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Page 1

RELDAN INSECTICIDE

Study Title

Validation Phase Report:

Validation of EnviroGard Chlorpyrifos-methyl (Reldan®)
Screening Kit

Data Requirements

Guideline Reference No. 171-4(C)

Author

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Study Completion Date

May, 1993

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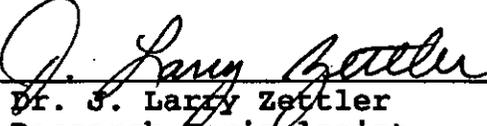
Gustafson, Inc.
1400 Preston Road, Suite 400
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Study Title: Validation Phase; Validation of EnviroGard
Chlorpyrifos-methyl (Reldan) Screening Kit.

STATEMENT OF COMPLIANCE

This study was designed and performed in accordance with Pesticide Assessment Guidelines promulgated by the U.S. Environmental Protection Agency in compliance with Good Laboratory Practices Standards (Fed. Reg., Vol. 54, No. 158 (1989)).

Phase Investigator:

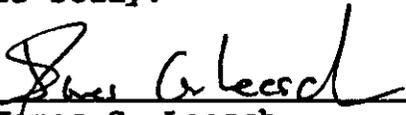

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6.4.93
Date

Quality Assurance Statement

Study Title: Phase Report; Validation of EnviroGard Chlopyrifos-methyl (Reldan) Screening Kit.

In compliance with Good Laboratory Practice Regulations, the Quality Assurance Unit has inspected this study. Inspection findings were reported to management, phase director, and the study director. Quality Assurance has reviewed the phase study report and certify that it accurately describes the methods and standard operating procedures and the report results accurately reflect the raw data of this study.

Signature:  5/28/93
Date
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<u>Inspection</u>	<u>Date Reported:</u>		
	<u>Study Director</u>	<u>Management</u>	<u>Phase Director</u>
Analytical	<u>3/10/93</u>	<u>3/10/93</u>	<u>3/10/93</u>
Test Kit	<u>3/15/93</u>	<u>3/15/93</u>	<u>3/15/93</u>
Final Report	<u>6/1/93</u>	<u>6/1/93</u>	<u>6/1/93</u>

Study Title: Phase Report; Validation of EnviroGard Chlorpyrifos-methyl (Reldan) Screening Kit.

USDA Project No. 921025

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Phase Study Time Table:

Start Date: February 8, 1993
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ABSTRACT

Grain which had been treated in a manner to simulate field applications was analyzed by gas chromatography (GC) to verify the target treatment rate. Samples of the grain were then extracted and evaluated with the EnviroGard Chlorpyrifos-methyl (Reldan) screening kit. The kit is used to determine if the grain has been treated with Reldan.

The GC analysis confirmed the grain was treated at 0.0, 0.10, 0.25, 1.0, and 6.0 ppm of Reldan. The EnviroGard Reldan test kit detected the absence of Reldan at the 0.0 ppm level and the presence of Reldan at the 0.1, 0.25, 1.0, and 6.0 ppm treatment on corn grain at the time of treatment (0 day) and 30 days post treatment.

INTRODUCTION

Corn grain samples which had been previously treated by ImmunoSystems, Inc., Scarborough, ME were received frozen by the USDA-SPIRD, Savannah, GA following overnight shipment and receipt (Table 1). In addition EnviroGard Reldan test kits (Lot No. 23432) were received from ImmunoSystems for the determination if grain had been previously treated with Reldan post-harvest protectant insecticide.

Residue determinations were conducted by gas chromatograph on the five samples of corn grain received (samples 921025-19 thru -23). Three replications of the treated corn grain samples were analyzed to determine the residues of Reldan on the corn grain (Table 2).

The limit of quantification was 0.03 ppm for the corn grain analysis. Fortified samples were analyzed and the percent recovery established (Table 3).

Samples of the corn grain were also analyzed by using the EnviroGard Reldan test kit. The test kit is designed to determine if grain has been previously treated with Reldan post-harvest protectant insecticide (Table 4).

MATERIALS

Test Substance - Reldan (Chlorpyrifos-methyl) with Lot No. 301 had been used to treat the grain. The test substance was GLP certified to contain 43.20% active ingredient. The analytical standard (Lot No. AGR 203113) was used in this phase study in making analytical determinations.

Test Commodity - The test commodity was corn grain, variety Funk's Blend. It was harvested and stored without the application of post-harvest insecticides.

Test Procedure - The grain and EnviroGard Reldan test kits were received from Immunosystems, Inc. on January 26, 1993. Analytical determinations of the parts per million of Reldan post-harvest insecticide on the grain samples were made with the GC on February 10 thru February 11 for the zero time analysis. The 30 post-treatment analysis were made on March 10 thru 11, 1993.

EnviroGard Reldan test kit evaluations were made on February 9 thru 10, 1993. The procedure is described in the brochure enclosed with the test kits entitled "EnviroGard (tm) Chlorpyrifos-methyl (Reldan) Screening Kit," which is included as an attachment to this report (see Appendix). The test kits used in this evaluation were from the production Lot No. 23432.

Analytical Method - The analytical method used to analyze the grain samples is entitled "Determination of residues of chlorpyrifos-methyl in grains" by A.W. Kuper, 1979. Calibration curves for Chlorpyrifos-methyl are shown in Figure 1. Analysis protocol is shown in the Appendix (p. 15).

Data Archival - Original raw data are archived at Gustafson, Inc., Plano, TX with the exception of log books and other similar information unique to this laboratory. This information is archived at the USDA-SPIRD, Savannah, GA.

RESULTS AND DISCUSSION

Analytical results show (Table 2) residue levels of Reldan on the grain were <0.03, 0.115, 0.234, 0.982, and 7.238 ppm at 0 time and <0.03, 0.062, 0.162, 0.782, and 4.817 ppm at 30 days post-application.

The EnviroGard test kit analysis resulted with a negative response to the untreated corn grain and a positive response to the Reldan treated grain. These results are shown in Table 4. The individual assays are shown in the Appendix (pp. 17 and 18).

CONCLUSION

The EnviroGard Chlorpyrifos-methyl test kit detected Reldan on treated corn grain at the 0.25, 1.0, and 6.0 ppm treatment level at the time of application and 30 days post-application. These treatment levels were validated by analyses with the Gas Chromatograph. This verifies the EnviroGard test kit is an accurate method to determine if grain has been previously treated with the post-harvest insecticide Reldan (Chlorpyrifos-methyl).

Table 1 - Sample Tracking Information

January 26, 1993	Received five (5) frozen samples from Immunosystems, Inc. Immediately logged in and placed in freezer
February 8, 1993	Subdivided five samples into three (3) fractions each A. 600 gm to ambient conditions to await 30-day analysis B. 600 gm to freezer for 0-day analysis C. 600 gm to freezer for potential reruns
February 9, 1993	Sample fraction B subdivided B1. 200 gm for immunoassay: one assay completed for 0 time B2. 400 gm to freezer for 0 day GC analysis
February 10, 1993	Sample B2 weighed, extracted, and fortified for GC analysis at 0 time B1. 4 assays of immunoassay completed for 0 time
February 11, 1993	B2. GC analysis completed for 0 time
March 10, 1993	Sample A for 30-day analysis subdivided A1. 150 gm for immunoassay; 5 assays completed A2. 400 gm for GC analysis weighed, extracted, and fortified
March 11, 1993	A2. GC analysis completed for 30 day time

Table 2 - GC Analysis in Parts per Million of Reldan on Treated Corn Grain Samples¹

Sample no.	Application rate	Residue at	
		0-days	30-day
921025-19	0	<0.03 ²	<0.03 ²
921025-20	0.1	0.115	0.062
921025-21	0.25	0.234	0.162
921025-22	1.0	0.982	0.782
921025-23	6.0	7.238	4.817

¹ Average of 3 replicates of duplicate samples

² Limit of detection

Table 3 - Percent Recovery of Reldan From Fortified Corn Grain Samples by GC Analysis¹

ppm added	Recovery at 0 days		Recovery at 30 days	
	ppm	percent	ppm	percent
0.137	0.131	95.4	0.150	109.2
1.37	1.45	105.6	1.44	105.1
6.56	6.73	102.6	6.78	103.3

¹ Average of 3 replicates of duplicate samples

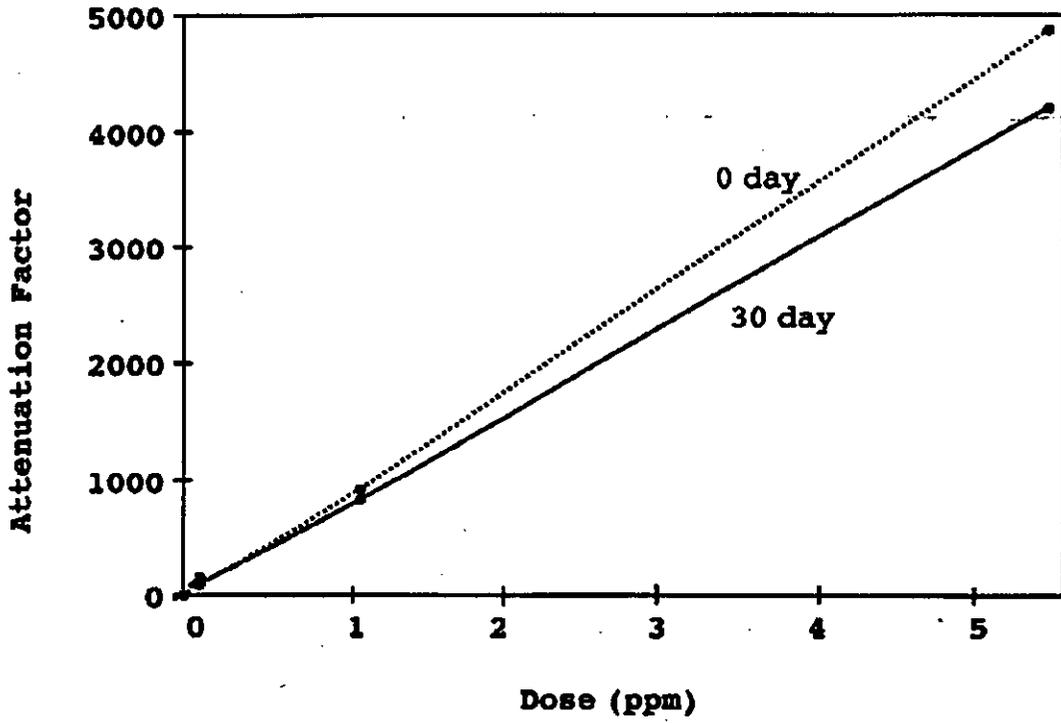
Table 4 - EnviroGard Chlorpyrifos-methyl (Reldan) Test Kit Results from Reldan Treated Grain¹

Dose	Determinations ² made at					
	0 days			30 days		
Visual	Photometer	interpre- tation	Visual	Photometer	interpre- tation	
0	-	0.74	-	-	1.08	-
0.1	+	-0.23	+	+	-0.02	+
0.25	+	-0.30	+	+	-0.21	+
1.0	+	-0.39	+	+	-0.34	+
6.0	+	-0.39	+	+	-0.43	+

¹ See appendix showing data for each of five individual assays

² Average of five replicate assays

Figure 1 - Linear Response of Reldan Fortified Corn Grain Samples



APPENDIX

RELDAN ON CORN-SPIRDL Residue Lab.

Analyst: J. M. Zehner

Jan. 26, 1993

Two packages received from ImmunoSystems, Inc. One contained corn grain samples frozen in gallon jars packed in dry ice. These were placed directly in freezer maintained at a minimum of -17°C . The other package contained 6 individually wrapped EnviroGard Screening Kits which were immediately stored in refrigerator maintained at 5°C .

Feb. 8, 1993

Samples were removed from freezer, thawed for approximately 7 hrs. Two subsamples removed from each of approximately 600 gms (1 quart). One subsample given to L. Zettler for 30-day immunoassay study (placed in environmental chamber maintained at 26.5°C and 60% RH), remainder of original sample returned to freezer. The second subsample to be used for GC analysis of day zero test, remainder to be kept for repeats if necessary.

Feb. 9, 1993

The second subsample removed and thawed. 200 gm given to L. Zettler for immunoassay test. The remainder ground in Waring Blender for G.C. analysis.

Feb. 10, 1993

40 gm of ground samples were placed in 250 ml Erlenmeyer flasks, 100 ml acetone added and shaken on Burrell wrist shaker for 4 hrs. Samples weighed in triplicate. Known amounts of reldan (equivalent to 0.137, 1.37 and 6.56 ppm) were added to untreated (rate=0) corn also in triplicate. Samples filtered through Whatman folded filter paper (cat. no. 1202 125). Solutions stored in freezer for GC analysis.

GC PROCEDURE

Instrument - Varian 3700, FPD detector
Integrator - Varian 4400
Column - 6 ft glass, 2mm id
Packing - 5% OV 101 on 80/100 mesh GCQ
Column temp - 210°C
 N_2 carrier - 30 ml/min
Detector - FPD (gas flows set at optimum response)
Detector temp - 300°C
Injector temp - 290°C
Volume injection - $4\mu\text{l}$ or $8\mu\text{l}$ depending on residue level

Each extract injected twice with standards injected between no more than 3 extracts.

Calculations made using both area and peak ht measurements. Sensitivities calculated and used between samples of $4\mu\text{l}$

injection and again between samples (& standards) of 8 μ l injection.

March 10, 1993

Samples removed from environmental chamber and split: approximately 150 gm to L. Fetzer (for Zettler) for immunoassay test; remainder ground and subjected to GC method as with zero day analysis.

Individual Assays for EnviroGard Chlorpyrifos-methyl (Reldan) Test Kit From Reldan Treated Corn Grain Samples at 0 Days Post Treatment

Assay No.	Sample ID	Dose (ppm)	Visual interpretation	Photometer reading	Photometer interpretation
1	5894	0	-	0.03	-
	5895	0.1	+	-0.23	+
	5896	0.25	+	-0.15	+
	5897	1	+	-0.32	+
	5898	6	+	-0.21	+
2	5894	0	-	1.05	-
	5895	0.1	+	-0.18	+
	5896	0.25	+	-0.31	+
	5897	1	+	-0.39	+
	5898	6	+	-0.39	+
3	5894	0	-	0.96	-
	5895	0.1	+	-0.2	+
	5896	0.25	+	-0.3	+
	5897	1	+	-0.4	+
	5898	6	+	-0.42	+
4	5894	0	-	1.06	-
	5895	0.1	+	-0.21	+
	5896	0.25	+	-0.31	+
	5897	1	+	-0.39	+
	5898	6	+	-0.45	+
5	5894	0	-	0.59	-
	5895	0.1	+	-0.32	+
	5896	0.25	+	-0.42	+
	5897	1	+	-0.43	+
	5898	6	+	-0.49	+

Individual Assays for EnviroGard Chlorpyrifos-methyl (Reldan)
Test Kit From Reldan Treated Corn Grain Samples at 30 Days Post
Treatment

Assay No.	Sample ID	Dose (ppm)	Visual interpretation	Photometer reading	Photometer interpretation
1	5894	0	-	1.25	-
	5895	0.1	+	-0.05	+
	5896	0.25	+	-0.19	+
	5897	1	+	-0.33	+
	5898	6	+	-0.38	+
2	5894	0	-	0.97	-
	5895	0.1	+	-0.01	+
	5896	0.25	+	-0.2	+
	5897	1	+	-0.28	+
	5898	6	+	-0.43	+
3	5894	0	-	0.92	-
	5895	0.1	+	0.00	+
	5896	0.25	+	-0.28	+
	5897	1	+	-0.37	+
	5898	6	+	-0.47	+
4	5894	0	-	1.13	-
	5895	0.1	+	-0.07	+
	5896	0.25	+	-0.13	+
	5897	1	+	-0.28	+
	5898	6	+	-0.34	+
5	5894	0	-	1.14	-
	5895	0.1	= ¹	0.01	+
	5896	0.25	+	-0.23	+
	5897	1	+	-0.46	+
	5898	6	+	-0.52	+

¹ Color difference between sample and standard indistinguishable

ENVIROGARD™ CHLORPYRIFOS-METHYL (RELDAN®) SCREENING KIT

Note: Read this instruction manual thoroughly before attempting to use this product.

For the on-site detection of chlorpyrifos-methyl pesticide residues in grain.

INTENDED USE

The EnviroGard Chlorpyrifos-methyl (Reldan) Screening Kit is a qualitative "positive/negative" test for the detection of chlorpyrifos-methyl residues in grain using a single calibrator equivalent to 0.25 ppm Reldan in grain. The test kit is designed to be used "on-site" with a minimum of training. Test results can be interpreted visually or with the use of a portable colorimeter.

TEST PRINCIPLES

The EnviroGard Chlorpyrifos-methyl (Reldan) Screening Kit is based on the use of polyclonal antibodies which bind either chlorpyrifos-methyl or chlorpyrifos-enzyme conjugate. chlorpyrifos-methyl in the sample competes with chlorpyrifos-enzyme conjugate for a limited number of binding sites. The antibodies which bind chlorpyrifos-methyl are immobilized to the walls of the test tubes.

1. A sample containing chlorpyrifos-methyl is added to an antibody-coated tube, followed by chlorpyrifos-enzyme conjugate. The chlorpyrifos-enzyme conjugate competes with the chlorpyrifos-methyl for the antibody binding sites.
2. After this mixture is incubated for 5 minutes, any unbound molecules are washed away.
3. A clear solution of chromogenic substrate is then added to the test tube. In the presence of any bound chlorpyrifos-enzyme conjugate, the clear substrate turns blue. One enzyme molecule can convert many substrate molecules to blue.

Since there are the same number of antibody binding sites on every test tube, and each test tube receives the same number of chlorpyrifos-enzyme conjugate molecules, a sample which contains a low concentration of chlorpyrifos-methyl enables the

antibody to bind many chlorpyrifos-enzyme conjugate molecules. Therefore a low concentration of chlorpyrifos-methyl will produce a darker blue sample. Conversely, a high concentration of chlorpyrifos-methyl will enable fewer chlorpyrifos-enzyme conjugate molecules to be bound by the antibodies, resulting in a lighter blue sample.

NOTE: Color is inversely proportional to concentration.

Darker Color = Lower Concentration ✓
Lighter Color = Higher Concentration

SPECIFICITY

The EnviroGard Chlorpyrifos-methyl (Reldan) Screening Kit will not differentiate between chlorpyrifos and structurally related compounds, but will detect their presence to varying degrees. The following chart shows approximate Least Detectable Dose (LDD) in parts per million for some structurally related compounds:

Compound	LDD (ppm)
Chlorpyrifos-methyl	0.25
Chlorpyrifos	0.50
Triclopyr	3.1
Fenchlorphos	25
Bromophos	25

The following compounds are not detectable at a concentration of 100 ppm:

Fenitrothion	Pirimiphos-methyl
Etrimphos	Malathion
Pyrethroids	Methoprene
Carbaryl	3,5,6-trichloro-2-pyridinol

PRECAUTIONS

- ◊ Store all kit components at 4 - 8°C (39 - 46°F) when not in use.
- ◊ Do not freeze kit components or expose them to temperatures greater than 37°C (99°F).

PRECAUTIONS (CON'T.)

- ◊ Do not store kit components for more than 8 hours at ambient temperatures (18 - 27°C or 64 - 81°F).
- ◊ Allow all kit components to reach ambient temperature (18 - 27°C or 64 - 81°F) before beginning test.
- ◊ Do not use kit components after the expiration date shown on the kit label.
- ◊ Do not use reagents or test tubes from a kit of one lot number with reagents or test tubes from a kit with a different lot number.
- ◊ Because of the rapid kinetics of the EnviroGard test kit, do not include more than 6 tubes in any single assay.
- ◊ Diluting or otherwise adulterating test reagents not specified in the test procedure may give inaccurate results.
- ◊ Use a fresh transfer pipet for each calibrator or sample.

BEFORE YOU START

Make sure that you have the following items in your test kit:

- ◊ Bag containing 20 antibody-coated tubes
- ◊ 1 dropper vial of assay diluent (Vial A)
- ◊ 1 glass vial of 0.25 ppm Chlorpyrifos-methyl calibrator (Vial B)
- ◊ 1 dropper vial of chlorpyrifos-peroxidase enzyme conjugate (Vial C)
- ◊ 1 dropper vial of enzyme substrate (Vial D)
- ◊ 1 dropper vial of stop solution (Vial E, used for optional photometric analysis)
- ◊ 1 6-place test tube rack
- ◊ Bag containing 20 transfer pipets

You will also need several other items:

- ◊ 70% Isopropyl Alcohol (Rubbing Alcohol)
- ◊ 50 mL screw-cap centrifuge vials with graduations
- ◊ timer or stopwatch
- ◊ tap or distilled water for washing tubes

- ◊ Millipore Differential Photometer (optional) ✓
- ◊ indelible marking pen for labelling tubes

Tips for Successful Analysis

- ◊ Be sure that all materials are ready (including grain extracts) and at room temperature before beginning to set up assay. The kinetics of the assay are temperature dependent.
- ◊ An underlying assumption with immunoassays is that all assay tubes are subjected to identical conditions except for the amount of analyte in the sample. This should be kept in mind when the assay is run i.e. avoid delays between additions of reagents to tubes in a single assay.
- ◊ Be sure to add reagents by dropping drops directly to the bottom of the tubes. Having drops run down the side of the tubes will affect results; this is particularly important with the sample. Hold the dropper bottles exactly vertically and approximately 1/2 inch above the tube when dispensing drops. When using the plastic transfer pipets it helps to fill and empty them before refilling to dispense drops. ✓
- ◊ Static charges on the tube (particularly when the humidity is very low) may affect drop size and will attract drop to side of tube rather than down the center, causing inaccurate results. If this problem is noted, it may help to grip the tube with the fingers of your free hand when dispensing drops.
- ◊ The differences in color development between samples may not be dramatic. Slight differences in color will need to be distinguished for some visual interpretations. Relatively high concentrations of Reldan on grain (> 4 ppm) may result in assay tubes which develop no detectable color. Use of the optional photometer will allow detection of differences finer than the human eye.
- ◊ There should always be color developed in the assay calibrator tube. Absence of color is an indication that the assay was run improperly and should be repeated.

GRAIN EXTRACTION

Before it can be analyzed in the test, chlorpyrifos-methyl needs to be extracted from the grain using methanol. *Note: The method described below has been validated for whole grain corn. Contact Millipore for Application Sheets for other grain types.*

1. Add grain to the extraction tube (50 mL centrifuge tube) up to the 15 mL mark. Be sure that level of grain is as close as possible to the mark
2. Add 70% isopropyl alcohol (Rubbing Alcohol) up to the 45 mL mark on the extraction tube. Be sure that level of alcohol is as close as possible to the 45 mark. Cap the extraction vial tightly to avoid leaking.
3. Shake the extraction bottle vigorously for 2 minutes. This extraction method has been shown to extract approximately 50% of the chlorpyrifos-methyl from the whole grain. This extraction efficiency has been taken into account in the preparation of the assay calibrator (= 0.25 ppm).
4. Allow any suspended solids in the extract to settle before removing sample for assay.

HOW TO PERFORM THE TEST

1. Remove up to 6 test tubes (number of samples being run plus one for calibrator) from the plastic bag, insert them in the test tube rack and label as follows:

(Note: Be sure to re-seal zip-lock bag with tubes and dessicant to protect them from moisture.)

<u>Tube Marking</u>	<u>Tube Contents</u>
"Cal"	0.25 ppm Calibrator
"S1"	Sample 1
"S2"	Sample 2
"S3"	Sample 3
"S4"	Sample 4
"S5"	Sample 5

2. Add 5 drops of Vial A (Assay Diluent) to each tube.
3. Using a clean dispenser, carefully add 3 drops of Vial B (0.25 ppm calibrator) to "Cal" tube. (Note: It is helpful to fill dispenser and empty back into vial before re-filling for dispensing into assay tube.)
4. Using a clean dispenser for each sample, carefully add 3 drops of each sample extract into the appropriately labeled tubes.
5. Shake tube holder for 2-3 seconds to mix contents.
6. Add 5 drops of Vial C (Enzyme Conjugate) to each tube.
7. Shake tube holder for 2-3 seconds to mix contents and incubate tubes undisturbed for 10 minutes.
8. Flick contents of tubes into a sink or other receptacle, fill tubes to overflowing with cool tap water and flick wash into sink.
9. Repeat Step 8 three more times for a total of four washes. After the final wash, tap inverted tubes on absorbant paper to remove as much wash as possible.
10. Add 10 drops of Vial D (Substrate) to each tube.
11. Visual interpretation of results can be made at any stage in the color development step when the color becomes intense enough to interpret. Color should be sufficient to interpret within 10 minutes.
12. If the optional photometric method is to be used, incubate tubes undisturbed for 10 minutes and then add 10 drops of Vial E (Stop Solution) to each tube and shake tube holder to mix contents. (Solution will change from blue to yellow.)

INTERPRETATION OF RESULTS

Visual Interpretation

1. Compare the color of each sample tube to the calibrator tube against a white background. The test tube rack in the kit is well suited for this purpose.
2. If a sample tube contains more color than the calibrator, the sample is **NEGATIVE** for Reldan(chlorpyrifos-methyl). If a sample tube contains less color than the calibrator tube, the sample is **POSITIVE** for Reldan.

Interpretation Using Millipore Differential Photometer

1. Place the calibrator tube in the left (reference) well of the photometer.
2. Place each sample tube, in succession, in the right (sample) well of the photometer. The difference in the absorbance of the two tubes (sample tube minus reference tube) will be shown in the readout. If the number shown is a negative number, the sample is positive for chlorpyrifos-methyl. If the number shown is a positive number, the sample is negative for chlorpyrifos-methyl.

Technical Assistance

For additional information about Millipore products, telephone toll-free (including Massachusetts): 800-225-1380.

In Western States, Alaska
& Hawaii: 800-632-2708
In Canada: 800-268-4881
In Toronto: 416-678-2161
In Puerto Rico: 809-747-8444

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