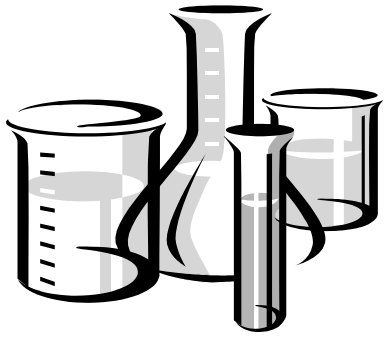
United States
Environmental Protection
Agency

Office of Chemical Safety
and Pollution Prevention
(7101)

EPA 712-C21002
[DATE 1/5/2021]

Product Performance Test Guidelines

OCSPP 810.3300: The Efficacy of Topically Applied Pet Products Against Certain Invertebrate Pests



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-2019-0161 is the docket number for the FIFRA SAP peer review record containing this guideline.

OCSPP 810.3300: The Efficacy of Topically Applied Pet Products Against Certain Invertebrate Pests

(a) Introduction.

(1) Scope. This guideline provides recommendations for the design and execution of laboratory studies to evaluate performance of pesticide products applied to pets to fulfill data requirements under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*). This guidance applies to products in any topically applied formulation, such as a spray, spot-on, collar, shampoo, or dust, if intended to be applied to pets for a pesticidal purpose such as to kill, repel, or control ticks, fleas, mosquitoes, and biting flies. This guideline does not apply to those products exempt from FIFRA Registration under 40 CFR 152.25, products applied to humans or livestock, or product performance testing described in other Agency guidelines. This guideline revises OCSPP Test Guideline 810.3300: Treatments to Control Pests of Humans and Pets (March 1998).

(2) Purpose. This guideline provides laboratory methods to evaluate product performance of pesticides applied to pets, against fleas, ticks, mosquitoes, and biting flies.

(3) Source. The main source material used in developing the flea and tick portion of this guideline was the European Medicines Agency Guideline, *Guideline for the testing and evaluation of the efficacy of antiparasitic substances for the treatment and prevention of tick and flea infestation on dogs and cats*. Other source materials are referenced throughout the guideline. A full list of references can be found in Section (o) at the end of this document.

(4) 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, 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, with the exception that studies will comply with 40 CFR 160.90 to ensure animal welfare, studies that do not otherwise comply with GLP standards may 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.

(5) New approach methodologies (NAMs). EPA encourages the development of new approach methodologies (NAMs) to reduce testing using vertebrate animals. At the time of finalizing this guideline, the Agency was unaware of valid study designs for supporting the reduction of animal use or the replacement of animal use for this guideline. However, the Agency acknowledges that such study designs may become available in the future as science advances. Protocols proposing new approach methods, especially those methods which do not

rely on utilizing vertebrate animals for testing, should be submitted to EPA for review before the study begins.

(b) Organization of the Guideline.

(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) Methods applicable to all tested pests;

(j) Specific guidance for testing of products for the treatment and prevention of flea infestations (all product types except insecticidal shampoos);

(k) Specific guidance for testing of products for the treatment and prevention of tick infestations (all product types except insecticidal shampoos);

(l) Specific guidance for testing of products for efficacy against mosquitoes and biting flies (all product types except insecticidal shampoos);

(m) Specific guidance for evaluating efficacy of pesticidal shampoo products (not including dry shampoos or dusts);

(n) Specific guidance for evaluating efficacy under simulated environmental conditions;

(o) References.

(c) Definitions. The following definitions are of special importance in understanding this guideline. They apply only in the context of this guideline and are not intended to be more generally applicable.

(1) Challenge/infestation/exposure is when pests are placed on or allowed to land on animal subjects.

(2) Comb out [count] refers to counting pests on an animal by running a comb through an animal's fur to determine the number of pests remaining on the animal. Live pests (which includes moribund) and dead pests are removed from the animal during a comb count.

(3) Control/residual efficacy refers to pesticidal activity at or above the minimum acceptable threshold resulting from an initial exposure of the target pest to a treated animal at least 24 hours after application.

(4) Hand count refers to counting pests on an animal by running a hand or thumb through an animal's fur to determine the number of pests remaining on the animal. Live and moribund pests are not removed during a hand count.

(5) An **insect growth regulator (IGR)** is a pesticide that inhibits maturation of an arthropod through its life cycle.

(6) **Kill** refers to the action of a pesticidal product to cause death or mortality to a pest (does not include moribund pests).

(7) **Knockdown** refers the action of rendering an arthropod incapable of coordinated movement or unable to right itself following exposure to a pesticide product.

(8) **Lowest labeled dose** refers to the lowest pesticide application rate to be applied to any pet per the label instructions. If a product is labeled for dosing by weight class (e.g., apply 0.34 fl. oz. tube of product to dogs weighing between 7 and 14 lb), the lowest labeled dose is the label dose (0.34 fl. oz.) divided by the maximum weight within the weight class (14 lb). Per the example above, the lowest labeled dose is 0.34 fl. oz./14 lb dog or 0.0243 fl. oz./lb.

If a product employs dose banding, wherein the product allows for variable dosing determined by the weight class of the pet, the lowest labeled dose should be calculated for each weight class. The lowest labeled dose for the product is the lowest dose across all the weight classes.

(9) **Moribund** refers to the state of an arthropod being incapacitated and exhibiting only uncoordinated twitching movements. Moribund arthropods should be considered alive.

(10) **Mortality** refers to death. A dead arthropod is an arthropod that does not move, even when poked or probed. Moribund arthropods should not be considered dead.

(11) **Pets** refers to species of animals which are commonly kept in domestic households (e.g., dogs and cats). Animal species that may be considered livestock (e.g., horses, cattle, swine, poultry, goats, sheep) are not considered pets.

(12) **Positive control** refers to animals treated with a product other than the test product that is applied via the same method for the same indication. Use of a positive control is not recommended.

(13) **Repellency** refers to products that typically result in pests avoiding or moving away from the treated animal or cause the pest to fall off the treated animal soon after contact. Other types of repellency based on the effect of the active ingredient on the arthropod pest may be justified.

(14) **Speed of kill efficacy/starts killing** refers to when a product begins killing, typically tested in fewer than 48 hours (e.g., "starts killing/kills in 60 minutes"). To obtain this claim, the same testing methods and infestation periods that will be used for general testing should be used at each infestation period (e.g., days 0, 7, 14 & 28 each at 60 min). Any pest found "moribund" should be counted as live at that time point and not removed to determine if it dies later. Exact timing should be recorded to ensure each count was conducted at the indicated timing. The same standards applied to the general testing should be applied to the "starts killing" claim as anything below those standards would not be considered clinically relevant (European Medicines Agency 2016).

(15) **Negative control** refers to animals in the untreated group.

(d) **Development of protocols for efficacy studies.** Testing pesticides for efficacy against invertebrate pests 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. Each of these topics is discussed in more detail in the sub-sections below. The product should be tested in a manner that reflects intended use and will provide data to support product performance. Additional considerations can be found in Sections (j) through (n).

(1) 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 (ix), given directly below.

i. Objectives. The objective of product performance testing is to determine efficacy of the lowest proposed labeled dose that has the intended effect on the claimed pest species or group of species. In all cases, the scientific objective and proposed label claims should be stated clearly, and all treatment groups should be compared to a negative control treatment.

ii. Test materials and treatments. End-use formulations should be tested using the lowest labeled dose for the tested pest. For products containing both IGR active ingredients (a.i.(s)) and other a.i.(s) see the flea section (j) for information on when testing should be conducted using the end-use product versus only the IGR. Test materials should be stored at ambient temperature and humidity for at least one day before use.

iii. Dose determination. The dose applied in product performance studies is the lowest labeled dose (as defined above in section (c)(8)) for the target pest from the proposed product label.

iv. Selection and allocation of animals. Animals should be qualified, blocked, and randomly allocated to treatment and control groups based on attractiveness to the pest species or group of species used for testing. After stratifying the qualified test animals based on attractiveness to the appropriate pest species, the animals in each stratum are allocated to control or treatment groups using randomization. For methods to determine attractiveness to specific pests, see sections (j) on fleas, (k) on ticks, and (l) on mosquitoes and biting flies.

v. Housing of test animals. Housing conditions should be selected in careful consideration of animal welfare aspects and prolonged isolation should be avoided as much as possible. For example, animals should be housed in individual cages during the period(s) of infestation with pests (i.e., from the day of infestation until the day of pest removal); for other time periods, animals may be housed together, but be separated by treatment groups to avoid confounding effects resulting from contamination of treatments. Housing conditions should not adversely affect the integrity of the study while meeting or exceeding GLP requirements outlined in 40 CFR 160.90, related to animal welfare. Temperature, relative humidity, and ambient light and photoperiod should be reported as well as the amount and type of food provided. Animals should be provided with water *ad libitum.* Enrichment should be provided while animals are housed alone.

vi. Choice of endpoints. Study endpoints should be appropriate for the specific objectives of the proposed research and likely to answer the research question. Commonly used endpoints include mortality and repellency. See specific study sections (j) through (n) for additional information on choosing endpoints for testing specific pests or claims.

vii. Test organisms. Testing should be conducted with individuals of the appropriate pest species or group of species to support efficacy claims on a proposed label. Pests should be sourced from laboratory colonies, except as noted in the fly section, and free of vector-borne pathogens. If available, information on strain and/or pesticidal resistance of test organisms should be reported. The minimum number of qualified animals per group should be determined to achieve sufficient power in addressing the study objectives.

viii. **Representative sampling**.

a. **Replication.** To ensure adequate statistical power and avoid unnecessary exposure of test animals to pesticides and biting pests, sample size should be justified using an analysis of power vs. sample size. Factors that may affect sample size and replication include the number of treatments, the experimental design, and the attractiveness of animals to the tested pest species or group of species.

b. **Negative control.** In all studies, an untreated control should be included. The number of negative control replicates should equal the number of replicates for each treatment. Controls should be similar in every way to the treatment group(s), except for application of the subject pesticide.

c. **Positive control.** To reduce the number of animals used during testing, a positive control is not recommended.

ix. **Quality Assurance/Quality Control 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 GLP regulations (40 CFR §160).

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

(3) Analyzing data. Study protocols should describe and explain the statistical methods used to analyze results of product performance tests. The proposed analysis should consider the specific study objectives and variables. If needed, a statistician should be consulted regarding the sample size vs. power of the study design and the proposed statistical analysis methods when developing test protocols. Analysis of data is recommended to determine if efficacy in the group treated with the product differs from the untreated control and if any within treatment effects were significant. Ninety-five percent confidence intervals should be reported around any estimated statistic. Using an asymptotic (large sample size) standard error of the estimates may be problematic for small sample sizes, therefore a bootstrapping approach is recommended for use to obtain the 95% confidence intervals of the estimates. Study protocols should explicitly describe the statistical model to be used and demonstrate whether 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, count, etc. – 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 (GLMMs) are recommended to analyze correlated non-normal data using an appropriate link function. Software for analysis using GLMs or GLMMs is widely available. One-way analysis of variance (ANOVA) or mixed-effects models can be used if their assumptions, such as normality, are justified.

(e) Review of protocols for efficacy studies. Protocols proposing novel testing methods or testing of other species of invertebrate pests on pets should be submitted to EPA for review before the study begins.

(f) Execution of efficacy studies.

(1) Execution of protocol. In cases where a protocol has been submitted to EPA for review,

testing should be initiated after the EPA review is complete and as applicable, EPA comments should be incorporated into the revised protocol.

(2) Quality Assurance (QA) oversight. Product performance testing is subject to Good Laboratory Practices (GLP) regulations pertaining to QA at 40 CFR §160. GLP regulations state that each testing facility 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 inspects each study at intervals adequate to ensure the integrity of the study and maintain written and properly signed records of each periodic inspection. See (a)(4)i. above for the discussion of the use of GLP laboratory methods when conducting product performance studies.

(3) 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.

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

(g) Reporting of completed efficacy studies to the Agency. For additional information on reporting, refer to EPA's Good Laboratory Practices regulations (40 CFR §160).

(1) Study identification. The 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.

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

(3) Testing conditions. Information on temperature, relative humidity, ambient light and photoperiod should be reported.

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

i. Identification of pest species, including where colonies were collected/obtained, and development stage.

ii. Methods for preparation of pest(s) for testing (feeding/starving), including rearing, handling, and maintenance of pest species.

iii. Description of test substance (i.e., product, % a.i., and formulation type to be tested). The negative control should also be described.

iv. Treatment dose and method of application should be consistent with label instructions. Dose for each animal should be reported in U.S. customary units as well as metric units as appropriate for the product. For products labeled for volumetric dosing by weight, doses should be reported in mg a.i./kg body weight in addition to U.S. customary and metric units for volume. For collar dosing, provide the total length and weight of each collar (after cutting) used for each animal used in the study.

v. Number of pests applied per infestation, number of treatments and number of animals (replicates) per treatment, including negative controls.

vi. Endpoints and time intervals of endpoint recordings, including a timeline for

qualification, treatment, and evaluations.

(5) Data/Results reporting. The following information should be reported:

i. Raw data. Include electronic and legible copies of all raw data, including count data for each animal and animal weight pre-application and post study completion.

ii. Results summary. Report summary test results and summary statistics on all aspects of research. See sections (j) through (l) for more details.

iii. Data analysis. Provide a copy of the statistical analysis plan and output including percent efficacy and associated 95% confidence intervals from statistical analysis. Refer to section (d)(3) for recommendations on analyzing data.

(6) 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 and how they apply to labeling and claims.

(7) Protocol with amendments and study deviations from the protocol. A copy of the 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) Methods applicable to all tested pests. The following sections apply to all efficacy studies testing topically applied treatments to control invertebrate pests of pets. For methods to test efficacy against specific pests see sections (j) on fleas, (k) on ticks, and (l) on mosquitoes and biting flies.

(1) Types of products. This guideline applies to topically applied treatments to control invertebrate pests of pets and includes spot-ons, sprays, dusts, collars, and shampoos.

(2) Laboratory testing. In a laboratory setting, testing should be conducted on-animal for each pest (e.g., fleas, ticks) for which efficacy of the product is claimed. To limit the number of animals used in testing, EPA recommends using multiple pest species per animal where practical. Flea and tick studies may be conducted with up to 2 species per animal at a time. For testing with mosquitoes, studies may be conducted with up to three species per animal at a time. For testing with flies, studies should only be conducted with one pest species per animal at a time. Field tests are not a preferred method to test efficacy of pet products but on occasion can be used to supplement laboratory data or to support specific claims; field studies should have protocols submitted to the Agency for approval prior to commencing the experiment.

(3) Dosing. Products should be applied to animals as noted in section (d)(1)iii. above.

(4) Test animals. Testing should be conducted only on the animal species appropriate for the labeled product. For example, dogs should be used for testing products intended for use on dogs; and cats should be used for testing products intended for use on cats. Animals should not be treated with any substance (e.g., pesticidal shampoo) that could affect the results of the study for at least 3 months prior to initiating the study, or for 30 days after the longest claimed period for products with efficacy claims lasting longer than 3 months. One exception is for products that are known to have very short acting periods and no residual efficacy, such as pills that kill fleas on pets within 24 hours. Products such as these may be given at least 1 week prior to the initiation of testing to be sure they have been cleared completely from the animals' bodies.

(j) Specific guidance for testing of products for the treatment and prevention of flea infestations (all product types except insecticidal shampoos). This section includes testing for flea mortality and IGR effects. Pesticide products applied to animals for use against fleas typically kill adult fleas and/or interrupt the life cycle by inhibiting the development of eggs or juvenile stages into the adult stage (e.g., IGRs). IGR efficacy can be mediated both indirectly by acting on egg development via a blood meal or contact with female fleas, or directly via contact with flea eggs in a treated animal's fur. IGRs are often combined with adulticides for treating flea infestations and in many situations, it is acceptable to test the marketed product. However, if an adequate number of viable specimens of the appropriate life stage (per (j)(5)ii.b.) cannot be collected from animals treated with a product containing both an adulticide and IGR component, then only the IGR a.i.(s) in the diluent should be tested to support efficacy claims related to the IGR component. Similarly, there may be certain claims related to the IGR a.i.(s) for which testing should be conducted using only the IGR a.i.(s) in diluent. Testing with either the formulated product or only the IGR ingredient in diluent should be conducted using the lowest labeled dose. For products containing both IGR a.i.(s) and other a.i.(s) making efficacy claims against only adult arthropods, the end-use formulation containing all a.i.(s) may be tested using the lowest labeled dose. Flea repellency may be tested; however, since currently there are no established methods for determining flea repellency, methods are not being provided in this document and it is recommended that a protocol be submitted.

(1) Test species. Testing should be conducted with laboratory-reared *Ctenocephalides felis* fleas of the appropriate life stage(s) (e.g., adults or eggs) to support proposed efficacy claims.

(2) Selection and allocation of test animals. To ensure adequate statistical power when evaluating efficacy against adult fleas, the minimum number of animals per group should be 7 for studies using cats and 6 for studies using dogs, unless justified by a power analysis. Animals should be qualified and stratified based on attractiveness to the pest species. After stratifying the qualified test animals, an equal number of animals in each stratum should be allocated to control or treatment groups using randomization. To determine attractiveness, prior to treatment (approximately between days -14 and -5), animals should be individually caged and exposed to 100 (± 10) unfed adult fleas for up to 24-48 hours. If at least 40% of fleas for cats and 60% of fleas for dogs are not retained on an animal during qualification, that animal should not be included in the study.

(3) Application method and exposure to fleas. Animals should be exposed to adult fleas based on the schedule in Table 1 for testing purposes. Animals in the treatment group should be weighed and treated on day 0 with the lowest labeled dose (as described in (c)(8) and (d)(1)iii. above) and applied as described on the product label. The negative control group should be untreated. For each infestation/re-infestation, test animals should be individually caged and infested with 100 (± 10) unfed adult fleas of similar age. Fleas should be distributed over the host animal; however, fleas should not be placed on or directly adjacent to the treated area. The methods for infesting the animals in the control group should be identical to those used for the treatment group. Flea tests may be combined with 1 species of tick testing, however it is not encouraged since grooming or scratching due to flea bites may detach ticks. Animals may wear cones (i.e., Elizabethan style collars) during the period between infestation and comb out for each infestation period. The control animals should retain a minimum of 40% of fleas on cats and 60% on dogs (based on group means) throughout the study to confirm they maintained adequate infestations. To test IGRs, animals should be kept in cages with trays underneath the cages after each infestation to collect flea eggs. For IGR testing, in the control group, a mean of at least 60% of fleas should emerge through the measured endpoint (e.g., egg hatch, adult emergence).

Table 1: Schedule for exposing animals to fleas based on the claimed duration of efficacy for the test substance.

Claimed Duration of Efficacy	Timing of infestations (treatment occurs on day 0)
< 7 days	Day -1, and the time corresponding to the longest labeled duration of efficacy
7 days to ≤ 4 weeks	Days -1 and 7, then weekly up to the longest labeled duration of efficacy
> 4 weeks	Days -1, 7, 14, 21 & 28 or 30, then monthly up to the longest labeled duration of efficacy

(4) Efficacy endpoints.

i. **Adult mortality.** Because dead fleas may fall off the animal during testing and not be found during on-animal counts, mortality should be determined indirectly by comparing live fleas on the treated animals to live fleas on the control animals. Moribund fleas should be considered alive.

ii. **IGRs.** Endpoints to measure efficacy of an IGR a.i.(s), depending on the specific claims, may include egg hatch and adult emergence. Other endpoints may be valid and should be justified.

(5) Data collection and recording for fleas.

i. **Adult mortality.** Multiple counts may be made after each infestation/re-infestation, particularly if a study is measuring the earliest efficacious time (e.g., 8 hours, 12 hours, etc.). All on-animal counts prior to the final count may be conducted using comb counts (and replacing the fleas) as long as the comber avoids the treated areas so not to mechanically spread the treatment, and the control group is treated in the same manner. The final count after an infestation should be conducted via comb out where all fleas are removed and counted. When multiple counts are made within an infestation period, each count should be recorded separately. Moribund individuals are considered alive.

It may take a few days after the initial treatment application for some types of products to spread throughout the animal's fur and skin and gain full efficacy (e.g., spot-ons and collars). Therefore, for products like these, the last pest count after the initial infestation may be recorded up to 72 hours post-treatment. However, for each subsequent re-infestation (e.g., the weekly challenges starting on day 7), the last pest count should be recorded by 48 hours post-infestation.

For speed of kill claims, see the definitions section (c)(14) for testing parameters.

ii. **IGRs.** For testing IGRs, animals should be individually caged and infested with adult fleas on the schedule listed in Table 1, and eggs should be collected from the animal and trays then moved to a clean container. The eggs should then be incubated to determine hatching and/or adult emergence depending on the claims desired.

When incubated under optimal conditions (i.e., at 21-29°C and 80% ± 10% humidity; Marchiondo et al. 2013), flea eggs will hatch approximately three days after being laid. Thus, to determine egg hatch rates, eggs should be observed by 96 hours of collections.

For adult emergence, eggs and hatched larvae should be evaluated to determine the number that emerged into adults. This should be done after 28-35 days (Marchiondo et al. 2013).

Other considerations:

a. Flea eggs may be collected overnight from the pans below each cage on multiple occasions to ensure enough eggs are collected after each infestation, then the adult fleas should be combed out of the animal after egg collection is complete.

b. Collected eggs can be divided into two groups to determine both larval hatch and adult emergence rates or can be combined into a single group if only one of the endpoints is being measured. At least 50 eggs total per animal should be collected so that both the larval hatch group and the adult emergence group each have a minimum of 25 eggs per animal in both the treated and control group at each time point for statistical comparisons between treated and control animals.

(6) Data analysis. If the study has only one measurement (one infestation) per animal, GLMs with logit link function for binomial or beta-binomial (when binomial data exhibiting over-dispersion) distribution data or other appropriate statistical methods should be used to estimate the survival or hatching proportion of each group. If the study has multiple measurements (multiple infestations) per animal (i.e., data are not independent), GLMMs with logit link function for binomial or beta-binomial (when binomial data exhibiting over-dispersion) distribution data or other appropriate statistical methods for correlated data should be used to estimate the survival or hatching proportion or adult emergence proportion of each group at each time point.

i. For adult mortality, the efficacy or effectiveness of the treatment and its 95% confidence interval should be calculated by the statistical model using Abbott's formula at each time point.

Adult Mortality Efficacy (%) = 100 × (proportion of live fleas on the control animals – proportion of live fleas on the treated animals) / proportion of live fleas on the control animals

= 100 × (1 – proportion of live fleas on the treated animals / proportion of live fleas on the control animals)

Proportion of live fleas on a control animal = # live fleas on the control animal / # fleas used to infest the control animal

Proportion of live fleas on a treated animal = # live fleas on the treated animal / # fleas used to infest the treated animal

ii. For IGRs, the efficacy or effectiveness of the treatment and its 95% confidence interval should be calculated using Abbott's formula at each time point.

IGR Efficacy (%) = 100 × (control hatching* proportion – treatment hatching* proportion) / control hatching* proportion

= 100 × (1 – treatment hatching* proportion / control hatching* proportion)

* may be substituted for adult emergence if that is the end point used for testing

(7) Data reporting. Refer to section (g) of this guideline for guidance on reporting results. Results should be reported separately for adult flea and IGR tests.

(8) Study conclusions. Summarize study outcomes for flea mortality and IGR results and discuss their implications for product labeling.

(k) Specific guidance for testing of products for the treatment and prevention of tick infestations (all product types except insecticidal shampoos). This section includes testing for tick mortality and repellency. Although there may be an effect of IGRs against ticks, testing of IGRs will not be included in section (k) because many of the ticks typically found in the U.S. are three-host ticks (Dantas-Torres 2010, Kocan et al. 2007) and therefore, IGRs are not appropriate for the prevention of tick reproduction (European Medicines Agency 2016).

(1) Test species. To ensure the product is efficacious against tick species that may vector agents causing tick borne illnesses, a laboratory study should be conducted on-animal with adults of three representative species of ticks. For more information regarding the specific representative species of ticks required for registration, consult with the Agency. If claims against other tick species are desired, those species may be tested in addition to the three representative species while using the same methods. Although testing should be conducted with the three representative tick species in total, no more than two species should be tested on the same animal simultaneously. If more than one species of tick is tested at the same time, the species chosen should be different in size and markings (before and after feeding) to be sure that counting can be done efficiently and precisely. For animal welfare reasons, if *Amblyomma maculatum* (Gulf Coast tick) is desired for a claim, it should be tested alone due to the effect of their bites.

(2) Selection and allocation of test animals. To ensure adequate statistical power when evaluating mortality or repellency against ticks, at least 11 animals (cat or dog) per group should be used, unless justified by a power analysis. Animals should be qualified and stratified based on attractiveness to the pest species. After stratifying the qualified test animals, an equal number of animals in each stratum should be allocated to control or treatment groups using randomization. To determine attractiveness, prior to treatment (approximately between days -14 and -5), animals should be individually caged and exposed to 50 (± 5) unfed adult ticks for 24-48 hours. At least 25% of the ticks should be retained per animal during qualification. If at least 25% of ticks are not retained on an animal, that animal should not be included in the study. If more than one species is going to be tested simultaneously, then only 1 of the species should be chosen to be used for the qualification process.

(3) Application method and exposure to ticks.

i. Mortality. Animals should be exposed to adult ticks based on the schedule in Table 2 for testing purposes. Animals in the treatment group should be weighed and treated on day 0 at the lowest labeled dose (as described in (d)(1)iii.) and applied as described on the product label. The negative control should be untreated. For each infestation/re-infestation, infest the test animals with 50 (± 5) unfed adult ticks for each species. Ticks should be of similar age post-molt to the adult life stage. Infestations should consist of a 50:50 ratio of female to male ticks (Dumont et al. 2015, Fourie et al. 2015, Stanneck et al. 2012, Young et al. 2003), however other ratios may be justified (e.g., 90:10 female to male ticks sex ratio for *Ixodes* spp.). Ticks should be distributed over host animal; however, ticks should not be placed on or directly adjacent to the treatment area. The methods for infesting the animals in the control group should be identical to those used for the treatment group. The use of double-sided sticky tape, petroleum jelly, or something similar around the openings of the cages and pans may be used to keep ticks from escaping. Animals may wear cones (i.e., Elizabethan style collars) during the period between infestation and comb out for each infestation period. The control animals should retain a minimum of 25% of ticks (Taenzler et al. 2016) throughout the study (based on group means) to confirm they maintained adequate infestations.

Table 2: Schedule for exposing animals to ticks for mortality testing, based on the claimed duration of efficacy for the test substance.

Claimed Duration of Efficacy	Timing of infestations (treatment occurs on day 0)
< 7 days	Day -2, and the time corresponding to the longest labeled duration of efficacy
7 days to ≤ 4 weeks	Days -2 and 7, then weekly up to the longest labeled duration of efficacy
> 4 weeks	Days -2, 7, 14, 21 & 28 or 30, then monthly up to the longest labeled duration of efficacy

ii. Repellency. Animals should be exposed to adult ticks based on the schedule in Table 3 for testing purposes. Animals in the treatment group should be weighed and treated on day 0 at the lowest labeled dose (as described in (d)(1)iii.) applied as described on the product label.

For each infestation/re-infestation, line individual pet carriers with a light-colored material, such as a light colored felt sheet. For each tick challenge, animals should be placed in the cage overnight or during a 12-hour dark period and 50 (± 5) unfed and similarly aged adult ticks of each species should be placed inside the cage but not on the animal. Infestations should consist of a 50:50 ratio of male to female ticks (Dumont et al. 2015, Fourie et al. 2015, Stanneck et al. 2012, Young et al. 2003), however other ratios may be justified (e.g., 90:10 female to male ticks sex ratio for *Ixodes* spp.). Carriers should be thoroughly washed, and flooring material discarded after each use (Young et al. 2003). The control animals should gain a minimum of 25% of ticks during each infestation throughout the study (based on group means) to confirm they acquired adequate infestations.

Table 3: Schedule for exposing animals to ticks for repellency testing, based on the claimed duration of efficacy for the test substance.

Claimed Duration of Efficacy	Timing of infestations (treatment occurs on day 0)
< 24 hours (1 day)	Day 0, immediately after application
>1 day to ≤ 7 days	Up to 24 hours post application, then the day of the longest claimed duration of efficacy
>7 days to ≤ 4 weeks	Up to 24 hours post application then weekly beginning on day 7 up to the longest labeled duration of the efficacy
> 4 weeks	Up to 24 hours post application then on days 7, 14, 21 & 28 or 30, then monthly up to the longest labeled duration of efficacy

(4) Efficacy endpoints. Endpoints (e.g., repellency, mortality) should be selected with respect to the intended use pattern for the product and effect on the arthropod pest for the a.i..

i. **Mortality.** Mortality claims (i.e., kills/controls) should be determined indirectly by comparing live ticks on the treated animals versus live ticks on the control animals.

ii. **Repellency.** A repellent effect is a reaction by a tick to avoid contact with a treated animal. Since repelled ticks will move away from the treated animal and possibly be unrecovered, repellency should be determined indirectly by counting the number of live or dead ticks (attached or unattached) on the animal compared to the control animals. Other methods of evaluating repellency against ticks (e.g., hot foot, inhibition of attachment, disruption of attachment; Halos et al. 2012) may also be considered but should be justified.

(5) Data collection and recording for ticks.

i. **Mortality.** It may take a few days after the initial treatment application for some types of products to spread throughout the animal's fur and skin and gain full efficacy (e.g., spot-ons and collars). Therefore, for products like these, the last pest count after the initial infestation may be recorded up to 72 hours post-treatment. However, for each subsequent re-infestation (e.g., the weekly challenges starting on day 7), the last pest count should be recorded by 48 hours post-infestation.

If earlier counts are desired within a time period (e.g., at 2 hrs., 12 hrs., etc.), they should be conducted with hand counts. The final count of the time period should be conducted via comb counts, removing all remaining ticks for that infestation period. Moribund ticks should be classified as "live."

For speed of kill claims, see the definitions section (c)(14) for testing parameters.

ii. **Repellency.** Tick counts should be taken within 24 hours of the infestation. The exact timing will depend on the effect on the arthropod pest of the a.i.(s) (e.g., volatile repellent, toxicant). Counts should be recorded as live or dead ticks on the animal (regardless if they are attached or unattached). Moribund ticks should be classified as "live." If another type of repellency test is desired, a protocol should be submitted

(6) Data analysis. If the study has only one measurement per animal (one infestation), GLMs with logit link function for binomial or beta-binomial (when binomial data exhibiting over-dispersion) distribution data or other appropriate statistical methods should be used to estimate the survival proportion of each group. If the study has multiple measurements (multiple infestations) per animal (i.e., data are not independent), GLMMs with logit link function for binomial or beta-binomial (when binomial data exhibiting over-dispersion) distribution data or other appropriate statistical methods for correlated data should be used to estimate the survival proportion of each group at each timepoint.

i. For adult mortality, the efficacy or effectiveness of the treatment and its 95% confidence interval should be calculated by the statistical model using Abbott's formula at each timepoint. For calculating mortality efficacy, the proportion of live ticks on the treated animals and proportion of live ticks on the control animals should be used in the following calculation:

Mortality Efficacy (%) = 100 × (proportion of live ticks on the control animals – proportion of live ticks on the treated animals) / proportion of live ticks on the control animals

= 100 × (1 – proportion of live ticks on the treated animals / proportion of live ticks on the control animals)

Proportion of live ticks on a control animal = # live ticks on the control animal / # ticks used to infest the control animal

Proportion of live ticks on a treated animal = # live ticks on the treated animal / # ticks used to infest the treated animal

ii. For repellency, the efficacy or effectiveness of the treatment and its 95% confidence interval should be calculated by the statistical model using Abbott's formula at each timepoint. For calculating repellency, compare live plus dead (attached/unattached) ticks on the treated animal versus on the control animals using the following calculation:

Repellency Efficacy (%) = 100 × (proportion of ticks on the control animals – proportion of ticks on the treated animals) / proportion of ticks on the control animals

= 100 × (1 – proportion of ticks on the treated animals / proportion of ticks on the control animals)

Proportion of ticks on a control animal = (# live ticks + # dead ticks) on the control animal / # ticks used to infest the control animal

Proportion of ticks on a treated animal = (# live ticks + # dead ticks) on the treated animal / # ticks used to infest the treated animal

(7) Data reporting. Refer to section (h) of this guideline for guidance on reporting results. Results should be reported separately for each tick species.

(8) Study conclusions. Summarize study outcomes for each species of tick for mortality or repellency and discuss their implications for product labeling.

(l) Specific guidance for testing of products for efficacy against mosquitoes and biting flies (all product types except insecticidal shampoos). This section includes testing for pesticides applied to animals to protect against mosquitoes and biting flies to repel, knockdown, or kill the adult life stage. Because the intent is to protect the pet from the biting pest, IGR ingredients are not relevant for these purposes; therefore, testing IGR ingredients against mosquitoes and biting flies is not considered in this section. Moreover, because mosquitoes and biting flies do not infest animals like fleas and ticks, products for application directly to mosquitoes and biting flies (e.g., sprays and shampoos for direct contact with the target pest) are not relevant for this section.

(1) Test species.

i. Mosquito species. To ensure the product is effective against mosquitoes that may vector heartworm (Nayar 1990, Tiawsirisup et al. 2007) and other animal parasites and diseases, tests should be conducted on three representative species of mosquitoes. For more information regarding testing specific species of mosquitoes, consult with the Agency.

ii. Biting fly species. Biting flies for pet product testing include, but are not limited to, *Lutzomyia spp.* or *Phlebotomus spp.* (sand flies) and *Stomoxys calcitrans* (stable flies). Evaluation of other biting flies would be considered with a protocol. Consult with the agency for more information regarding testing specific species and representative species of biting flies. Fly species desired for testing which do not have lab colonies may be obtained from wild populations.

(2) Selection and allocation of test animals. To ensure adequate statistical power, at least 6 animals per group should be used for assessing efficacy against sand flies or when testing a single mosquito species that will utilize 100 adult pests per infestation, unless justified by a power analysis. For studies assessing efficacy against stable flies, at least 8 animals with 50

adult pests per infestation or at least 12 animals with 25 adult pests per infestation should be used per group to ensure adequate statistical power. For studies testing three species of mosquitoes simultaneously, at least 8 animals with 50 of each species should be tested (150 total), unless justified by a power analysis.

Animals should be qualified and stratified based on attractiveness to the pest species used for testing, then an equal number of animals in each stratum should be allocated to control or treatment groups using randomization. If more than one species is going to be tested simultaneously, then only one of the species should be chosen to be used for the qualification process. To determine attractiveness of each animal, prior to treatment (approximately between days -14 and -5) animals should be individually caged and exposed to 25 – 100 adults of the appropriate pest species. For mosquitoes, exposure should be for one hour. For biting flies, exposure should be for 30 minutes. Animals should be sedated for the exposure period during qualification testing. For infestations using painful or aggressively biting mosquitoes or biting flies, the animals should be administered pain medications or a drug that has proven analgesic and sedative properties. Cages should be sized to comfortably house the sedated animal; typical cages are approximately 24 inches x 24 inches x 24 inches, but larger animals may need larger cages (Meyer et al. 2003, Ross et al. 1997). After the exposure period, animals should be removed from cages and insects should be aspirated and knocked down (e.g., freezing, exposed to CO_2), after which time insects should be assessed for blood feeding. During qualification, if at least 60% of insects do not take a blood meal on a specified animal, that animal should not be included in the study.

(3) Application method and exposure to mosquitoes and biting flies. Animals should be weighed and treated on day 0 with the treatment at the lowest dose (as described in (d)(1)iii. above) and applied as described on the product label. Animals should be exposed to insects based on the schedule in Table 4. Except for applying the treatment, animals in the untreated control group should be handled and exposed to the same conditions as animals in the treatment group. To ensure insects are healthy, at least 60% of insects should take a blood meal on control animals throughout the course of the study (based on group means) (Franc et al. 2012, Hayasaki et al. 2009, Liénard et al. 2013, Meyer et al. 2003).

i. Exposure to mosquitoes. Animals should be sedated and placed for one hour into cages (as detailed in section (l)(2)) containing 150 (± 15) adult female mosquitoes of three species concurrently (50 ± 5 of each of species) aged between three and ten days. The rearing and handling, including feeding of mosquitoes used in the control group should be the same as the treated groups. Mosquitoes should not be blood fed prior to testing but can be provided sugar water up until trials, however, 4-24 hours of sugar water starvation is recommended (Franc and Bouhsira 2009, Tiawsirisup et al. 2007).
If only testing against a single species, cages should contain approximately 100 (± 10) adult female mosquitoes (Franc et al. 2012, Hayasaki et al. 2009, Meyer et al. 2003) three to ten days of age. After one hour, animals should be removed from the cage, given an anti-sedative, and allowed to recover.

ii. Exposure to biting flies. Animals should be caged and exposed in the same manner as detailed in section (l)(2). Biting fly species should be tested individually and not combined within a cage. If testing against sand flies, 100 (± 10) individuals should be placed into each cage (Franc and Bouhsira 2009, Halbig et al. 2000, Molina et al. 2006). If testing against stable flies, 50 (± 5) individuals should be placed into each cage (Fourie et al. 2006), however testing with only 25 (± 2) stable flies per time period could be justified with the use of 12 animals per group.

For animal welfare reasons, during tests using aggressive or painful biting flies such as stable flies, animals should be sedated and given pain medication for the duration of the

infestation period. All medications given should be noted in the final study report. Biting flies should also be starved prior to testing and the length of starvation should be justified (e.g., citing peer reviewed studies).

Table 4: Schedule for exposing animals to mosquitoes and biting flies based on the claimed duration of efficacy for the test substance.

Claimed Duration of Efficacy	Post-application timing for exposing animals to insects (application occurs on day 0)
< 7 days	The time corresponding to the labeled duration for efficacy
7 days to < 4 weeks	Days 1 and 7 and again at the time corresponding to the labeled duration for efficacy
1 month	Days 3, 14, and day 28 or 30
> 1 month to ≤ 3 months	Days 3, 28 or 30, and then one exposure occurring at the longest labeled duration of efficacy
> 3 months	Days 3, 28 or 30, then once every two to three months with the last exposure occurring at the longest labeled duration of efficacy

(4) Efficacy endpoints. Endpoints (e.g., blood feeding, mortality) should be selected with respect to the intended use pattern for the product and effect on the arthropod pest for the active ingredient.

i. **Mortality.** Mortality may be considered a viable endpoint for a study if it occurs prior to the insect taking a blood meal. Mosquitoes and biting flies that take a blood meal are not considered to have been repelled and can transmit agents that may cause disease; therefore, if more than 10% of mosquitoes blood fed, mortality should not be considered a relevant endpoint.

ii. **Repellency.** The recommended endpoint for assessing repellency is to evaluate if insects took a blood meal. Insects that take a blood meal are not considered to have been repelled. More conservative endpoints such as insect landings may also be considered but should be justified. Assessing repellency may take place within the same study after mortality is determined.

(5) Data collection and recording for mosquitoes and biting flies.

i. **Mortality.** The number of live and dead insects should be recorded by species no later than 24 h post exposure and subsequently assessed for blood-feeding using the same methods as those used to evaluate repellency.

ii. **Repellency**. To determine repellency only, immediately after removing animals from the cage all insects should be aspirated from the cage, knocked down (e.g., using CO_2, or frozen), identified to species, and assessed for blood-feeding. It is preferred that blood-feeding is assessed and confirmed for each individual insect using an enzyme-linked immunosorbent assay (ELISA) (e.g., Beier et al. 1988) or a polymerase chain reaction (PCR) based method (e.g., Fitzpatrick et al. 2019); however, other methods such as squishing may also be appropriate as long as the method chosen is used consistently throughout the study and applied the same to treated and control groups.

(6) Data analysis. If the study has only one measurement (one exposure) per animal, GLMs with logit link function for binomial or beta-binomial (when binomial data exhibiting over-dispersion) distribution data or other appropriate statistical methods should be used to estimate the survival proportion of each group. If the study has multiple measurements (multiple exposures) per animal (i.e., data are not independent), GLMMs with logit link function for binomial or beta-binomial (when binomial data exhibiting over-dispersion) distribution data or other appropriate statistical methods for correlated data should be used to estimate the survival proportion of each group at each timepoint.

i. For mortality, the efficacy of the treatment and its 95% confidence interval should be calculated by the statistical model using Abbott's formula at each timepoint.

Mortality Efficacy (%) = 100 × (proportion of live pests in control group – proportion of live pests in treated group) / proportion of live pests in control group

= 100 × (1 – proportion of live pests in treated group / proportion of live pests in control group)

Proportion of live pests on a control animal = # live pests on the control animal / # pests used to infest the control animals

Proportion of live pests on a treated animal = # live pests on the treated animal / # pests used to infest the treated animals

ii. For repellency, the efficacy of the treatment and its 95% confidence interval should be calculated by the statistical model using Abbott's formula at each timepoint.

Repellency Efficacy (%) = 100 × (proportion of pests that blood fed on the control animals – proportion of pests that blood fed on the treated animals) / proportion of pests that blood fed on the control animals

= 100 × (1 – proportion of pests that blood fed on the treated animals / proportion of pests that blood fed on the control animals)

Proportion of pests that blood fed on a control animal = # pests that blood fed on the control animal / # pests used to infest the control animals

Proportion of pests that blood fed on a treated animal = # pests that blood fed on the treated animal / # pests used to infest the treated animals

(7) Data reporting. Refer to section (g) of this guideline for guidance on reporting results. Results should be reported separately for each species of mosquito or biting fly.

(8) Study conclusions. Summarize study outcomes for mortality or repellency results and discuss their implications for product labeling.

(m) Specific guidance for evaluating efficacy of pesticidal shampoo products (not including dry shampoos or dusts).

(1) Test species. See sections (j) for fleas, (k) for ticks, and (l) for mosquitoes and biting flies.

(2) Selection and allocation of test animals. See sections (j) for fleas, (k) for ticks, and (l) for mosquitoes and biting flies.

(3) Application and exposure methods.

i. Application methods. After allocation, animals should be divided into untreated control group (i.e., not shampooed) or treatment group (i.e., shampooed with product). Animals

in the treated group should be placed in a tub and shampooed on day zero with the treatment shampoo. Shampooing should consist of wetting animals, then shampooing and lathering the entire body and head per label directions. While in the tub, animals should be rinsed until all soap is removed.

ii. **Exposure methods for testing pesticidal shampoos for direct contact efficacy against *current* flea and tick infestations.** Animals should be infested on day -1 for flea claims and day -2 for tick claims using the exposure methods described in sections (j) and (l). When testing efficacy of shampoos against a current flea or tick infestation, animals infested with pests should be shampooed in a tub with a screen over the drain to keep fleas and/or ticks in the tub. Immediately following rinsing, all fleas and/or ticks found in the tub should be removed and moved to a clean container to dry in environmental conditions conducive to their survival for up to 24 hours for fleas and 48 hours for ticks. Any arthropod that is dead or does not recover in that time (i.e., probed with no movement) should be counted as dead. Pests on the animals should also be counted by 24 hours for fleas and up to 48 hours for ticks. The total number of live pests (on the animal and in the containers) after the time indicated above is what should be used for statistical analysis. Because mosquitoes and biting flies do not infest animals like fleas and ticks, products for use against mosquitoes and biting flies (e.g., pesticidal shampoos for direct contact efficacy) are not relevant against current infestations.

iii. **Exposure methods for testing pesticidal shampoos for *residual efficacy* against fleas, ticks, mosquitoes or biting flies.** Testing fleas and ticks should be conducted with the appropriate pests as indicated in sections (j) and (k) beginning with methods for the re-infestations (i.e., after the treatment). Animals should be completely dry before their infestation. For testing against mosquitoes and biting flies, testing should be conducted for the appropriate pests after shampooing, as indicated in section (l).

(4) Efficacy endpoints.

i. **Endpoints for testing pesticidal shampoos for direct contact efficacy against current flea and tick infestations.** To determine efficacy for shampoo products for existing infestations the endpoints for mortality should be used as found in sections (j) and (k).

ii. **Endpoints for testing pesticidal shampoos for residual efficacy against fleas, ticks, mosquitoes or biting flies.** To determine whether a shampoo product has residual efficacy, re-infestations (e.g., the infestation after treatment) should use, for the appropriate pests, the endpoints in sections (j), (k) or (l).

(5) Data collection and recording.

i. **Data collection and recording for testing pesticidal shampoos for direct contact efficacy against current flea and tick infestations.** Counting live pests in the treated group (both on animal and ones found in the tub that recover within time period) vs. the control group should be conducted as found in sections (j) and (k). The animals' fur should be completely dry before counts.

ii. **Data collection and reporting for testing pesticidal shampoos for residual efficacy against fleas, ticks, mosquitoes or biting flies.** The same methods for data collection and recording for testing residual efficacy as found in sections (j), (k) or (l) should be used for the appropriate pests.

(6) Data analysis. Calculate as you would in section (j), (k) or (l).

(7) Data reporting. Refer to section (g) of this guideline for guidance on reporting results. Results

should be reported separately for each species tested.

(8) Study conclusions. Summarize study outcomes and discuss their implications for product labeling.

(n) Specific guidance for evaluating efficacy under simulated environmental conditions. Shampooing/bathing, swimming, exposure to rainwater, and exposure to sunlight can impact efficacy. The methods presented below are intended for testing to support optional claims regarding the durability of products for residual efficacy (including residual shampoo products) after animal is exposed to water, bathing, or sunlight. The methods in this section may be utilized within a study assessing mortality/repellency conducted, as above in sections (j), (k), (l), (m), or may be used to conduct studies separate from those studies. The product application and infestation methods above in sections (j), (k), (l) or (m) (with some exceptions described below), along with the endpoints, data collection, and data analysis sections, can be used and applied to the following tests:

(1) Testing for product stability after bathing or water exposure. When the treated animal is exposed to water or is bathed, especially for products with a claimed duration of efficacy for 2 or more weeks, the mortality or repellent effect after the exposure should be evaluated (European Medicines Agency 2016). Infestation and counting should be conducted at a minimum after the first shampoo or water exposure and at the end of the claimed residual period (e.g., on day 28 for a monthly product). Infestation and counting can also occur after each shampoo/wetting, if desired. When infestations follow shampooing/wetting they should occur within 48 hours of shampooing/wetting. Animals should be dry before pest infestations.

i. Testing for product stability after shampooing/bathing. To determine efficacy of a product after shampooing/bathing, animals should be divided into treated and untreated control groups. Both groups should be shampooed. The entire body, including the head should be shampooed and massaged using a non-pesticidal, non-medicated shampoo and rinsed until all residual shampoo is removed (Schuele et al. 2008, Taenzler et al. 2016). For products intended to be efficacious against pests for up to 2 weeks, animals should be shampooed no later than 5 days after treatment with the product. For products with efficacy claims > 2 weeks but < 4 weeks, animals should be shampooed on days 5 & 19. For products with claims > 4 weeks, the animals should be shampooed no later than day 5, then again on day 19 and monthly thereafter.

ii. Testing for product stability after water exposure (e.g., waterproof, water-resistant, swimming)**.** To determine efficacy after water exposure, animals should be divided into treated and untreated control groups. The entire body of each animal in both groups should be exposed to water long enough to soak the fur and skin through submerging (except for the head) or showering the animal (Schuele et al. 2008, Taenzler et al. 2016). If animals are submerged, water should be poured over the back of animal's head to ensure the head is wetted (Taenzler et al. 2016). For products intended to be efficacious against pests for up to 2 weeks, animals should be exposed to water 5 and 12 days after treatment. For products with efficacy claims for >2 weeks but ≤ 4 weeks, animals should be exposed to water once a week starting at 5 days after treatment. If the product label contains claims that the product is efficacious for greater than 4 weeks, the animals should be exposed to water weekly the first month, then once every two weeks thereafter.

(2) Testing for product stability after sun exposure. Sunlight can degrade some a.i.s used in products applied to pets. To determine efficacy after animals have been exposed to daily sunshine, animals should be divided into treated and untreated control groups. For both groups, animals should be allowed regular daily outdoor time in an area exposed to direct sunlight. Typical studies include indoor/outdoor runs with covered areas allowing the animals

to have exposure to sunshine but also a place to escape the heat or inclement weather conditions, however other study designs may be submitted. Preferably, animals should be exposed to sunshine for an average of 2 hours per day. Submitting additional records of daily sunshine/rain amounts should be provided for the duration of the study. For products for which efficacy has been determined prior to testing efficacy after sun exposure, infestation and counting should only be conducted at the end of the claimed residual period (e.g., on day 28 for a monthly product).

(o) References. The following publications were consulted for supporting guideline recommendations:

Beier, J.C., Perkins, P.V., Wirtz, R.A., Koros, J., Diggs, D., Gargan II, T.P., and D.K. Koech. 1988. Bloodmeal identification by direct enzyme-linked immunosorbent assay (ELISA), tested on Anopheles (Diptera: Culicidae) in Kenya. Journal of Medical Entomology. 25: 9-16.

Bouhsira, E., Fysikopoulos, A., and M. Franc. Efficacy of fipronil-(S)-methoprene metaflumizone combined with amitraz and pyriprole commercial spot-on products in preventing *Culex pipiens pipiens* from feeding on dogs. 2009. Veterinary Record. 165: 135-137.

Dantas-Torres, F. Biology and ecology of the brown dog tick *Rhipicephalus sanguineus.* 2010. Parasites & Vectors. 3:26.

Dumont, P., Liebenberg, J., Beugnet, F., and B. Fankhouser. Repellency and acaricidal efficacy of a new combination of fipronil and permethrin against *Ixodes Ricinus* and *Rhipicephalus sanguineus* ticks on dogs. 2015. Parasites & Vectors: 8: 531.

European Medicines Agency. Guideline for the testing and evaluation of the efficacy of antiparasitic substances for the treatment and prevention of tick and flea infestation in dogs and cats. 2016. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC500210927.pdf

Fitzpatrick, D.M., Hattaway, L.M., Hsueh, A.N., Ramos-Niño, M.E., and S.M. Cheetham. 2019. PCR-based bloodmeal analysis of *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) in St. George Parish, Grenada. Journal of Medical Entomology. 56: 1170-1175.

Fourie, L.J., Stanneck, D., and I.G. Horak. The efficacy of a topically applied combination of imidacloprid and permethrin against *Stomoxys calcitrans* on dogs. 2006. International Journal of Applied Research in Veterinary Medicine. 4: 29-33.

Fourie, J. J., Horak, I.G., de Vos, C., Deuster, K., and B. Schunak. Comparative speed of kill, repellent (anti-feeding) and acarcidal efficacy of an imidacloprid/flumethrin collar (Seresto®) and a fipronil/(S)-methoprene/Eprinomectin/praziquantel spot-on (Broadline®) against *Ixodes ricinus* (Linné, 1758) on cats. 2015. Parasitology Research. 114 (suppl 1): S109-S116.

Franc, M., and E. Bouhsira. Efficacy of a combination of a fipronil-(S)-methoprene spot-on formulation and a deltamethrin-impregnated collar in controlling fleas and sandflies on dogs. 2009. Veterinary Therapeutics. 10: 71-77.

Franc, M., Genchi, C., Bouhsira, E., Warin, S., Kaltsatos, V., Baduel, L., and M. Genchi. Efficacy of dinotefuran, permethrin and pyriproxyfen combination spot-on against *Aedes aegypti* mosquitoes on dogs. 2012. Veterinary Parasitology. 189: 333-337.

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