# AN ELISA IMMUNOASSAY METHOD FOR THE DETERMINATION OF RESIDUES OF METHOMYL IN WATER, SOIL, AND SEDIMENT

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## INTRODUCTION/SUMMARY

### *Scope*

Methomyl is used as a foliar spray and controls many insects including: Alticinae, Aphidiae, and Lepidoptera in cereals, citrus, cotton, and many other crops. The method described here has been developed to measure residues of methomyl in water, soil, and sediment.

The method is an enzyme-linked immunosorbent assay (ELISA) for measurement over a range of 0.05 to 5 parts per billion (ppb). The Limit of Quantitation (LOQ) in the assay is 0.1 ppb, which initiates the dynamic operating range of the assay. This translates to a LOQ in a water sample of 0.2 ppb. The LOQ of a soil or sediment sample is set at 10 ppb. Mean recoveries of spiked methomyl in water, soil, or sediment are within 100 ± 30%.

### *Principle of Method*

(1) Polyclonal anti-methomyl antibodies (Ab) and buffer are added to sample containing an unknown amount of methomyl and incubated. The antibodies bind to any methomyl 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 a methomyl derivative-ovalbumin conjugate. Any excess Ab not bound to methomyl in the sample will bind to methomyl immobilized on the microtiter plate. The plate is then washed to remove any Ab not bound to the plate. The amount of Ab bound to each microtiter well is an inverse measure of the amount of methomyl in the sample.

(3) To detect the Ab bound to each microtiter well, an anti-rabbit antibody conjugated to an enzyme, alkaline phosphatase (Ab-E), is added to each well and incubated

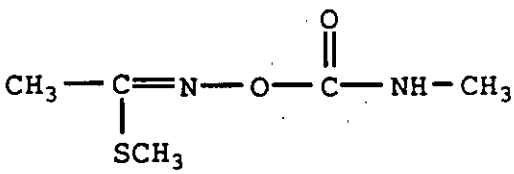
This Ab-E will bind to any anti-methomyl antibodies bound to the microtiter plate. The microtiter plate is then washed to remove any unbound Ab-E.

(4) An alkaline phosphatase substrate, para-nitrophenylphosphate, is added to the microtiter wells. The enzyme-substrate reaction produces a yellow color which is inversely proportional to the concentration of methomyl 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 methomyl concentrations for each unknown on the plate.

## MATERIALS AND METHODS

### *Compound Identity*

*Structure*



*Chemical Name*

S-methyl N-[(methylcarbamoyl)oxy]thioacetimidate

*Common Names*

methomyl

*DuPont IN Code No.*

X1179

### *Equipment*

Disposable glassware or plasticware is recommended for all possible steps to minimize contamination. Polypropylene is preferred for plasticware, but high-density polyethylene is also acceptable. In addition, comparable equipment may be substituted for all equipment; however, note any specifications in the following descriptions before substituting other equipment.

(1) Tubes:

Centrifuge, polypropylene; 15 and 50 mL: Falcon Blue Max #2097 & 2098, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035.

Assay tubes, 12 x 75 mm polypropylene culture tubes: VWR #60818.

Cube 2ubes®, polypropylene: DBM Scientific Corporation, 511 Fifth Street, San Fernando, CA 91340, (818) 360-3610, Catalog No. CT-205

(2) Pipettes:

Eppendorf Repeater 4780, with reservoir tips of 0.5-, 2.5- and 5-mL capacity: Brinkman Instruments, Cantiaque Road, Westbury, NJ 11590.

edp™ motorized pipettes, 0.25, 1.0, 2.5, and 10.0 mL: Rainin Instrument Co., address above.

Titertek 12-channel adjustable volume (0.050-0.300 mL) multipipettes with tips: Flow Labs, 7655 Old Springhouse Rd., McLean, VA 22102.

Biohit Proline® Electronic Multichannel Pipettors; 1.0 mL, 50 to 1000 µL, and a Multichannel (12) 25 to 250 µL. The 2.5-mL capacity is not available at this time. These are made in Finland and distributed by Vangard International, Inc., IIII-A Green Grove Road, P. O. Box 308, Neptune, NJ 07754-0308, 1-(800)-922-0784.

(3) Reagent Reservoirs: disposable, #4870: Costar Corp. 206 Broadway, Cambridge, MA 02139.

(4) ELISA Microtiter Plates: NUNC-Immunoplate, IF with certificate, Cat. No. 439454: Distributed in U.S.A. by Vangard International, Inc., 1111-A Green Grove Rd., P.O. Box 308, Neptune, NJ 07753. Other plates from this vendor (e.g., stripwell plates) or plates from other vendors must give equivalent performance in terms of accuracy, precision, and recovery in order to be substituted.

(5) ELISA Plate Washer: Ultrawash II Dynatech Laboratories, Inc. 14340 Sullfield Circle, Chantilly, VA 22021.

(6) ELISA Plate Reader: Titertek Multiskan MCC/340 Plate Reader, equipped with a 405-nm filter: Flow Labs, address above, or UV max Kinetic Microtiter Plate Reader, Molecular Devices Corporation, 3180 Porter Drive, Palo Alto, CA 94304 or comparable equipment.

(7) Computer and Software:

IBM Personal System 2 Model 50, with one hard drive, one floppy drive, IBM Proprinter II printer and IBM Disc Operating System Software Version 3.30: IBM Corporation, 1001 Jefferson St., Wilmington, DE 19801.

TiterCalc Software Version 2.1: Hewlett-Packard, Rt. 41 - Starr Rd., P. O. Box 900, Avondale, PA 19311 or

Macintosh SE/30 with LaserWriter Plus printer and Macintosh Disk operating system 6.07 or comparable equipment.

Microsoft® Mouse and Microsoft® Windows Software Version 2.0:

Microsoft Corporation, 16011 NE 36th Way, Box 97017, Redmond, WA 98073-9717.

(8) pH Meter: Corning Model 120 pH Meter: Corning Glass Works, Medfield, MA 02052.

(9) Balance: Mettler PM 460 Balance: Mettler Instrument Corp., P. O. Box 71, Hightstown, NJ 08520.

(10) Vortex Mixers and Stir Plates: VWR brand: distributed by VWR Scientific, P. O. Box 626, Bridgeport, NJ 08014.

(11) Filters: Millex-HV Unit, 0.45-µm pore size, 25-mm diameter: Millipore Products Division, Bedford, MA 01730. Other filters may only be substituted if assay performance is validated and found to be comparable to the performance with these filters.

(12) B.D. Disposable Syringes: 20-cc Plastipak without needles, VWR Rutherford, NJ 07070.

(13) Tumbler: Design of tumbler for soil extraction (Appendix B).

(14) Sorvall RC5C Centrifuge: A comparable refrigerated model with capacity for 50-mL centrifuge tubes at 5000 RPM may be substituted. Du Pont/Sorvall Marketing Services, Barley Mill Plaza, Wilmington, DE 19898.

(15) Infrared Moisture Determination Balance: Model AD-4712 or a comparable moisture determination balance. A&D Engineering, Inc., 1555 McCandless Drive, Milpitas, CA 95035

***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.

(A) 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. In some cases, it may be desirable to use U.S.P. grade pyrogen-free sterile water to make up plate coating and blocking buffers. Unless otherwise specified, "water" in this method refers exclusively to Milli-Q™ water or water of equivalent quality, i.e. HPLC-grade water.

B) Phosphate-Buffered Saline Solution (PBS):

120 mM NaCl
2.7 mM KCl
10 mM Phosphate Buffer
pH 7.4 at 25°C
0.02% Sodium Azide (Optional)

Add one bottle Sigma Diagnostics Phosphate Buffered Saline powder (PBS, Catalog #1000-3, Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178) to 1-liter water and stir until dissolved. 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. If preservative is required for longer storage, add 0.2-g sodium azide (Sigma #1000-3, or equivalent).

(C) 10 x PBS/1% BSA:

1.2 M NaCl
27 mM KCl
100 mM Phosphate Buffer
1% BSA (Bovine Serum Albumin)
0.02% Sodium Azide (Optional)
pH 7.4 at 25°C

Add one bottle of Sigma PBS (see B) to 70 mL of water. Stir to dissolve and then add 1 g of Bovine Serum Albumin (BSA), Fraction V, or BSA giving equivalent performance in the assay, and stir until all clearly dissolves. Add 0.02-g sodium azide if desired. Make up to 100-mL total volume with water, and store 10mL aliquots in 15-mL polypropylene tubes at 2 to 8°C for a maximum of one week without azide and for one month

with azide. Equilibrate at room temperature with stirring before use, to dissolve crystals which may have formed.

(D) PBS/0.5% BSA:

120 mM NaCl
2.7 mM KCl
10 mM Phosphate Buffer
0.5% BSA
0.02% Sodium Azide (Optional)
pH 7.4 at 25°C

Add one bottle of Sigma PBS (see B) to 900 mL of water and stir until dissolved. Add 5 g of Bovine Serum Albumin (see C) and stir until dissolved. Make up to 1000-mL total volume, with water. Store at 2 to 8°C for a maximum of one week without azide and for one month with azide.

(E) Wash Buffer (PBS-Tween):

120 mM NaCl
2.7 mM KCl
10 mM Phosphate Buffer
0.05% Tween-20
pH 7.4 at 25°C

Add four bottles Sigma PBS (see B) to 4 L water. Add 2-mL Tween-20. Stir for approximately 20 minutes. Store at room temperature for a maximum of 36 hours. Make fresh daily.

(F) Coating Antigen Master Stock Solution:

Methomyl Hapten B3-ovalbumin conjugate (Figure 1), Lot No. E6298, made Jan 15, 1990, 5.8 mg/mL protein, stored frozen at -20°C or -70°C.

(G) Coating Antigen Intermediate Stock Solution:
1000 µg/mL

Prepare a dilution of an aliquot of Coating Antigen Master Stock Solution in PBS to give a concentration of 1000 µg/mL (1.0 mL Master Stock Solution + 4.8 mL PBS) and freeze, 1.0-mL aliquots at -20°C. Freeze the remainder as 0.1-mL aliquots.

The 0.1-mL aliquots containing 100 µg may then be further diluted (0.1 mL + 9.9 mL PBS) to prepare a coating antigen reagent solution of 10 µg/mL which may be stored at 2 to 8°C.

(H) Coating Antigen Reagent: 0.1 µg/mL

To prepare 20 plates, add 4 mL of the 10 µg/mL antigen reagent solution to 396 mL PBS while stirring. This solution now contains 0.1 µg/mL. Use immediately. Do not store.

(I) Blocking Buffer (3% BSA) (Use of this reagent is not necessary for this assay):

Add 15 g BSA to 500 mL PBS. Stir without heat until all is clearly dissolved (about 1 hr). Use immediately. Do not store. Makes 20 plates.

(J) Anti-Methomyl Antibody Master Stock (Neat):

This is a polyclonal Anti-Methomyl antibody. This material is stored at -70°C for longest shelf-life (approximately 5-10 years), but may be stored at -20°C for short periods (months) in a manual defrost freezer if necessary. Do not store in a frost-free freezer.

The current lot in use is from Rabbit 1413, Hapten A1 (Figure 1), bleeds from Fall/91. Other lots of comparable performance may be used after method performance is validated with that lot.

(K) Anti-Methomyl Antibody Tablets

Each tablet delivers the equivalent amount of antibody as the liquid formulation when hydrated as described. The current tablet lot, MeP-74610-38-1, contains tris buffer, BSA, trehalose, and polyethylene glycol with 1 µL of anti-methomyl antibody per tablet. Hydrate the tablet in 5 mL of 10XPBS/1% BSA and rock gently until tablet is completely dissolved. 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.

X2 for 1 plt ?

(L) Second Antibody-Enzyme Conjugate Stock:

Use an affinity-purified goat anti-[rabbit IgG (H + L)]-alkaline phosphatase conjugate. Recommended vendor is Jackson Immunoresearch Laboratories, Inc., 872 West Baltimore Pike, P.O. Box 9, West Grove, PA 19390, Catalog #315-055-003. Reconstitute each vial of lyophilized powder with 1-mL 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 the conjugate insert sheet for reconstitution and storage. Store hydrated stock at 2 to 8°C for up to one month or 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.

(M) Second Antibody-Enzyme Conjugate Reagent:

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. This is enough for two plates. This gives a substrate development time of 40 to 60 minutes. The titer may need to be adjusted in order to keep the substrate development time in the optimal 40- to 60-minute range. New lots or new vendors' materials may 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.

(N) Substrate Buffer Stock:

10% Diethanolamine
0.1 g/L $MgCl_2 \cdot 6H_2O$
0.02% Sodium Azide

Order from New England Nuclear, 549 Albany Street, Boston, MA 02118, DEA Buffer 5X Concentrate, Code #77544. This is part of the DuPont EBV ELISA Kit but only order the buffer. If it is not available, a 10% diethanolamine solution can be made and used as follows:

Add 97-mL diethanolamine, 0.2-g sodium azide and 0.1-g magnesium chloride ($MgCl_2 \cdot 6H_2O$) to 800-mL water.

Adjust the pH to 9.8 with concentrated HCl. Make up to 1000 mL with water.

(O) Enzyme Substrate Reagent: 1 mg/mL p-nitrophenylphosphate (PNPP)

1-mg/mL p-nitrophenylphosphate (PNPP)
2% Diethanolamine
0.02 g/L $MgCl_2 \cdot 6H_2O$

Make fresh before use. For two plates, add 32 mL of water to 8 mL of Substrate Buffer Stock and mix. Add two 20-mg substrate tablets and mix until dissolved in a 50-mL disposable polypropylene tube. A better grade of substrate tablet, which comes as 20-mg tablets in individual foil wrappers (Sigma Catalog No. N-2765), is recommended. These tablets are made to tighter specifications and designed for ELISA use.

(P) Soil/Sediment Extraction Solvent: 90% acetone/10% PBS

Dispense 20 mL/10-g soil. Use the EDP 10-mL pipette.

(Q) Methomyl Analytical Standard (powder):

Methomyl analytical standards are nominally 99% pure. The current lot in use is IN# X1179-357, 99.7% pure, available from E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Wilmington, DE 19880-0402.

(R) Methomyl Stock Solution:

0.1 mg/mL methomyl in methanol (100,000 ng/mL)

Weigh out 10.0 mg of methomyl and place in a 100-mL volumetric flask. Dissolve in 100-mL, HPLC-grade methanol. Store preferably in glass vial with solvent-resistant cap or high-density polyethylene (HDPE) 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 0.1 mg/mL (recheck at six-month intervals). Rinse stir bars with acetone to avoid contamination of other solutions with standard.

(S) Methomyl Intermediate Stock Solutions:

These are generally used to make further dilutions in order to make standards, but can also be used for control sample fortifications if desired.

10,000 ng/mL Stock: Add 1 mL of Methomyl Master Stock Solution to 9 mL PBS and mix. Store at 2 to 8° for one month.

1000 ng/mL Stock: Add 1 mL of 10,000 ng/mL Stock to 9 mL PBS (or equivalent proportions) and mix. Store at 2 to 8°C for one month.

(T) Methomyl Spiking Stock Solutions:

These are used to prepare standards. They are stored at 2 to 8°C for up to one month. They are all 100x the desired final concentration so that the spiking volume for the standards is minimal and the same at each level.

These spikes may also be used for fortified controls.

(1) Preparation of Spiking Stock Solutions:

| Volume (mL) of 1000 ng/mL Stock | Volume of PBS (mL) | Spiking Stock Concentration (ng/mL) |
|---|---|---|
| 0.00 | 10.00 | 0.0 |
| 0.05 | 9.95 | 5.0 |
| 0.1 | 9.9 | 10.0 |
| 0.25 | 9.75 | 25.0 |
| 0.50 | 9.5 | 50.0 |
| 1.0 | 9.0 | 100 |
| 5.0 | 5.0 | 500 |

(2) Preparation of Standards:

*Water Assay*

Normally, a large volume (40 mL) of each standard is prepared in 50-mL polypropylene tubes. Add 400 µL of each Spiking Stock Solution to a tube containing 39.6-mL 50/50 PBS/water. Preparation of 40 mL of each standard reduces the potential for variability when making standards fresh each day. It also improves

precision and accuracy by eliminating the need to pipette extremely small amounts of spiking solution for smaller volumes of standards. The zero standard should be from the same PBS/water solution used to dilute samples. The standards are stable for up to one month stored at 2 to 8°C.

*Soil/Sediment Assay*

Prepare standards the same as above except use 100% PBS instead of 50/50 PBS/water.

| Spiking Solution Used (ng/mL) | Standard Concentration (ppb) |
|---|---|
| 5.0 | 0.05 |
| 10.0 | 0.1 |
| 25.0 | 0.25 |
| 50.0 | 0.5 |
| 100 | 1.0 |
| 500 | 5.0 |

## ANALYTICAL METHOD

### *Microtiter Plate Preparation*

An analog (see Figure 1) 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 in 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.

Nunc-Immuno Plate, MaxiSorp F96 (or equivalent) microtiter plates are coated with the Coating Antigen Reagent (200 µL/well), dispensed from a disposable reagent reservoir with a 12-channel pipettor. Plates are covered and incubated at room temperature (20° to 25°C) overnight on laboratory bench. As an alternative, plates may also be incubated at 2° to 8°C

overnight. The following morning, the plates are washed two cycles of three, 400-µL washes each (2 x 3), turning the plate 180° between wash cycles. Note that 180° rotation of the plate on the plate washer after each wash ensures that each well is washed at least once in case of a blocked tube in the dispensing head. 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***

To remove foreign materials, or as a routine precaution, filtering all samples through a Millipore-HV, 0.45-µm pore size, 25-mm diameter filter is recommended. These filters contain Durapore membranes, which are made from polyvinylidine difluoride (an inert, high purity, hydrophilic polymer) and do not retain methomyl. A minimum dilution of 1:2 (one part sample plus one part PBS) is recommended. Since natural waters may contain a wide variety of minerals, organic substances, acids, bases, 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 1:2 recommended dilution of the sample in the assay reduces the matrix effect. If control water samples are available, it is necessary to validate the method by performing recovery studies of spiked samples 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, to validate the quantifiable range in that matrix.

If a sample needs to be rerun at a dilution greater than 1:2, a spiked control for that new dilution level needs to be run at least once per study site to verify impact of each matrix. Running a spiked control at each new dilution once per study site is sufficient.

***Soil Sample Preparation***

Assay 30 samples/day, n=3, over two plates. On each plate, include seven standards, two controls, and 15 samples.

*Step 1*

Thaw soil samples overnight in sample containers at 2° to 8°C.

*Step 2*

Transfer 5.0 g of each sample to moisture balance to determine % Moisture and record on worksheet. (This will be used to correct the soil wet weight in the final calculations.) Transfer 10 g of each to 50-mL tubes for extraction. Carefully transfer sample I.D. At this time also set-up fortified soil controls.

*Step 3*

Add 20-mL acetone/PBS to each of the 10-g soil samples. For fortified soils, add the spike and then acetone/PBS to 20-mL total volume. Vortex and place on tumbler for one-hour extraction. Design of tumbler is shown in Appendix I.

*Step 4*

Remove samples from tumbler and centrifuge at ~4000 RPM, 0°C, for 30 minutes. Decant supernatant and filter it through a Millipore-HV, 0.45-µm pore size, 25-mm diameter filter into a 50-mL polypropylene tube, transferring label information onto that tube. These filters contain Durapore membranes, which are made from polyvinylidine difluoride (an inert, high purity, hydrophilic polymer) and do not retain methomyl. These extracts may be held overnight at 4 to 8 °C.

*Step 5*

Pipette 1.0 mL of each filtered extract into a 15-mL polypropylene tube and take to near dryness under nitrogen or filtered, compressed air, 40 psi, in a 50°C water bath. Do not over dry. Reconstitute with 10 mL of PBS and then vortex each tube vigorously. Store at 2 to 8°C until analysis. CAUTION: The 1.0 mL of extract must be dispensed accurately.

Each day, another batch of samples can be prepared for analysis the following day. It is likely that there will be some repeats each day from the previous day at different dilutions. For that reason, samples should be retained at 2 to 8°C until reportable results are obtained.

***Sediment Sample Preparation***

*Step 1*

Thaw 35 grams each of sediment sample overnight at 2° to 8°C in a 50-mL polypropylene centrifuge tube. The following morning, centrifuge at 4000 RPM, (3100 G) and 0°C for 30 minutes to remove excess water. Decant supernatant and proceed to Step 2 above.

***Assay Procedure***

*Step 1: Reagents*

Take reagents, plates, standards, and samples out of the refrigerator to allow them to warm up to room temperature before starting the assay. For 10XPBS/1% BSA, take out the 10-mL aliquot in the 15-mL polypropylene tube to prepare the antibody reagent. Inspect the tube to make sure there are no precipitates present. If it is necessary to warm the tube to solubilize any precipitates, a 37°C water bath is recommended. Allow the contents of the tube to equilibrate to room temperature **before** adding the antibody tablets.

*Step 2: Anti-Methomyl Antibody Tablets*

Visually inspect the integrity of the tablets you are going to use. Sometimes a piece of a tablet may be broken off, for example. In that case, discard the tablet. Do not try to use any tablets that are not perfect. Dissolve two tablets in 10 mL of 10XPBS/1% BSA. Rock tube gently until tablets completely dissolve. This reagent combines the antibody with the protein buffer for one-step addition. Add 0.1-mL antibody solution to each 0.9 mL of sample. Note that 10-mL antibody solution provides enough reagent for analysis of ~100 samples (1.0 mL each). 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, sediment, or water with appropriate concentrations across the quantifiable range using the Spiking Stock Solutions and 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.

For aqueous samples which are diluted 1 in 2, simply multiply measured ppb x 2 to equal ppb in the sample. For soil and sediment samples, after drying one mL of extract and reconstitution in 10-mL PBS, each mL = 0.1-mL supernatant. This means that the measured ppb in the assay must be multiplied by 10 to determine ppb supernatant. All samples and controls are diluted to minimize potential matrix effects. For further calculations for ppb soil or sediment sample, see Table I.

*Step 4: Assay Set-Up*

Set up two rows of 12 cube 2ubes® for each plate. See Figure 2 for plate layout. Cube 2ubes® are designed to use with a multichannel pipette so the 12 samples can be handled 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 12 tubes simultaneously using a reagent reservoir and the multichannel (12) pipette. Mix by aspiration and dispense X 5. Change tips and dispense antibody into the second row of tubes immediately following the antibody addition to the first set. *NOTE: You do not use reverse pipetting mode for this step.* Incubate at room temperature for 60 minutes. To allow for completion of each step in the assay and keep the time the same for each plate, a 15-minute delay is suggested between each plate throughout the assay.

*Step 5: Addition of Reaction Mixture Aliquots to Microplate*

After a 60-minute incubation in the assay tubes, 200-µL aliquots of the standards, controls, and samples are pipetted into wells of the antigen-coated microplate plate according to the typical plate format shown in Figure 2. Use the multichannel pipette in the reverse pipetting mode. Change tips or have another multichannel pipette ready for the second row of 12 samples. Set timer for another 60-minute incubation on the plate.

Alternatively, the 12 x 75 polypropylene culture tubes may be used if preferred to the cube 2ubes® and a sample size of

1.8 mL (vs. 0.9 mL). If the assay is set up this way, the multichannel pipette cannot be used for antibody addition or plate loading. The EDP pipette, set for a multi-dispense mode, is used for each individual tube.

*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-µL freshly prepared conjugate to all wells with standards, controls, samples and Row H (about 25 mL/plate). Use a multichannel pipette and a disposable reagent reservoir. Load the plate from top to bottom 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 for 60 minutes after the plate is loaded.

*Step 7: Enzyme Substrate Addition*

During the plate incubation, prepare the enzyme substrate reagent. After the plate incubation, wash plate 3 X 3 cycles on plate washer and pound as described above. Add 200-µL substrate reagent to all wells in all rows in the same manner as described in the conjugate addition except now end with Row A. Note that reaction begins immediately when substrate is added; therefore, timing differences between wells must be minimized. This is the reason for the order of addition. 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.

Alternatively, begin with Row B, and then Row E, and so forth. This is recommended for soil samples to minimize the timing between standards and samples. It is a good idea to mark the plate by Row B and Row E and to draw a line across the plate between Row D and E as a guide to expedite loading of substrate.

A satisfactory standard curve and sample data may be obtained when the 0 absorbance is at least 1000 ± 100 mAU if the substrate has been on the plate at least 45 minutes or the final readings can be taken when the 0 absorbance is 2000 mAU (2.0) O.D.

CAUTION: The UVMax® 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.

***Instrumentation***

Data are collected on a microtiter plate reader with an IBM PS/2-50 computer running Titercalc 2.1 software in Microsoft Windows, or on a Molecular Devices UVMax plate reader with a Macintosh SE/30 computer running SOFTmax version 2.01. Equivalent equipment and software can be used. In general, the following parameters apply:

| | |
|---|---|
| Control Mode: | Computer controls reader |
| Assay Read Mode: | Endpoint |
| Wavelength: | 405 nm |
| Standard Curve Fit: | 4-parameter logit |
| Automix: | On |
| Computer Report Contents: | Run date, disc file number, plate format, plate absorbance readings, standard curve data, curve fit parameters and graph, control and/or blank data, sample data, and calculated concentration results |

***Data Collection and Analysis***

The absorbance of each of the 96 wells of the microtiter plate is read on a microtiter plate 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 standard four-parameter logit curve generated from this type of assay 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:

$$y = a + \frac{(d-a)}{(1 + e^{b(c-z)})}$$

where z = LN (x)

Initial estimates of the coefficients may be made as follows:

a = lower asymptotic absorbance

b = slope of the curve at c

c = ln(x) at the inflection point of the curve

d = upper asymptotic absorbance

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 in ppb 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***

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 calculation of % inhibition of each of the standards, the correlation coefficient (R-Sqr), standard error (Sterr), and residuals (% difference between actual and calculated standard concentrations). The % inhibition (0 mAU minus standard mAU / divided by 0 mAU) X 100 = % Inhibition) should be constant from plate to plate and day to day. Any significant change (>10%) suggests a problem which should be investigated. The R-Sqr correlation coefficient usually runs between 0.95 and 0.99 for a curve with good precision and fit. Precision, as measured by % CV (% coefficient of variation = standard deviation/mean x 100), is another measure of the reliability of the assay. Typical precision data are shown in Table II. A typical curve fit is shown in Figure 3.

For quality control, monitoring the substrate blank (three or more wells in Row A which received substrate only), and 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 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.

The absorbance precision as % CV 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 concentration % CV for results will be about 20% or less.

The measured methomyl concentration in the fortified control must be within specifications (± 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 methomyl concentrations corresponding to that control level must be rerun.

Parallelism of the method in the matrix under study was investigated by running more than one dilution of a sample, with all results falling within the quantifiable range of the assay, to assure that the same result is obtained at the different dilutions, 30%.

Two-replicate aliquots of a sample must show methomyl concentrations within ± 30% of each other to show reproducibility of the method.

***Limits of Detection and Quantitation***

Limit of Detection (LOD): The minimum concentration which is statistically significantly distinguishable from 0. This can be assessed by running multiple replicates at 0 and the LOD, and performing a t-test to determine whether the absorbances are statistically different to a confidence level of 95% (p-value ≤0.05). It has also been defined as the concentration at which 15% inhibition of the "0" standard absorbance reading occurs.

Limit of Quantitation (LOQ): The minimum concentration at which the method meets the specifications for precision, accuracy, spiking recovery, and parallelism (if applicable).

***Final Data Acceptability, Calculating, and Reporting***

Apply the Quality Control criteria to the plate and the calculated individual sample results as described above. See Table I for calculation of assay results. Refer to Appendix III for a summary of guidance on acceptable data.

Determine whether any sample falls below the LOQ and report concentration as "<LOQ." Determine whether any sample falls above the range. This should be rerun at an appropriate dilution factor which can be estimated from the result, 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.

Refer to Appendix III to determine whether an entire run is acceptable.