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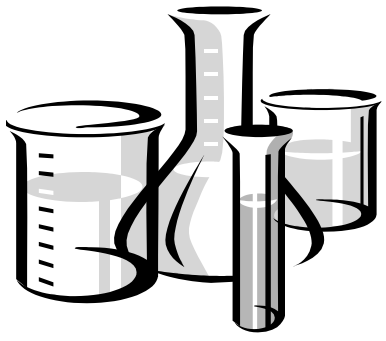
Draft Product Performance Test Guidelines

OCSPP 810.3100: Treatments for Red Imported Fire Ants



NOTICE

This guideline is one of a series of test guidelines established by the Office of Chemical Safety and Pollution Prevention (OCSPP) [formerly the Office of Prevention, Pesticides and Toxic Substances (OPPTS) prior to April 22, 2010], United States Environmental Protection Agency (US EPA) for use in testing pesticides and chemical substances to develop data for submission to the agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a), referred to hereinafter as the harmonized test guidelines.

The OCSPP test guidelines serve as a compendium of accepted scientific methodologies for research intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting appropriate tests, and is also used by EPA, the public, and the companies that are required to submit data under FIFRA. These guidelines are not binding on either EPA or any outside parties, and the EPA may depart from them where circumstances warrant and without prior notice. The methods described in these guidelines are strongly recommended for generating the data that are the subject of the guidelines, but EPA recognizes that departures may sometimes be appropriate. You may propose alternatives to the methods described in these guidelines, with supporting rationale. The agency will assess them for appropriateness on a case-by-case basis.

For additional information about the harmonized test guidelines and to access the guidelines electronically, please go to *https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances*. You may also access the guidelines in *http://www.regulations.gov* grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, EPA-HQ-OPPT-2009-0576, and EPA-HQ-OPP-2011-1017. ***EPA-HQ-OPP-2017-0693*** is the docket number for the FIFRA SAP peer review record containing this draft guideline.

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(a) Introduction

a. Scope. This guideline provides recommendations for the design and execution of laboratory and field studies to evaluate the performance of pesticide products for the treatment of red imported fire ants (*Solenopsis invicta*) in connection with registration of pesticide products under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.). This guidance applies to products in any formulation, such as a liquid, aerosol, granular or bait, if intended to be applied for control of red imported fire ant colonies (i.e., workers, queen(s) and brood) or as a barrier treatment. This guideline does not apply to those products exempt from FIFRA Registration under 40 CFR 152.25 or to product performance testing described in other agency guidelines. For example, tests for additional formulations of products targeting red imported fire ant individuals (rather than colonies), such as direct spray testing and indoor/outdoor residual applications targeting workers/foragers, should refer to the Premises Guideline for appropriate testing.

b. Purpose. This guideline provides laboratory and field study methods to evaluate product performance of pesticides against red imported fire ant colonies and includes statistical analysis and reporting recommendations.

(b) Organization of the OCSPP 810.3100 Guideline.

a. (a) Introduction;

(b) Organization of the Guideline;

(c) Definitions;

(d) Development of protocols for efficacy studies;

(e) Review of protocols for efficacy studies;

(f) Execution of efficacy studies;

(g) Reporting of completed efficacy studies to the agency;

(h) Retention of records;

(i) Specific guidance for field studies for testing area-applied pesticide products;

(j) Specific guidance for field studies for testing mound-applied pesticide products;

(k) Specific guidance for laboratory studies for testing bait products;

(l) Specific guidance for laboratory studies for testing barrier and/or repellent products;

(m) Specific guidance for laboratory studies for testing insect growth regulator (IGR) products;

(n) References.

b. General Considerations. Any protocol and/or study developed using this guidance must meet the provisions set forth in several statutes and regulations, including, but not limited to, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA, 7 U.S.C. 136, *et seq.*) under which EPA regulates pesticides. This guideline does not supersede or overrule the regulations governing

research conducted with human subjects such as those contained in 40 CFR Part 26, or any other agency regulations. To the extent there are any unintended conflicts between this guideline and any EPA regulation, the regulation at issue governs.

i. Good Laboratory Practice Standards. Good Laboratory Practice (GLP) Standards set forth in 40 CFR Part 160 apply to laboratory studies evaluating pesticide product performance. Part 158 specifies that "applicants must adhere to the good laboratory practice (GLP) standards described in 40 CFR Part 160 when conducting studies" [40 CFR 158.70(b)]. However, studies that do not comply with GLP standards may nonetheless be considered if, in the agency's judgment, the design and conduct of the study provide results that are scientifically reliable. 40 CFR 160.12(b) states that with any submitted research data "[a] statement describing in detail all differences between the practices used in the study and those required by this part" must be submitted to aid in making that determination.

ii. State requirements. Investigators and Sponsors should ensure research is conducted in compliance with any applicable state laws or regulations, which are independent of and additional to those cited in this guideline.

(c) Definitions

a. ***Application rate*** refers to the amount of product applied per unit area or volume (e.g., oz/ft^2). It can also be expressed in seconds of spray per unit area or volume.

b. ***Brood*** refers to the immature members of a colony collectively, including eggs, larvae, and pupae (Torre-Bueno 1989).

c. ***Colony*** refers to a group of related individuals, which constructs nests and rears offspring in a cooperative manner (Torre-Bueno 1989); mature colonies contain brood.

d. ***Field study*** refers to a scientific investigation that occurs in the red imported fire ant's typical habitat or dwelling space.

e. ***Food lure*** refers to a food item, such as hot dog pieces, used to attract foraging ants.

f. ***Forager*** refers to, in social insects, a member of the worker caste that gathers food and brings it back to the colony (Torre-Bueno 1989).

g. ***Method of application*** refers to the way a pesticide can be delivered (applied) to a pest or site. Examples of application methods include aerosol spray, liquid spray, watered-in granulars, and bait.

h. ***Midden*** refers to the pile of trash which is located away from the colony and contains solid waste and dead individuals.

i. ***Monogyne*** refers to colonies containing a single fertile queen.

j. ***Moribund*** refers to ants that are on their backs with only a single appendage twitching. Ants exhibiting this behavior may not be considered dead.

k. ***Mortality*** refers to ant death. A ***dead ant*** is an ant that does not move, even when poked or probed.

l. ***Mound*** refers to the above ground structure constructed for thermoregulation of a colony; an active mound contains all or part of a colony.

m. ***Mound density*** refers to the number of red imported fire ant mounds present per unit of area.

n. ***Negative control*** refers to the group of specimens in an experiment that receive no treatment or a treatment with the diluent only; no response is expected.

o. ***Pest exposure period*** refers to the amount of time a pest comes in contact with a pesticide.

p. ***Photoperiod*** refers to the relative amount of time during the day in which it is light or dark (Borror et al. 1981)

q. ***Polygyne*** refers to colonies containing multiple fertile queens.

r. ***Product performance testing*** refers to scientific studies that are designed to test the effectiveness of a pesticide product against the specific target pest.

s. ***Queen*** refers to a mated, egg-laying female member of the reproductive caste.

t. ***Residual efficacy*** refers to a surface or space treated with a pesticide product continuing to provide the intended pesticidal effect at an acceptable level for an extended length of time after application. The product's residues should be effective for at least 24 hours post application.

u. ***Social form*** refers to the number of egg laying queens in a colony; a monogyne colony has a single fertile queen and a polygyne colony has multiple fertile queens.

(d) Development of protocols for efficacy studies. Testing pesticides for efficacy against red imported fire ants begins with development of a study protocol. General considerations in developing a study protocol for efficacy studies include scientific design of the study, data collection, data analysis, and reporting. The product should be tested in a manner that reflects intended use and that will provide data to support product performance. Both field and laboratory tests are necessary to adequately address efficacy. Laboratory tests should be conducted in conjunction with field tests to provide supportive data for workers, queen(s) and in some cases, brood. Each of these topics is discussed in more detail in the sub-sections below. Additional study-specific considerations can be found in Sections (i) through (m).

a. Scientific design of study. The experimental methods should be likely to provide a definitive answer to the research question and should include a detailed description of the experimental design, addressing topics (i) through (viii), given directly below.

i. Objectives. For products that kill red imported fire ants (i.e., members of a colony), the objective of product performance testing is to determine the lowest proposed label rate that kills ants and/or a colony/mound/nest. For products that control red imported fire ants, the objective is to determine if the pesticide application has residual efficacy. In all cases, the scientific objective and intended label claims should be stated clearly and all treated ants/colonies/mounds/nests should be compared to ants/colonies/mounds/nests that have received no treatment or a diluent-only treatment.

ii. Test materials and treatments. End-use formulations should be tested using the lowest labeled application rates for use on red imported fire ants. Test materials should be stored at ambient temperature and humidity for at least one day before use.

1. Products that target adults or colonies/mounds/nests should be tested using workers unless colonies are specifically targeted, in which case brood and queen(s) should also be included.

2. Products that target ant development should be tested using brood, queen(s), and workers.

iii. Dose determination. The test dose in product performance studies is the lowest application rate on the target pest from a proposed product label, typically expressed as amount of

product per unit area for surface area treatments (e.g., oz/ft^2). Rates may also be expressed as seconds of spray per unit area or volume and amount of product per mound (with a maximum amount per acre). The amount of active ingredient applied per unit area or time should also be given. Label rate should match the rate tested in the efficacy study. While rates should be reported in units according to the US traditional systems of weights and measures, units may also be reported using metric system measurements. The method to measure test dose can vary among studies, however, the following are common methods of measurement:

1. Weigh the container holding the pesticide before and after application, and divide the difference by the unit area treated.

2. Measure the amount of test product, such as a liquid or bait (and alternate food source), in a container before and after application, and divide the difference by the unit area treated.

3. To determine the quantity sprayed per second, spray five panels for three seconds each. The product container should be weighed before and after each spray and the difference recorded. The mean value of the five replicates should be determined and that result divided by three to determine the average amount of product applied per second of spraying. The same procedure should be conducted to evaluate dust product formulations except that application should be made from a height of six inches or as directed by the product label.

iv. **Testing conditions.** During product performance testing in the laboratory, temperature should be kept at 25-30° C (unless otherwise indicated), with a relative humidity (RH) of 70-90%, and a 16:8 (L:D) photoperiod. The temperature during the test should be kept as constant as possible because changes can affect the performance of the product treatments. A food and water source should be provided for all test organisms throughout the study. Field studies should be conducted in weather that is realistic for use. Extreme weather (temperature, wind and/or precipitation) should be avoided.

v. **Choice of endpoints.** Study endpoints should be appropriate for the specific objectives of the proposed research and likely to provide a robust answer to the research question. Endpoints such as kill should be evaluated at the lowest labeled application rate for red imported fire ants and/or colonies/mounds/nests. The endpoint selected should be claimed on the proposed label. The following are examples of commonly used endpoints; see specific study sections (i) through (m) for additional information or variations.

1. **Mortality.** Observations of mortality should be reported based on recommendations provided in the specific study design. The number of dead worker ants and queen(s) in each replicate should be recorded separately at each time point tested, as practically possible. A mortality count should include only dead, but not moribund, ants.

2. **Number of active mounds.** Numbers of active mounds on plots should be reported based on recommendations provided in the specific study design. The number of active mounds in each replicate should be recorded separately at each time point tested.

3. **Forager numbers.** Numbers of foragers collected at food lures should be reported based on recommendations provided in the specific study design. The number of foragers collected in each replicate should be recorded separately at each time point

tested.

4. **Presence/absence of brood.** Observations of presence/absence of brood should be reported for all colonies/mounds/nests based on recommendations provided in the specific study design.

5. **Food consumption.** The amount of food consumed should be reported based on recommendations provided in the specific study design.

vi. **Test organisms.** Testing should be conducted with adult red imported fire ants unless the product is intended to target ant development and/or immature stages. All sources of red imported fire ants should be listed in the study methods along with species.

vii. **Representative sampling**.

1. **Replication.** The protocol should fully describe how sample size and replication were determined, and a power versus sample size analysis should be performed to support the proposed sample size. Other factors that may affect sample size and replication are the number of treatments, the experimental design, and the heterogeneity in the sample pest population (e.g., social form) and the environment (e.g., different habitat population densities).

2. **Rearing, handling, and maintenance of red imported fire ants.** When applicable, a description of the laboratory colony rearing practices should be included. Collection details and maintenance procedures for field-collected ants should be described.

3. **Negative control.** In most studies, a negative control should be included. The number of control replicates should equal the number of replicates for each treatment. When appropriate, a negative control is typically treated with diluent only or receives no treatment at all.

viii. **Quality assurance (QA) plan.** Protocols should provide for periodic quality assurance inspections that are adequate to ensure the integrity of the study and consistency with the provisions of EPA's Good Laboratory Practices regulations (40 CFR §160).

b. Data collection and reporting. Study protocols should provide for collection and reporting of data covering all aspects of the research including those discussed in section (g) of this guideline. GLP regulations specify that each study protocol should provide for collecting and reporting all elements provisioned by the GLP regulation at 40 CFR §160.120.

c. Data analysis. Protocols should include a full description and explanation for the statistical methods proposed to analyze product performance test results, taking into account the specific study objectives and variables. A statistician may be consulted regarding the sample size vs. power of the study design and the statistical methods for data analysis when developing test protocols. Analysis of data is recommended to determine if the mortality rate of the group treated with the product differs from the negative control mortality and if any within treatment effects were significant. Ninety-five percent confidence intervals should be reported around any estimated statistic. Protocols should explicitly describe the statistical model to be used and demonstrate that the assumptions underlying the model can be met for all proposed analyses. Restrictions on randomization of any testing components should be documented clearly and should be accounted for correctly in the statistical analyses. Generally, generalized linear models (GLMs) are recommended to fit models directly to non-normal (e.g., binomial – which describes many of the collected product performance data sets) data using an appropriate link function.

GLMs do not involve transforming the response variable, thereby allowing data to remain on the original scale of measurement. Generalized linear mixed-models (GLMM) may also be appropriate for correlated data. Software for analysis using GLMs or GLMMs is available in many widely sold statistical analysis packages. If survival analyses, such as the Kaplan-Meier Estimator, are used, justification should be provided for use of the median value to characterize product performance and demonstrate that the underlying assumptions of these analyses have been met. One-way analysis of variance (ANOVA) or mixed-effects models can be used if their assumptions, such as normality, etc. are justified.

(e) Review of protocols for efficacy studies. Protocols proposing novel testing methods (i.e., non-guideline testing methods) or testing of other species of ants for which the Agency requires data to support product performance should be submitted to EPA for review before the study begins.

(f) Execution of efficacy studies.

a. Execution of protocol. In cases where a protocol has been submitted to EPA for review, testing should be initiated when the EPA review is complete and as applicable, EPA comments should be incorporated into the revised protocol.

b. QA oversight. Product performance testing is subject to GLP regulations at 40 CFR §160. GLP regulations state that each testing facility should include an independent QA unit. The QA unit monitors and documents execution of each protocol in accordance with the GLP regulations (40 CFR §160.35). The QA unit should inspect each study at intervals adequate to ensure the integrity of the study and maintain written and properly signed records of each periodic inspection. Please see (b)(b)(i) above for the discussion of the use of GLP laboratory methods when conducting pest product performance studies.

c. Protocol amendments. Amendments are planned changes to the protocol and should be made before the study is executed. All amendments to the protocol should be noted in the written report to the agency.

d. Deviations from protocol. Even when executing the best-designed and most comprehensive protocols, unanticipated deviations from the protocol may occur. All such deviations from the protocol and their impact on the research should be fully reported in the study report submitted to EPA (40 CFR §160.185).

(g) Reporting of completed efficacy studies to the agency.

a. Study identification. Title, identifying study number(s), sponsor, study director, investigators, name and location of the testing facility, and dates of the study should be reported. If tests are conducted outside the U.S., the relevance of the study for U.S. regulatory purposes should be justified in the study report.

b. Study objective(s). The purpose of the study should be stated.

c. Testing conditions. Information on temperature, relative humidity (RH), ambient light and photoperiod (L:D), and air flow (where applicable) should be reported.

d. Testing system. Testing system information, including but not limited to the following, should be reported:

i. Identification of social form and field populations if applicable; where colonies were collected/obtained; development stage; and methods for preparation of ants for testing (feeding/starving) should be identified.

ii. Rearing, handling, and maintenance of ants.

iii. Description of test substance (i.e., product, % active ingredient, and formulation to be tested). Negative control should also be described, including the application, if applicable.

iv. Description of the experimental unit.

v. Treatment application rate and method of application (rate and method of application should be consistent with label instructions).

vi. Number of product treatments if greater than one.

vii. Number of negative control and treatment replicates.

viii. Number of replicates per treatment.

ix. Number of individuals or mounds/plots per replicate for each treatment including controls.

x. Length of ant exposure to each treatment.

xi. Endpoints and time intervals of endpoint recordings.

e. Data/results reporting

i. **Raw data.** Include legible copies of all raw data.

ii. **Results summary.** Report summary test results on all aspects of research. The amount of product applied and active ingredient delivered per replicate should be reported. See sections (i) through (m) for more details.

iii. **Data analysis**. Provide a copy of the statistical analysis plan and results from statistical analysis. Refer to Section (d)(c) for recommendations on data analyses, unless otherwise indicated in a study-specific Data analysis and reporting section.

f. Study conclusions. The report should include a discussion of the study results and conclusions based on treatment endpoints. Conclusions should state why and how the study results do or do not support the tested hypothesis.

g. Protocol with amendments and study deviations from the protocol. A copy of the study protocol should be included with amendments and deviations. Deviations should be justified and described together with their impact on the validity of the study. The study should align with the protocol.

(h) Retention of records. The record-keeping provisions of 40 CFR §160.190 and §160.195 apply to records of any study conducted under the GLP rule.

(i) Specific guidance for field studies for testing area-applied pesticide products.

a. Study objective: The field studies described in this section are designed to determine product performance of area-applied pesticide products, such as, but not limited to, broadcast baits, bait stations, and area-applied barriers, against red imported fire ants in a colony/mound/nest. In addition, laboratory tests should be conducted for baits (see section (k)); for barrier and/or repellent products (see section (l)); and for products containing an IGR (see section (m)).

b. Materials and methods

i. **Site selection.** Sites should be selected from at least two localities within the range of the red imported fire ant and should not have been exposed to pesticide treatment for at least

one year prior to the study. One site should include monogyne colonies and the other should include polygyne colonies. Refer to section (c) for definitions of colony type.

ii. **Experimental units.** The treatment should be applied to a plot. Plots may be any size, so long as all plots from both sites are the same size, and should contain a minimum density of active fire ant mounds equivalent to 20 active mounds per acre to ensure sufficient numbers of foraging fire ants are present for testing of area-applied treatments (Drees et al. 2013). Plots within a site should be separated from each other by at least 17 meters or more (Colby et al. 2008, Stringer et al. 2011). All plots within a site should be as similar as possible with respect to density of active fire ant mounds, relative numbers of active foragers, and environmental conditions such as hydrology and vegetation cover.

iii. **Number of replicates per treatment.** Depending on the results of power vs. sample size analysis, the study should include a minimum of four plots per treatment per site. The design should be balanced with an equal number of treated and negative control plots per site.

iv. **Application method.** The lowest application rate to be supported on the product label should be applied using methods consistent with the product label. If bait stations are used, the number of deployed bait stations and the amount of bait in each station corresponds to the lowest number of stations per unit area proposed on the label. Bait station products should be tested as they would be deployed by a consumer.

v. **Ant exposure to product treatments.** Area-applied product studies should be conducted for a minimum of 60 days post-application at each location. A longer study duration may be needed based on desired label claims and the product's mode of action.

vi. **Data collection and endpoints**. All sampling should be conducted when soil temperatures are between 18.3 – 36 °C (Porter & Tschinkel 1987, Helms & Vinson 2005, Drees et al. 2007). A pre-treatment sampling of mounds and forager activity should be conducted 1-7 days prior to treatment application. The number of active mounds should be used to establish homogeneity among plots on a site. Sampling should be conducted over a minimum of 60 days with a minimum of four sample collections at days 15, 30, 45, and 60 post-treatment.

The primary endpoint for measuring efficacy of area-applied products in this design focuses on forager numbers. The number and timing of sampling intervals for foragers should be based on the pesticide's mode of action and desired label claims. If uncertain about suitable establishment of plots and/or sampling regime, consult the Agency for appropriate intervals. Ideally, data should be collected simultaneously from treated and control plots.

1. **Sampling of foragers.** The number of foragers present in the plot at each sampling interval should be estimated. This may be accomplished through the use of an attractive food lure (e.g., hot dog pieces) and vials. Place a minimum of 9 vials containing the attractive food lure equidistant from the plot edge and each other within the plot for an established sampling period, 30 minutes or 1 hour. At the end of the sampling period, cap the vials. The number of ants in each vial is to be counted and reported for the plot.

2. **Number of active mounds**. The number of active mounds per plot should be used to establish homogeneity among plots on a site. A mound is considered active when

at least 20 workers exit the mound immediately following disturbance (Oi & Oi 2006). To determine if mounds on each plot are active, the surface of each mound should be scratched with a pair of forceps or similar object. It is optional to compare numbers of active mounds pre- and post-treatment for specific mound claims, such as “no new mounds.” In this case, at each sampling interval, all mounds in each plot per site should be assessed for activity.

3. **Insect growth regulators (IGRs).** For products containing an IGR, an additional sampling assessment to confirm the presence of brood in all mounds in each plot should be conducted. To assess for the presence of brood during pre- and post-treatment sampling, using a small-headed shovel or hand trowel, a small portion of the mound (about the size of a fist) should be removed to observe brood. Record whether brood is present or absent in all mounds in each plot.

c. Data analysis and reporting. Refer to sections (iii) – (v) for guidance on statistical analyses. Refer to Section (g) of this guideline for guidance on data/result reporting. In addition, the following information should be reported:

i. Social Form. Report the social form of imported fire ant colonies on each site and how social form was determined (e.g., mound density, worker head size, genetics, etc.).

ii. Treatment data. The amount of product applied, expressed as weight of product (and active ingredient) per unit area, should be reported for each replicate. If reapplication/rebaiting occurred, specify methods and thresholds for reapplication/rebaiting.

For bait stations, the amount of bait per bait station and the duration of baiting events needed to achieve product efficacy should be specified. The number of bait stations per unit area and location of bait stations, including distance from the mound/colony and other bait stations should be reported. Bait removal by the test species as the difference in pre- and post-weights and accounting for water loss/gain should be documented.

iii. Forager numbers. For each assessment, the date, time, temperature, and weather should be reported. Numbers of fire ants per vial should be recorded. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of ants. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

iv. Number of active mounds. For each assessment, the date, time, temperature, and weather should be reported. The number of active and inactive mounds per plot per treatment within a site should be reported for each assessment. If product application is intended to prevent new mounds from forming on treated plots, then the number of all active mounds per plot per treatment within a site should be reported for each assessment. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of active mounds. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

v. Presence/absence of brood if using an IGR. For each assessment, the date, time, temperature, and weather should be reported. The number of mounds with and without brood per plot per treatment within a site should be reported for each assessment. Generalized linear mixed effects models for Poisson distribution should be used to analyze

the number of mounds with brood. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

d. Study conclusions. Summarize study outcomes for area applied product testing against red imported fire ants in a colony/mound/nest, and/or mounds and discuss their implications for product labeling.

(j) Specific guidance for field studies for testing mound-applied pesticide products.

a. Study objective: The field studies described in this section are designed to determine the product performance of mound-applied pesticide products, such as but not limited to baits, dusts, and injectable aerosols, against red imported fire ants in a colony/mound/nest. In addition, laboratory tests should be conducted for baits (see section (k)); for barrier and/or repellent products (see section (l)); and for products containing an IGR (see section (m)).

b. Materials and methods

i. Site selection. Sites should be selected from at least two localities within the range of the imported fire ant, and should not have been exposed to pesticide treatment for at least one year prior to the study. One site should include monogyne colonies and the other should include polygyne colonies. Refer to section (c) for definitions of colony type.

ii. Experimental units. The treatment should be applied to individual mounds on designated plots. Each plot should contain a minimum of 10 active mounds for treatment. Plots may be any size. Control plots should be approximately 17 meters or more from treated plots (Colby et al. 2008, Stringer et al. 2011). All plots within a site should be as similar as possible with respect to density of active fire ant mounds and environmental conditions such as hydrology and vegetation cover.

iii. Number of replicates per treatment. Depending on the results of power vs. sample size analysis, the study should include a minimum of four plots per treatment per site. The design should be balanced with an equal number of treated and negative control plots per site.

iv. Application method. The lowest application rate recommended on the product label should be the treatment application rate and the product should be applied using methods consistent with the product label.

v. Ant exposure to product treatments. Treatments should be applied no less than 24 hours after disturbance of the mounds, unless required for product application. Studies testing mound-applied products should be conducted for a minimum of 30 days at each location. A longer study duration may be needed based on desired label claims and the product's mode of action.

vi. Data collection and endpoints. All sampling should be conducted when soil temperatures are between 18.3 – 36 °C (Porter & Tschinkel 1987, Helms & Vinson 2005, Drees et al. 2007). A pre-treatment assessment of mounds should be conducted 1-7 days prior to treatment application. Sampling should be conducted over a minimum of 30 days with a minimum of four sample collections, days 7, 14, 21, and 30 post-treatment. The number and timing of sampling intervals should be based on the pesticide's mode of action and desired label claims. If uncertain about a suitable sampling regime, consult the Agency for appropriate intervals. Ideally, data should be collected simultaneously from treated and

control plots.

1. **Number of active mounds.** The number of active mounds per plot is used to establish homogeneity among plots on a site and to compare numbers of active mounds pre- and post-treatment. A mound is considered active when at least 20 workers exit the mound immediately following disturbance (Oi and Oi 2006). To determine if mounds on each plot are active, the surface of each mound should be scratched with a pair of forceps or similar object. If product application is intended to prevent new mounds from forming on treated plots, then the number of all active mounds per plot per treatment within a site should be sampled for each assessment.

2. **Insect Growth Regulators (IGRs).** For products containing an IGR, an additional sampling assessment to confirm the presence of brood in all mounds in each plot should be conducted. To assess for the presence of brood during pre- and post-treatment sampling, using a small-headed shovel or hand trowel, a small portion of the mound (about the size of a fist) should be removed to observe brood. Record whether brood is present or absent in all mounds in each plot. Once treatment is applied, data collection should be conducted over a minimum of 60 days with a minimum of four sample collections at days 15, 30, 45, and 60 post-treatment or more depending on desired claims.

c. Data analysis and reporting. Refer to sections (iii) – (iv) for guidance on statistical analyses. Refer to Section (g) of this guideline for guidance on data/result reporting. In addition, the following information should be reported:

i. Social Form. Report the social form of red imported fire ants on each site and how social form was determined (e.g., mound density, worker head size, genetics, etc.). For each assessment, report the date, time, temperature, and weather.

ii. Treatment data. The amount of product applied, expressed as weight of product per unit area, should be reported for each replicate. If reapplication/rebaiting occurred, specify methods and thresholds for reapplication/rebaiting. For bait, specify the duration of baiting events needed to achieve product efficacy.

iii. Number of active mounds. For each assessment, the date, time, temperature, and weather should be reported. The number of active treated mounds per plot per treatment within a site should be reported for each assessment. If product application is intended to prevent new mounds from forming on plots with treated mounds, then the number of all active mounds per plot per treatment within a site should be reported for each assessment. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of active mounds. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence interval should be adjusted for baseline values and reported for each time point.

iv. Presence/absence of brood if using an IGR. For each assessment, report the date, time, temperature, and weather. The number of treated mounds with/without brood per plot per treatment within a site should be reported for each assessment. If product application is intended to prevent new mounds from forming on plots with treated mounds, then brood assessments should be conducted on all mounds per plot per treatment within a site. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of mounds with brood. Estimated count ratio (i.e., mean ratio) between treatment and control and its 95% confidence intervals should be adjusted for baseline values and reported for each time point.

d. Study conclusions. Summarize study outcomes for mound applied product testing against red imported fire ants in a colony/mound/nest and/or mounds and discuss their implications for product labeling.

(k) Specific guidance for laboratory studies for testing bait products.

a. Study objective: The laboratory studies described in this section are designed to determine product performance of bait products against all life stages of red imported fire ants. In addition, a field test(s) (see sections (i) and/or (j)) should be conducted in conjunction with laboratory tests for baits.

b. Materials and methods

i. Experimental units. An experimental unit consists of an individual nest arena containing fire ant brood, workers, and queens connected to two foraging-arenas via plastic tubing (e.g., Tygon® tubing). The nest arena should contain a water source for the ants and harborage for queen(s) and brood. One foraging arena should contain the test material (treated bait) and the other should contain an alternate food source. The length of plastic tubing connecting foraging arenas to the nest arena should be equal. Arenas should consist of an open-top box where interior vertical surfaces are coated with a non-stick material (e.g., Fluon® or talcum powder) to prevent escape of ants. All arenas used in this study should have the same dimensions.

ii. Number of replicates per treatment. Depending on the results of power vs. sample size analysis, a minimum of five replicates per treatment, with a minimum of 100 workers, a queen, and a recorded and consistent amount of brood per replicate arena, should be tested. Design should be balanced with an equal number of treated and control arenas. All arenas should be held under the same environmental conditions: 25°-30° C; photoperiod of 16:8 (L:D), and between 70 – 90% RH (Porter & Tschinkel 1987).

iii. Application method. Treatment should be product specific and applied at the lowest labeled rate and in the manner and duration as directed by the product label. This study design should consist of a choice test to demonstrate acceptability of the bait product and the alternate food source should be palatable, established in the literature, and consistent across trials. The alternate food source should be available *ad libitum* to the ants throughout duration of the study. Control arenas should receive the alternate food source only.

iv. Ant exposure to product treatments. Ants should be acclimated to the arenas for a minimum of 24 hours, during which time they are provided with the alternative food source and water. Uneaten food and dead individuals (not to exceed 10% of the original number) should be removed prior to treatment. Tests should be conducted for a maximum of 14 days post-treatment.

v. Data collection and endpoints. The total amount of treated bait used, expressed as weight of product per unit area, should be documented for each replicate. If re-baiting occurs, how re-baiting is conducted and thresholds for re-baiting should be recorded. The number of workers per replicate should be reported. Mortality counts should be conducted at intervals $\leq$ 48 hours through the duration of the study. Dead individuals should be removed and counted. Control groups should be assessed in the same manner as those receiving treatment. Following the final assessment, all nest arenas should be frozen to determine the number of surviving individuals and for calculation of the total number of workers.

c. Data analysis and reporting. Refer to Section (g) of this guideline for guidance on data/result

reporting. Control mortality should not exceed 25% for workers and there should be no queen mortality.

i. **Reduction in worker numbers.** Mortality counts of workers should be reported and generalized linear mixed effect models for Poisson distribution should be used to analyze the number of survival workers (number of survival workers = total workers – mortality counts of workers). Survival rate per treatment group and survival rate ratio between treatment and control groups should be calculated with 95% confidence limits per assessment.

ii. **Queen mortality.** Mortality counts of queen(s) should be reported and mortality rate should be calculated with 95% confidence limits across replicates per assessment.

d. **Study conclusions.** Summarize study outcomes for bait products against imported fire ant colonies (i.e., workers and queen(s) and discuss their implications for product labeling.

(l) Specific guidance for laboratory studies for testing barrier and/or repellent products.

a. **Study objective:** The laboratory studies described in this section are designed to determine product performance of barrier/repellent products (other than premises treatments) against red imported fire ants. In addition, a field test(s) (see sections (i) and/or (j)) should be conducted in conjunction with laboratory tests for barrier and/or repellent products.

b. **Materials and methods**

i. **Experimental units.** An experimental unit consists of an individual nest arena containing fire ant workers and optional queen(s) plus brood (depending upon product claims) connected to two foraging-arenas via plastic tubing (e.g., Tygon® tubing). The nest arena should contain a water and food source for the ants and harborage for the queen(s) and brood if included in the study. The food source should be palatable, established in the literature, and consistent across trials, and should be available *ad libitum* to the ants throughout duration of the study. Foraging arenas should contain equal amounts of food placed at the far end of each arena. One foraging arena should contain substrate (e.g., sand, soil, or impregnated material) treated with the product and the other should contain untreated substrate (same as in treated arenas but without the active ingredient). The length of plastic tubing connecting foraging arenas to the nest arena should be equal. Arenas should consist of an open-top box where interior vertical surfaces are coated with a non-stick material to prevent escape of ants. All arenas used in this study should have the same dimensions.

ii. **Number of replicates per treatment.** Depending on the results of power vs. sample size analysis, a minimum of five replicates per treatment with a minimum of 100 workers should be tested. Design should be balanced with an equal number of treated and control arenas. All arenas should be held under the same environmental conditions: 25°-30° C; photoperiod of 16:8 (L:D), and between 70 – 90% RH (Porter & Tschinkel 1987).

iii. **Application method.** Treatment should be product specific and applied at the lowest labeled rate and in the manner as directed by the product label. Testing should be conducted on substrates aged to the maximum residual time point specified on the label. Aging of substrates should occur outdoors or if indoors using simulated temperature, rain, and sunlight. This study should consist of a choice test of two foraging arenas: one containing treated substrate (e.g., sand, soil or impregnated material) and the other containing untreated substrate.

iv. **Ant exposure to product treatments.** Ants should be acclimated to the arenas for a minimum of 24 hours, during which time they are provided with the alternative food source and water. Uneaten food and dead individuals (not to exceed 10% of the original number) should be removed prior to treatment. Tests should be conducted for a maximum of 14 days post-treatment prior to providing access to the foraging arenas. Foraging arenas should contain a minimum of ~1.0 cm (½") depth of substrate, if sand/soil is used. Equal amounts of food should be available for the ants to forage at the far end of each the foraging arenas from the entrance. Control colonies should also have access to two foraging arenas, both containing untreated substrate only. Tests should be conducted for a maximum of 14 days post-treatment.

v. **Data collection and endpoints.** The proportion of food consumed in treated arenas should be used as an indirect measure of repellency. Food should be replenished in each arena every 48 hours through 14 days, recording pre- and post- weights at each time point. Any observations of foraging behavior or lack of foraging behavior within the arenas should be reported. The total number of workers per replicate should be collected. Mortality should be evaluated at intervals ≤ 48 hours through the duration of the study. Dead individuals should be removed and counted. Controls should be assessed in the same manner as those receiving treatment. Following the final assessment, all nest arenas should be frozen to determine the number of surviving individuals and for calculation of the total number of workers.

c. **Data analysis and reporting.** Refer to Section (g) of this guideline for guidance on data/result reporting. Control mortality should not exceed 25% for workers.

i. **Food consumption**. The amount of food consumed in each foraging arena per replicate should be reported by each time-point and by total of all time-points. The total amount of food consumed for treated and untreated arenas per replicate should be calculated by each time-point or by total of all time-points. The proportion of food consumed in treated arena (= amount food consumed in treated arena/(amount food consumed in treated arena + amount food consumed in untreated arena)) for each replicate at each time point or by total of all time points should be calculated and then analyzed using generalized linear mixed effects models (GLMMs) with logit link function or other appropriate statistical methods. The estimated proportions of amount food consumed in treated arenas and their 95% confidence intervals should be reported.

ii. **Reduction in numbers of workers**. Mortality of workers should be reported as the number of dead individuals per replicate at each time point. Generalized linear mixed effects models for Poisson distribution should be used to analyze the number of surviving workers. Estimated survival rate and its 95% confidence interval per group, and survival rate ratio between treatment group and control group and its 95% confidence should be reported for each time point.

d. **Study conclusions.** Summarize study outcomes for barrier and/or repellent products against red imported fire ants and discuss their implications for product labeling.

(m) Specific guidance for laboratory studies for testing IGR products.

a. **Study objectives:** The laboratory studies described in this section are designed to determine product performance of IGR products against immature life stages of red imported fire ants. In addition, a field test(s) (see sections (i) and/or (j)) should be conducted in conjunction with laboratory tests for IGRs.

b. Materials and methods.

i. Experimental Unit. Guidance for setting up experimental units to test products containing an IGR may be found in (k)(b)(i) of this guideline for baits or (l)(b)(i) for barriers and/or repellents. In addition, for an IGR laboratory study, harborage in the nest arenas should contain a covered plastic Petri dish measuring 150 mm in diameter and filled 6 mm deep with plaster. After the plaster has set, one or more access holes (1-2 mm diameter) should be soldered into the side of the dish, just above the plaster, to allow access into the nest cell. Transparent yellow acetate paper affixed over the lid will filter ambient light allowing for observation of brood inside the nest cell with minimal disturbance (Williams 1990).

ii. Number of replicates per treatment. Depending on the results of a power vs. sample size analysis, a minimum of five replicates per treatment with approximately 10,000 workers, a queen, and a minimum of 10 ml of brood per replicate arena should be tested (Banks et al. 1983). Specific methods establishing larger colonies in the laboratory for extended studies are explained in Banks et al. 1981. Additional guidance for setting up replicates to test products containing an IGR may be found in (k)(b)(ii) of this guideline for baits or (l)(b)(ii) for barriers and/or repellents.

iii. Application method. Guidance for application of test products can be found in (k)(b)(iii) of this guideline for baits or (l)(b)(iii) for barriers and/or repellents.

iv. Ant exposure to product treatments. Ants should be acclimated to the nest arenas for a minimum of 72 hours and provided with food and water prior to being granted access to the foraging arenas. Tests with IGRs should be conducted for a minimum of 30 days post-treatment. Additional guidance for ant exposure to products may be found in (k)(b)(iv) of this guideline for baits or (l)(b)(iv) for barriers and/or repellents.

v. Data collection and endpoints. For IGRs, data should be collected at intervals ≥ 7days through the duration of the study. A minimum of three time points for collection of data should include the following: presence or absence of brood, changes in caste structure and/or deformities of the brood, or presence of dead brood in the colony midden (trash pile). If the test product contains additional active ingredients that are not IGRs, then data collection corresponding to (k)(b)(v) for baits or (l)(b)(v) for barriers/repellents should be conducted. Controls should be assessed in the same manner as those receiving treatment.

c. Data analysis and reporting. Refer to Section (g) of this guideline for guidance on data/result reporting. In addition, the following information should be reported:

i. Brood. For treated and control replicates, presence or absence of brood, changes in caste structure and/or deformities of the brood, and any dead brood observed should be reported.

ii. Additional reporting. If the test product contains additional active ingredients that are not IGRs, then results and data analysis corresponding to (k)(c) for baits or (l)(c) for barriers/repellents should be conducted.

d. Study conclusions. Summarize study outcomes for IGR product testing against red imported fire ants and discuss implications for product labeling.

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