PROTOCOL FOR

EVALUATION OF TOXICITY

OF RESIDUES OF <*TEST SUBSTANCE*>

ON ALFALFA TO HONEY BEES (*Apis mellifera*)

EPA Guideline Requirement

SUBDIVISION L, FIFRA GUIDELINE NO. 141-2
& OPPTS 850.3030 (PUBLIC DRAFT)

Protocol Number

Prepared By:

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EVALUATION OF TOXICITY
OF RESIDUES OF *<TEST SUBSTANCE>*
ON ALFALFA TO HONEY BEES (*Apis mellifera*)

SPONSOR:
<*Name*>
<*Address*>
<*Phone*>

TESTING FACILITY:
LANDIS INTERNATIONAL, INC.
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STUDY DIRECTOR:
<Name>
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ANALYTICAL TESTING FACILITY:
<Name>
<Address>
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Phone <number>

PROTOCOL NUMBER:

Trial No.	Location (EPA region)	Field Cooperator

TEST SYSTEM: Alfalfa/Honey Bee (*Apis mellifera*)

TEST SUBSTANCE: *<Pesticide Trade Name>*
Formulation: *<Percent active ingredient, type>*
Lot No.: Will be recorded in the study file
CAS No.: *<Number>*
CAS Name: *<Name>*
Common Name: *<Name>*
EPA Reg. No: *<Number>*
IUPAC Name: *<Name>*

EVALUATION OF TOXICITY
OF RESIDUES OF *<TEST SUBSTANCE>*
ON ALFALFA TO HONEY BEES (*Apis mellifera*)

PROTOCOL RECIPIENTS:
(1) Sponsor
(2) Study Director
(3) Quality Assurance Unit
(4) Field Cooperator
(5) Analytical Laboratory

GLP NOTEBOOK RECIPIENT:
(1) Field Cooperator

SAMPLE ACTIVITY I.D. LOG RECIPIENTS:
(1) Sponsor
(2) Study Director
(3) Field Cooperator
(4) Analytical Laboratory

PROPOSED EXPERIMENTAL START DATE: *<Expected date of application at earliest test site>*

PROPOSED EXPERIMENTAL COMPLETION DATE: *<Date>*

OBJECTIVE

The objective of this study is to evaluate the toxicity of test substance residues on alfalfa plant foliage to the honey bee (*Apis mellifera*). The methods, species used, and route of administration described in this protocol are based upon procedures specified in Section 141-2 of the Environmental Protection Agency's Registration Guidelines, Pesticide Assessment Guidelines, FIFRA Subdivision L, Hazard Evaluation: Non-target Insects (1) and OPPTS 850.3030 Ecological Effects Guidelines; Honey Bee Toxicity of Residues on Foliage (Public Draft).

JUSTIFICATION OF THE TEST SYSTEM

The honey bee (*Apis mellifera*) is useful in evaluating the potential hazards of agricultural chemicals to non-target insects since the honey bee is an important pollinator of various agricultural crops. Exposing honey bees to treated foliage simulates a route of exposure that could be encountered under field conditions through foraging activities of honey bees.

SUMMARY

A single application of the test substance, formulated as the representative end-use product, will be made to each of nine alfalfa plots, each approximately 1 m^2 in size. The target rate of <*application rate*> lb active ingredient (a.i.) will be applied in a spray volume of <*number*> gallons per acre (GPA), using appropriate CO_2 hand spraying equipment. Three control and three "formulation blank" treated alfalfa plots will be included in a randomized complete block test design. Alfalfa foliage from each treatment and control plots will be collected at 3, 8 and 24 hour intervals and placed in six replicate test chambers. Twenty-five worker honey bees will be introduced into each of six test replicate chambers and maintained under controlled conditions for a period of at least 24 hours. Worker bees will be observed for mortality and any other toxicological responses. If mortality rates of bees exposed to 24 hour-old residues is greater than 25%, additional samples will be taken at 24 hour intervals until mortality of bees is not different from the control.

EXPERIMENTAL DESIGN

Test Site Location: The trial location will be in <*state*>. Any age alfalfa plants may be used as long as they are producing foliage. To avoid bias, the plots will be located in areas representative of the entire alfalfa field. Animals will not be allowed to graze the alfalfa.

Plot Size and Design: The trial will consist of a total of 15 plots. Of the 15 plots, nine will be treated with the test substance, three plots will be treated with the formulation blank (formulation carrier without the active ingredient) and three will be control plots. Each plot will be at least 1 m^2 in size. The 12 treated plots will be arranged in a randomized complete block design. Each plot will be separated by a 1 m buffer (alley) on all sides. The control plots will be located a minimum of 100 feet upwind from the treated plots.

APPLICATION OF TEST SUBSTANCE

Test Substance: The test substance will be the end-use formulation of <*product and formulation*>. A complete description (including batch number, purity, percent active ingredient, identity, physical properties, and stability) of the compound will be the responsibility of the Sponsor.

Application: A single application of the test substance will be made to each of the nine alfalfa plots approximately 1 m^2 in size which have been grown according to standard agronomic practices. The target rate will be <*application rate*>. A spray volume equivalent to <*number*> GPA will be applied. A "formulation blank", containing only water and formulation components (no active ingredient), will be included on three ~1 mt^2 plots. The three control plots will be sprayed with water only, from the same water source used to prepare the spray mix for the test substance and formulation blank treatment. The spray application equipment will be calibrated prior to use.

SAMPLING

Two composite samples from each of the non-treated, formulation blank and treated plots will be collected at each sampling interval, one to be used for bee toxicity testing and one for residue analysis. Each composite sample will be collected from a separate pass through each plot. Additional alfalfa samples will be collected at 24 hours to be employed as fortification samples (see page 5, **Field Fortification Samples**. The sample collected for residue analysis will be shipped frozen to the following analytical laboratory:

<*Laboratory Name*>
<*Address*>
<*Address*>

Alfalfa Foliage for Bee Toxicity Testing: Alfalfa test samples will be collected at 3, 8 and 24 hour intervals after application. If mortality rates of bees exposed to 24 hour-old residues are greater than 25%, sampling at 24 hour intervals will continue until mortality of bees exposed to foliage is not significantly greater than control mortality. Three <*test substance*> treated plots, one "formulation blank" plot and one control plot will be randomly assigned for each of the three sampling intervals. If needed, the 48 hour sampling interval and all subsequent samples will be taken by random assignment. The method used to avoid biased sampling will be recorded in the field notebook. The samples will consist of whole plant tops (without roots). At each sampling event, the control plots will be sampled first followed by the "formulation blank" plot and finally the treated plots. At each collection interval, <*number*> plants (approximately 50 g of alfalfa) will be hand harvested from each treated plot and <*number*> plants (approximately 150 g) each from the "formulation blank" and control plot. Samples will be placed in separate labeled containers and individually weighed. The three treated samples will be combined to create one composit sample. The control, "formulation blank" and treated foliage will then be individually chopped, mixed and divided into six 15 gram portions per treatment. Each 15 gram sample will be placed into a single appropriately labeled test chamber. **Table I** shows the description and number of field samples collected for the bee toxicity testing phase of the study.

FIELD PROCEDURES (CONT'D)

Samples for Residue Analysis: In order to analytically determine the residues of <*test substance*> on alfalfa foliage, plant samples will be taken after the spray residue has dried (within 3 hours of spray application) on all plots. Foliage samples will be taken as described for bee toxicity testing; however, replicate foliage samples will be sent to the analytical lab rather than being chopped, mixed and divided. The sampling method used to generate non-biased, representative crop samples will be recorded in the Field Notebook. The weight of all samples will be recorded. Special care will be taken to prevent contamination of the samples. After collection and preparation, samples will be placed in appropriately labeled sample bags and placed in containers with a coolant, such as blue ice. These samples will be transferred to freezers (maintained at 32^{o}F or colder) as soon as possible. See **Table II** for number of analytical samples to be collected.

Field Fortification Samples: The analytical laboratory will prepare spike solutions of known concentration and ship them <*condition*> to LANDIS for use in field spikes. Quality control (field spike) samples will be prepared once (i.e., at 24 hours) using 10 g portions of plant material from a non-treated plot. The samples will include: control (2); formulation blank (2); <*test substance*> at <*concentration*(s)> ppm (2). A total of <*number*> samples will be prepared in <*describe container*>

Sprayate Samples: Immediately prior to or immediately after spray application, six 10 ml samples of the spray mixture will be obtained from the sprayer reservoir and placed in amber sample vials. Reference LANDIS INTERNATIONAL SOP #4.8-Current Revision, *Sprayate Samples*, for the specific preparation of these samples. The sprayate samples will be frozen as soon as possible after collection and labeling. Three of the sprayate samples will be shipped frozen to the analytical laboratory within 14 days of collection; the remaining three samples will be maintained frozen by the Field Study Scientist as reserve samples.

Source Water and Test Product Samples: Prior to or immediately after spray application, one liter from the source of the water used to mix the spray application will be collected. Also, two samples of test substance (approximately 15 ml each) will be collected. These samples will be shipped to the analytical laboratory at ambient temperature along with a copy of the calibration records from the GLP notebook and a photocopy of the Application Verification Product Sample Information form. Reference LANDIS INTERNATIONAL SOP #4.20-Current Revision, *Sprayate Method Validation and Recovery,* for further guidance in handling of these samples.

SAMPLE HANDLING

Samples will be stored in freezers set to maintain temperatures below freezing. Samples must be placed in the freezer as soon as possible following collection. If possible, samples will be shipped on the same day of sampling. All samples will be packed and shipped frozen to the analytical laboratory.

Prior to each shipment of samples, **Packing/shipping** forms provided by LANDIS INTERNATIONAL, INC., will be completed and the appropriate documents included for shipment (see LANDIS INTERNATIONAL SOP #5.12-Current Revision, *Cooperator and Laboratory Instructions for Sample Packing/Shipping Forms*). The "**white**" and "**yellow**" copies of the Packing/Shipping form will be sent with the samples to the selected analytical laboratory. Upon receipt by the analytical laboratory, the laboratory will complete the section at the bottom of the form, retain the "**yellow**" copy for their files, and return the "**white**" copy to LANDIS INTERNATIONAL, INC. The Field Study Scientist will mail the "**pink**" copy directly to LANDIS INTERNATIONAL, INC., and retain the "**gold**" copy for inclusion in the GLP Field Notebook. All samples will be packed and shipped according to LANDIS INTERNATIONAL SOP #4.14-Current Revision, *Packing and Shipping Samples*.

LABORATORY PROCEDURES
Exposure of Bees to the Test Substance

Treatment Groups: Honey bees will be exposed to foliage from each of the three treatment groups (1. test substance, 2. "formulation blank" and 3. control) harvested at each post-treatment time interval (3, 8 & 24 hr.). A sufficient number of honey bees will be immobilized with $CO_{2,}$ and at least 25 bees will be assigned to each of six replicates in each of the three treatment groups (18 replicates for each time interval) by random draw.

Honey Bee Test Duration: Honey bees will be exposed to treated and control alfalfa foliage collected at each of the sample periods for at least 24 hours. Mortality results will continue to be recorded every 24 hr thereafter until evidence of treatment-related mortality has ceased or until 20% mortality is observed in the control group. A study may be considered invalid and will be repeated if mortality in the control group exceeds 20% within the first 24 hours of the study.

Test Bees: All test bees (*Apis mellifera*) will be seven days of age or less and apparently healthy at initiation of the study. Procedures for collecting and rearing honey bees are described in LANDIS INTERNATIONAL, INC. SOP #4.25-Current Revision, *Collecting and Rearing Honey Bees*. Brood frames containing only worker bees in the pupal stage will be obtained from Rossman Apiaries (Moultrie, GA) or from another reputable supplier. Worker honey bees of the correct age will be obtained by isolating brood frames in an enclosed cage. Enough frames will be collected to run one complete test. Within seven days after the frames have been isolated, young adult bees will be immobilized with CO_2, collected, and randomly assigned to treatment and control groups as described above.

LABORATORY PROCEDURES (CONT'D)
Exposure of Bees to the Test Substance (Cont'd)

Bee Diet: During the incubation phase, the bee colony will be allowed to forage freely on brood frames. While in test chambers, bees will be fed *ad libitum* a 50% sugar/water food supply prepared in distilled water.

Specifications for acceptable levels of contaminants in dietary material (table grade sugar) for foliage residue toxicity studies with *A. mellifera* have not been established. Concurrent controls serve to monitor factors that might adversely impact the study.

Housing and Environmental Conditions: Test bees will be housed in test chambers that consist of disposable, one pint, rolled paper containers measuring approximately 9 cm in diameter and 15 cm in height. Test chambers will be covered with plastic petri dishes approximately 10 cm in diameter. A small plastic cup (approximately 20 ml) containing the sugar/water mixture will be affixed to the bottom of each chamber. Each test chamber will contain at least 25 worker bees. Each test chamber will be identified by protocol number, dosage group, and replicate number. Bees will be maintained at a temperature range of approximately 25-35°C and relative humidity between 50-80%. The test chambers will be maintained in the dark except when tests are being initiated or observed.

Observations: All bees will be observed for mortality, behavior, and toxicological responses at four hours after exposure and then at 24 hour intervals (from the time of exposure) until evidence of treatment related mortality has ceased or until 20% mortality is observed in the control group. Clinical signs such as lethargy, hypersensitivity and ataxia will be recorded. Abnormal behavior will be determined by comparing signs in the treatment groups with control group observations. To avoid unnecessary disturbances, dead bees will not be removed until the test is terminated.

STATISTICAL ANALYSIS

The raw data will be analyzed by analysis of variance (ANOVA 2-Way) to determine any significant mortality difference between the response of control and treated bees. The data may be transformed to obtain experimental errors normally distributed with homogeneous variance. If a significant F-Test at $p = 0.05$ is found between the treatment concentrations and the mortality observations, then a Least Significant Difference test will be used to separate the means.

Residue Analysis: Foliar, test substance and source water samples will be analyzed as soon as possible after receipt by the analytical lab. The samples will be analyzed for <*test substance*> and its degradates <*name*>, expressed as total <type> residues. The method of analysis will be added to the protocol by amendment. Concurrent laboratory fortified samples will be prepared and assayed for each sample set.

RECORD KEEPING

The following records and site specific information will be collected:

(1) Copy of the signed protocol

(2) Honey bee source, date received, and approximate numbers of pupae received

(3) Husbandry and environmental conditions

(4) Dosage calculation, preparation, and administration

(5) Daily observations

(6) Statistical calculations

(7) For the field portion of the study, weather data (min/max temperature and rainfall) will be collected from an NOAA weather station located within 20 miles of the test plots or from an on-site weather station. During the laboratory phase, a hygrothermograph will be used to record temperature and relative humidity. On-site weather information will be recorded on the days of application.

(8) A description of each trial site, including a map of the test plots indicating their location, topography and size, slope, and location and size of the control plots in relation to the test plots will be provided.

(9) Crop and pesticide use history on the plots at each trial site for at least three years, and preferably five years preceding this study will be provided.

(10) Cultural agronomic practices during the course of each trial will be provided.

(11) Description of any post-treatment maintenance, such as use of fertilizers and pesticides, irrigation, and weeding, will be provided for each trial.

(12) A description of the test material (lot number, purity, and identifying codes) will be provided for each trial.

(13) The date of each sampling and a description of the sampling technique, including weight and condition of the sample taken, will be provided for each trial.

REPORTING

A draft report of the study results will be prepared by LANDIS INTERNATIONAL, INC. and submitted to the Sponsor or Sponsor representative. Upon approval, the final field report will be prepared. The report will include, but not be limited to the following:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and completed.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods employed for analyzing the data.

(4) The test substances identified by name, chemical abstracts service (CAS) number or code number, strength, purity, and compositions or other appropriate characteristics, as provided by the Sponsor.

(5) Stability and when relevant to the conduct of the study, the solubility of the test and control articles under conditions of administration, if provided by the Sponsor.

(6) A description of the methods used.

(7) A description of the test system used.

(8) A description of the dosage, dosage route of administration, and duration.

(9) A description of all circumstances that may affect the quality or integrity of the study.

(10) The name of the Study Director, and other scientists or supervisory personnel involved in the study.

(11) A description of the calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

(12) The location where all specimens, raw data, and the final field report will be stored.

(13) A statement prepared and signed by the QA Officer listing the dates inspections were made and findings reported to the Study Director/Management.

Table I. Alfalfa Sample Types and Number

Harvest Interval (Hr.After Treatment)	Plot Description*	Sample Type	Number of Samples	Sample Size
3	Treated (3 plots)	Residue	3	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Residue	1	<*number*> plants/1 plot
	Control (1 plot)	Residue	1	<*number*> plants/1 plot
3	Treated (3 plots)	Bee Toxicity	3#	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
	Control (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
8	Treated (3 plots)	Residue	3	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Residue	1	<*number*> plants/1 plot
	Control (1 plot)	Residue	1	<*number*> plants/1 plot
8	Treated (3 plots)	Bee Toxicity	3#	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
	Control (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
24	Treated (3 plots)	Residue	3	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Residue	1	<*number*> plants/1 plot
	Control (1 plot)	Residue	2	<*number*> plants/1 plot; 2 independently collected samples

Table I. Alfalfa Sample Types and Number (Cont'd)

Harvest Interval (Hr.After Treatment)	Plot Description*	Sample Type	Number of Samples	Sample Size
24	Treated (3 plots)	Bee Toxicity	3#	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
	Control (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
48 (if needed)	Treated (3 plots)	Residue	3	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Residue	1	<*number*> plants/1 plot
	Control (1 plot)	Residue	1	<*number*> plants/1 plot
48 (if needed)	Treated (3 plots)	Bee Toxicity	3#	<*number*> plants each of 3 plots
	Blank Form. (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot
	Control (1 plot)	Bee Toxicity	1	<*number*> plants/1 plot; 2 independently collected samples

* From plots randomly assigned for each of the sampling intervals

Combine the 3 treated samples into a single sample after weighing each individually

Table II. Sample Type and Number of Samples to be Analyzed During the Exposure of Honey Bees to Residues of <*Test substance*> on Alfalfa.

Harvest Interval (Hr.)	Plot Description			Field Fortification Sample*
	Control	Form. Blank	Treated	
3	1	1	2	NA
8	1	1	2	NA
24	1	1	2	<*number*>
48	1	1	2	NA

* See **Field Fortification** section, page 5, for a description of samples.

PROTOCOL SIGNATURE PAGE

Sponsor	Date
Study Director	Date
Auditor, Quality Assurance Unit	Date