaction Mixture to Microtiter plate

After incubation in the assay tubes, 200- μ L aliquots of the standards, controls, and samples are pipetted into wells of the antigen-coated microtiter plate. Use the multichannel pipette to transfer up to 12 samples simultaneously; three replicate rows. Begin with Row B. Change tips or have a second multichannel pipette ready for the next set of samples.

Alternatively, use the EDP (or comparable) pipette set for a multi-dispense mode to dispense the three replicates. Change tips between samples. Set timer for another incubation on the plate.

Step 6: Second Antibody-Enzyme Conjugate Addition

During the sample incubation, prepare the second antibody enzyme conjugate as described in the reagent section. After the plate incubation, wash the plate using 2 x 3 cycles on the plate washer and then pound on several thicknesses of paper towels to blot and remove any remaining droplets. Add 200- μ L freshly prepared conjugate to all wells with standards, controls, samples and to Row H (about 20 μ L/plate). Use a multichannel pipette and a disposable reagent reservoir. Load the plate as shown in Figure 1, starting with Row B. Row A is reserved for the substrate blank. Row H is used for the non-specific binding blank.

Incubate at room temperature after the plate is loaded.

Step 7: Enzyme Substrate Addition

While the plate is incubating, prepare the enzyme substrate reagent. After the plate incubation is completed, wash plate 3 X 3 cycles on plate washer and pound as described above.

Add 200- μ L substrate reagent to all wells in all rows. Begin with row B, C, D, E, F, G, H, and then A. The reaction begins immediately when substrate is added, therefore, timing differences between standards and samples must be minimized. Set clock and monitor yellow color development. Note that the enzyme-substrate reaction is temperature sensitive so the reaction time may vary from day to day.

A satisfactory standard curve and sample data may be obtained when the 0 absorbance is at least 1.0 ± 0.1 AU if the substrate has been on the plate at least 30 minutes or the final readings can be taken when the 0 absorbance is near 2.0 AU.

CAUTION: The VMax[®] microtiter plate reader has an automix function which should be turned on. Each time you read a plate, the reaction mixture in the wells is mixed which is desirable for assay precision. However, this also has the effect of accelerating the reaction kinetics. Therefore, the second plate should be read as often and at approximately the same time intervals as the first plate to minimize any differences between the two plates. It is also recommended that an initial read should be done right after substrate addition to insure good mixing.

DATA COLLECTION AND ANALYSIS

Instrumentation

The absorbance of each of the 96 wells of the microwell plate is read on a microplate reader equipped with a 405-nm filter and processed by a computer program which generates a standard curve based upon a four- or five-parameter logit function. The microplate reader set-up conditions are as follows:

Control Mode:	Computer
Assay Read Mode:	Endpoint
Wavelength:	405 nm
Data Display:	Raw
Standard Curve Fit:	4-parameter logit
Auto Mix:	On
Auto Calibrate:	On

The standard four-parameter logit curve generated from this type of assay on semi-log paper is sigmoidal in shape with absorbance (AU, mAU or O.D.) on the Y axis and the log of the concentration of the compound on the X axis. Such curves can fit the following equation:

$$y = \frac{A - D}{1 + (x/C)^B} + D$$

Initial estimates of the coefficients may be made as follows:

- A = upper asymptotic absorbance, approximated by the absorbance of the "zero" standard
- B = slope of the curve at the inflection point, approximated by the decrease in absorbance per 10-fold increase in analyte concentration
- C = value of x at the inflection point of the curve
- D = lower asymptotic absorbance, approximated by the absorbance of the highest standard

The computer program makes an iterative adjustment of A, B, C, and D until the residual sum of the squares of the differences between measured standard points and their calculated concentrations is minimized (a nonlinear regression). The computer then uses the fitted equation to calculate concentrations for the samples and controls from their absorbance readings. For more information, see your software manual for the exact equation form and iteration algorithm used.

Quality Control Criteria

A visual inspection of the curve shows whether the line fits the points, the degree of scatter of the data and whether a gross error such as switching two standards has occurred. A more quantitative evaluation of the curve fit is shown by the correlation coefficient (R-Sqr), standard error (Sterr) and residuals (% difference between actual and calculated standard concentrations). Note that the R-Sqr correlation coefficient usually runs between 0.95 and 0.99 for a curve with good precision and fit. Calculation of % Inhibition (% I) of each of the standards provides additional quantitative information on curve fit. The % Inhibition (% I = ((A-B)/A) x 100, where A is the absorbance of the negative control and B is the absorbance

of the sample) should be similar from plate to plate and day to day. Any significant change ($> 10\%$) suggests a problem which should be investigated. Typical curve fits for the SUs are shown in the Appendices.

For quality control, monitoring the substrate blank (three or more wells in Row A which received substrate only) and the conjugate blank (three or more wells in Row H which received conjugate and substrate only) on each microtiter plate is recommended. A significant change in mAU for any of these blanks, plate to plate, may indicate a problem. A change in reagent lots may also cause a shift in one or more of the blank readings. If there is an apparent problem, fresh reagents should be prepared and the samples rerun.

Precision, as measured by % Coefficient of Variation ($\% CV = (\text{standard deviation}/\text{mean}) \times 100$), is another measure of the reliability of the assay. The absorbance precision should be 10% or less (i.e., $< 10.5\%$). If any set of triplicates is over 10.5% CV, inspect the data to see whether one of the triplicates is an outlier (> 2 standard deviations above or below the closest of the two remaining readings, with the standard deviation being that of the remaining two readings). Outliers may be deleted and the data recalculated, provided the % CV of the two remaining readings is $< 10.5\%$. If no outlier is found, the sample must be rerun. Following these criteria assures that the %CV of the analyte concentration will be about 20% or less. Typical precision data can be found in the Appendices.

Also note that the measured SU concentration in the fortified control must be within specifications ($\pm 30\%$ of target) in order to accept data from that plate. If more than one fortified control was run on a plate, and one does not meet specifications, then all samples with SU concentrations corresponding to that control level must be rerun.

Duplicate samples must also show SU concentrations within $\pm 30\%$ of each other to show reproducibility of the method.

Final Data Acceptability, Calculating, and Reporting

Apply the Quality Control Criteria to the plate and the calculated individual sample results as described above.

Determine whether any samples fall below the Limit of Quantitation (LOQ) and report concentration as " $< LOQ$ ". The Limit of Quantitation is the minimum concentration at which the method meets the specifications for precision, accuracy and

spiking recovery. This was determined to be 0.5 ng/mL for water samples and 0.0 ng/mL for soil samples.

For results within the quantifiable range, see Table II, Calculation of Assay Results, to determine the concentration in the original undiluted sample.

Determine whether any samples fall above the quantifiable range. These should be rerun at an appropriate dilution factor so that the rerun result will fall within the quantifiable range. A control, spiked to a similar concentration and diluted by the same factor, should also be run.

Finally, if there is any doubt about the response in the assay attributable to anything other than the compound being measured, the test of parallelism should be applied. Serial dilutions of the questionable sample should show linearity on dilution, or 100 +/-30% recovery of the calculated concentration.