SECTION G

Title

OX5034 *Aedes aegypti*: Proposed Field Trial Protocol for an Experimental Use Permit

Data Requirement

Not applicable

Author

Oxitec Ltd.

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Prepared By

Oxitec Ltd.
71 Innovation Drive Milton Park
Abingdon, Oxfordshire OX14 4RQ
United Kingdom

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CLAIM OF CONFIDENTIALITY

Information claimed as confidential has been removed to a confidential attachment.

Submitter:_____ Date:_April 30, 2020_____

Nathan Rose, DPhil

Head of Regulatory Affairs

Oxitec Ltd.

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Good Laboratory Practice Compliance Statement

Good Laboratory Practice Standards, 40 CFR Part 160, are not applicable to this protocol.

Sponsor/Submitter:

____ _____ Date _April 30, 2020_____

Nathan Rose, DPhil

Head of Regulatory Affairs

Oxitec Ltd.

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1. Introduction

Oxitec has developed genetically engineered (GE) male *Aedes aegypti* of the OX5034 strain for use in mosquito control. *Aedes aegypti* is a known vector for human diseases associated with Zika, dengue, and chikungunya viruses. Oxitec's novel approach to mosquito control uses the release of male OX5034 mosquitoes carrying a "female-specific self-limiting gene" to mate with wild females. When male OX5034 *Aedes aegypti* homozygous for the self-limiting gene (carrying two copies of the gene) are released into the environment and mate with wild *Aedes aegypti* females, their offspring inherit a single copy of the self-limiting gene (so are hemizygous). The self-limiting gene kills only female offspring (carrying one copy of the self-limiting gene), which die at early larval stages of development, while hemizygous males will survive to pass the OX5034 genes on to subsequent generations. Laboratory tests show that 100% of the resulting female offspring will die before reaching adulthood. Hence the OX5034 mosquito can be considered a sex- and species-specific larvicide targeting only female *Aedes aegypti.*

Expression of tTAV-OX5034 is regulated by tetracycline or one of its analogues. Tetracyclines bind to tTAV protein, preventing it from activating transcription. Thus, when either tetracycline or one of its analogues is absent from the OX5034 mosquito larval diet, tTAV-OX5034 protein causes lethality in females carrying at least one copy of the construct, including the progeny of mating between OX5034 homozygous males and wild *Ae. aegypti* females.

In addition to the gene that confers the self-limiting trait, OX5034 also express DsRed2-OX5034, a red fluorescent marker protein, which aids identification of OX5034 under laboratory conditions at the larval, pupal, and adult stages.

2. Proposed Claims to be Supported by Field Trials

Information on page 4 of this volume is confidential business information and, therefore, has been removed to a confidential attachment.

Cross-reference number 1

This protocol is designed with the primary goals of demonstrating efficacy and aspects of biosafety relevant to the use of OX5034 as a vector control tool for killing female larval progeny of wild female *Ae. aegypti*. Mortality rates will be evaluated by comparing rates of survival to adulthood between treated female larval progeny (those fathered by OX5034 males) and untreated female larval progeny (those fathered by wild males). A single breeding site can thereby contain a mixture of treated (parented by OX5034) and untreated (parented by wild) individuals. All treated individuals express at least one copy of the self-limiting gene, which is sufficient to confer lethality. Progeny will be sampled and collected using egg traps (ovitraps). Geographically distinct untreated control areas (at least 500 m distant from treatment areas) will also be utilized to estimate percentage efficacy, as described below.

Mating fraction data will also be collected under the proposed field trial protocol, as requested by EPA, but as described here and elsewhere, efficacy is most appropriately defined by the effect (percentage mortality) of the pesticide on treated individuals, compared to untreated individuals.

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The Experimental Use Permit is being requested for the testing of OX5034 in two field trial locations (Monroe County, Florida and Harris County, Texas). These have been selected to conduct potential field trials in Climate Zones 1 and 2. A phased field trial approach, encompassing single-point application (Trial A) and multiple-point application (Trial B) is anticipated; however, if enough confidence in the application strategy (i.e. application rates and coverage) has been generated prior to the EUP trials being initiated (for example from pilot studies in Brazil), Trials A and B may be conducted simultaneously. Further discussion regarding trial locations can be found in Section 4.1.

Information on page 5 of this volume is confidential business information and, therefore, has been removed to a confidential attachment.

Cross-reference number 2

2.1.1. Product Coverage/Dispersal

Product coverage is most appropriately defined by the distance the product reaches from the point of application. This field protocol will measure mean dispersal distance in the field, and proposes using this metric to describe the pesticide coverage. This metric is not dependent on females or mating behaviour, and thus more accurately reflects the area where males have a significant presence. Product coverage is anticipated to be in the region of 1-2 acres (equivalent to ~50m mean male dispersal), but this will be measured during the EUP field trial.

The dispersal of the transgene (i.e. dispersal of fluorescent progeny, whether male or female) and the maximum dispersal of males are both considered unsuitable (Figure 4). Maximum dispersal of males may be due to extrinsic factors, e.g. wind, human contact, etc. Maximum dispersal of the transgene, i.e. maximum dispersal of mated females, may also be much further, due to blood-feeding and oviposition behaviour of mated females.

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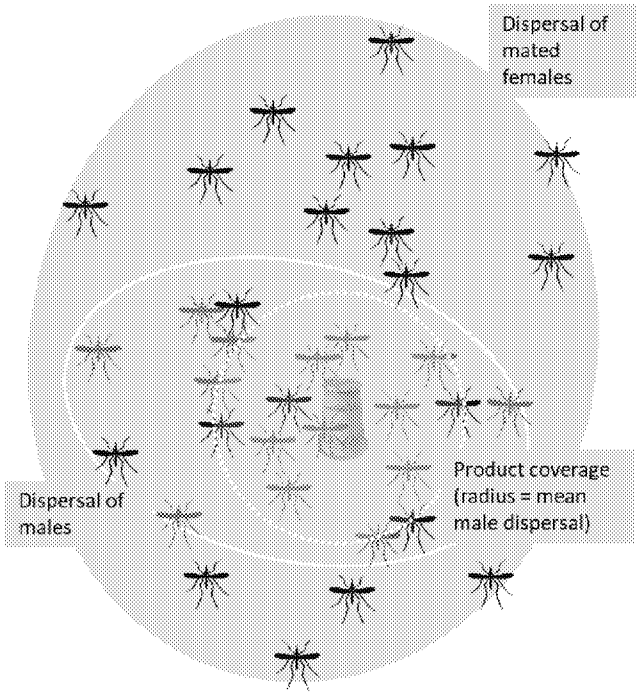

Figure 4. Male dispersal from a Mosquito Rearing Box (green cylinder) is shown by green mosquito icons. The large green oval indicates maximum male dispersal, while the pale green circle indicates mean distance travelled by male OX5034 mosquitoes. Mated females (black mosquito icons) may disperse much further than the dispersal of OX5034 males, due to blood-feeding/oviposition behaviour of mated females.

3. Names, qualifications and contact details of individuals who will supervise experimental work

Information on page 7 of this volume is confidential business information and, therefore, has been removed to a confidential attachment.

Cross-reference number 3

4. States in which the pesticide will be used and the acreage to be treated in each State

A minimum of one trial will be performed. The precise location(s) of the trial site(s) and related plots are yet to be determined and will be reported to the EPA before the protocol is initiated. Trial sites will be selected from a total of 30 locations in two states as shown in Table 2 and Table 3. Detailed explanations of Trial A and Trial B are given below. The dose rates, expressed per state (FL or TX) per year, for male OX5034 mosquitoes and for active ingredient tTAV-OX5034 protein, are given in Table 4, and the annual acreages per state are given in Table 5.

Table 1. Proposed field trial locations, maximum numbers of sites, and maximum acreages for OX5034 *Aedes aegypti* trials including both treated and untreated sites. For the purposes of clarification, it should be noted that field trial acreages do not vary between life-stages deployed. Whether eggs or adults are deployed, field trials use the same overall areas.

State	County	Number of proposed sites	Maximum acreage per trial site	Maximum total acreage including untreated sites over both years of the trial
Florida	Monroe County	12 (Trial A) comprising 9 treated and 3 untreated comparators	200	4800 (3600 treated)
Florida	Monroe County	9 (Trial B) comprising 6 treated and 3 untreated comparators	80	1440 (960 treated)
Texas	Harris County	9 (Trial B) comprising 6 treated and 3 untreated comparators	40	360 (240 treated)

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Table 2. No of sites, application rates (doses 1-3 i.e. lowest - highest), and replicates for Trials A and B, including the treated acreages and life-stages assessed. Please note that both locations, or only one location (FL or TX) may be used for Trial B.

Trial	Location	Number of untreated areas (Required)	Number of treated areas (dose 1 - low) (Required)	Number of treated areas (dose 2 - medium) (Optional)	Number of treated areas (dose 3 - high) (Optional)	Maximum acreage per trial site	Maximum total acreage over both years of trial	Life stage assessed
Trial A	Florida - Monroe County	3	3	3	3	200	4800	Eggs or adults (one life-stage only)
Trial B*	Florida - Monroe County	3	3	3	N/A	80	1440	Eggs only
	Texas - Harris County	3	3	3	N/A	40	360	Eggs only

**One (either FL or TX) or both locations may be used.*

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Table 3. Dose rates of male OX5034 mosquitoes and of active ingredient (tTAV-OX5034 protein) per state per year, for both Trials A and B, on the basis of 52 weeks of releases per year. Note that the trial is planned for two years' duration in Florida and one year in Texas.

Male OX5034 mosquitoes / year in Florida		
Trial A	9,360,000	Male OX5034 mosquitoes
Trial B	499,200,000	Male OX5034 mosquitoes
Total (Trial A and Trial B)	508,560,000	Male OX5034 mosquitoes
Active ingredient tTAV-OX5034 / year in Florida		
Trial A	38.38	mg tTAV-OX5034 protein
Trial B	2,046.72	mg tTAV-OX5034 protein
Total (Trial A and Trial B)	2,085.10	mg tTAV-OX5034 protein
Male OX5034 mosquitoes / year in Texas		
Trial B	249,600,000	Male OX5034 mosquitoes
Total (Trial B)	249,600,000	Male OX5034 mosquitoes
Active ingredient tTAV-OX5034 / year in Texas		
Trial B	1,023.36	mg tTAV-OX5034 protein
Total (Trial B)	1,023.36	mg tTAV-OX5034 protein

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Table 4. Maximum annual acreage per state for the duration of the EUP, including treated and control sites.

State	Acres (Trial A + Trial B)
YEAR 1	
Florida	3,120
Texas	0
TOTAL (YEAR 1)	3,120
YEAR 2	
Florida	3,120
Texas	360
TOTAL (YEAR 2)	3,480
OVERALL PERMIT TOTAL	6,600

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4.1. Number of Trial Locations

In the 75-day deficiency letter dated 28 Jan 2020, BPPD indicated that more than one trial location would likely be required to support a Section 3 registration of OX5034 *Aedes aegypti*. Oxitec believes that a single trial in a suitable climate zone should be sufficient to provide efficacy data to support the proposed label claims and registration throughout the United States under FIFRA Section 3, provided that sufficient experimental replicates were included in that geographic location. The rationale for this assertion is provided below.

Monroe County, Florida is in Climate Zone 1

Monroe County, FL, is one of only three counties in the continental United States in Climate Zone 1 (Figure 5).

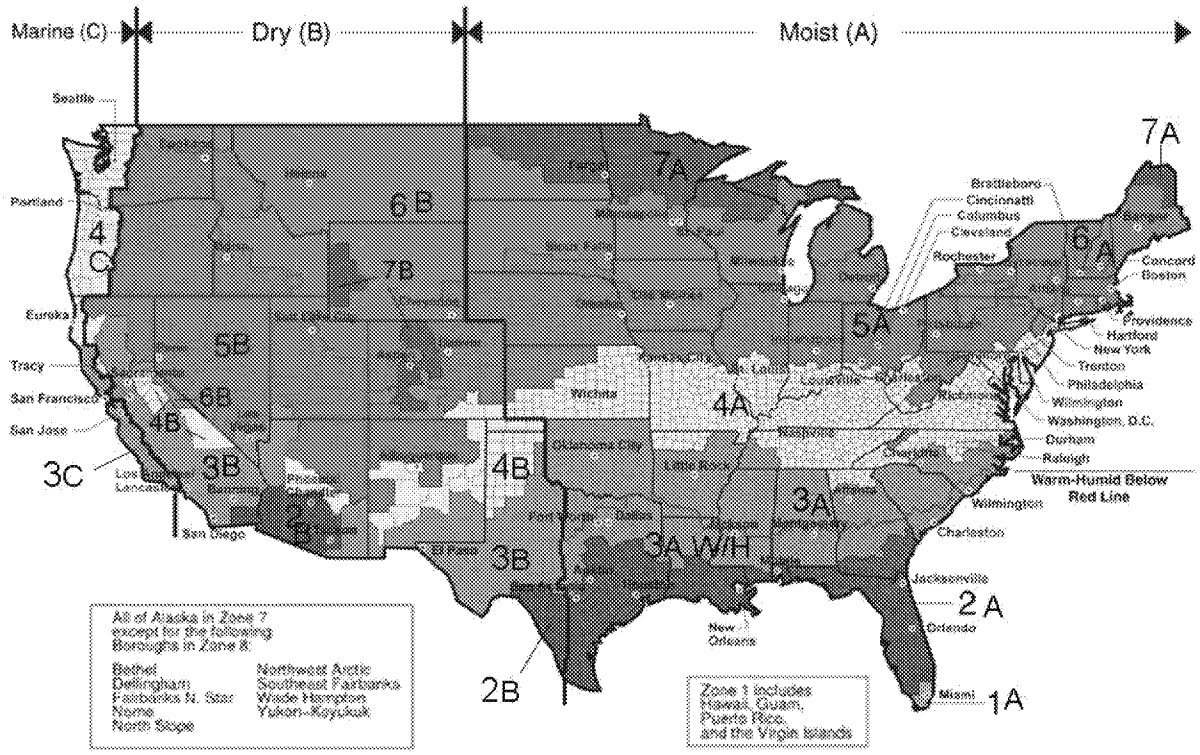

Figure 5. 2012 International Energy Conservation Code® (IECC) climate zone map for the United States.

Information on page 12 of this volume is confidential business information and, therefore, has been removed to a confidential attachment.

Cross-reference number 4

4.2. Test facility

The facility site(s) will be confirmed to the EPA before protocol is initiated.

5. Details of the Proposed Program including pests, crops, sites of application, and major geographic areas where material is to be used. Specify use pattern, plot sizes, number of plots, number of replicates, dosage rates, methods of application, season for use.

5.1.1. Use Pattern

Terrestrial, non-food.

5.1.2. Pest

Aedes aegypti mosquito.

5.1.3. Study Sites and Anticipated Timelines

The EUP trial design is divided into two study designs (Trial A and Trial B). These may take place simultaneously if enough confidence in our application strategy (i.e. application rates and coverage) has been generated prior to the EUP trials starting (for example from pilot studies in Brazil). It might also be sufficient to run only Trial A, with suitable replication, to produce sufficient data for pesticide registration.

At each study site, either of the two study designs may be deployed. The details of which study design will be undertaken at which site will be reported to the EPA before initiation. The objectives of the Field Trial A will be to quantify various parameters from a single release point (see section 5.2). The objectives of the Field Trial B will be to quantify various parameters across multiple release points (see section 5.8). See Table 2 for details of trial application sites and plot sizes. It should be noted that Table 2 includes the untreated sites that will be used for comparative purposes.

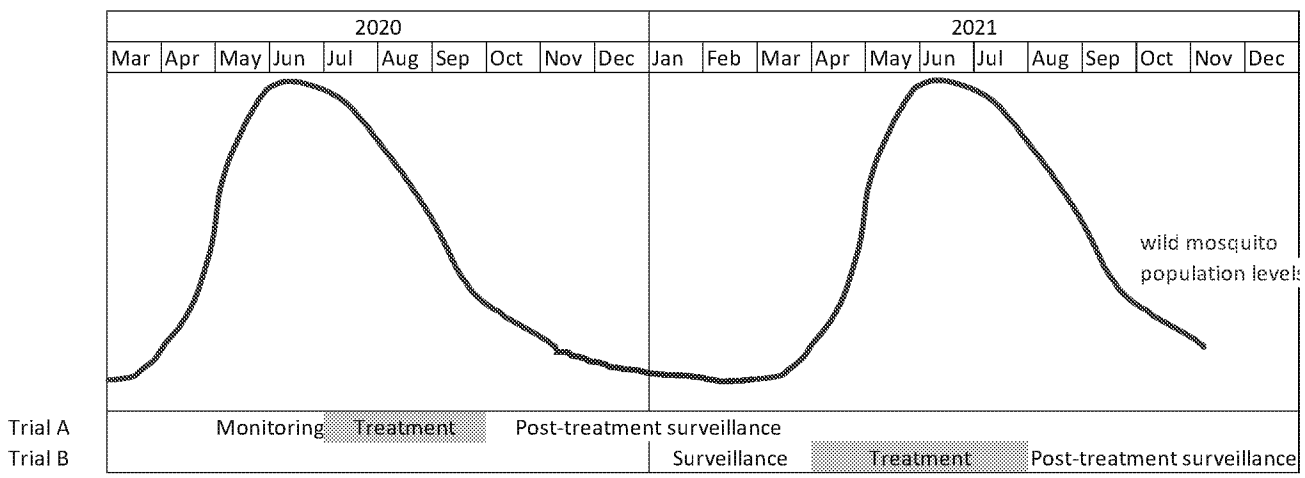

Figure 6. Anticipated Trial A and B timelines including associated mosquito surveillance periods. The red line indicates the anticipated *Aedes aegypti* seasonality. Please note that a total of 24 months for the completion of the studies has been requested as start dates, treatment periods, and post-release surveillance periods are subject to change.

5.2. Trial A

The objectives of the Field Trial A will be to quantify various parameters from a single release point.

Site selection will be based on specific criteria.

5.2.1. Trial A Site selection criteria

All sites will comply with the following criteria:

- Total study area: minimum of 25 and maximum of 200 acres. These areas are based on the expected distribution of male *Aedes aegypti* from a single release point. For example, if the maximum distance travelled is ~180 m, that equates to a circle with an area of 25 acres based around a single mosquito release point. If the maximum distance travelled is ~500 m, that equates to a circle with an area of 200 acres based around a single mosquito release point. Most studies have found the maximal distance travelled by wild *Aedes* to be between 82 and 400m, this being heavily influenced by climatic conditions, presence of vegetation and the availability of breeding sites (Reiter et al., 1995; Muir & Kay, 1998; Russell et al., 2005). Previous field releases of OX5034 male mosquitoes in Brazil have recorded maximum dispersal distances of 198 m. Hence it is likely that OX5034 male mosquitoes will not travel further than the 400 m perimeter described in Section G.
- As requested by EPA, trial areas will be separated by at least 500m and if mosquitoes are trapped at the perimeter of a Trial A area, then additional ovitraps will be installed at distances greater than 400 m from the release site to capture any mosquitoes that may travel further. These additional traps may be constrained in terms of direction by available land mass, although every effort will be made to accommodate the distances required. Water bodies such as canals or ponds up to approximately 100m across will be considered not to impact dispersal.
- Confirmed presence of *Aedes aegypti* (based on surveillance data).
- Available documentation of mosquito abatement (other than experimental treatment) during the period of study. Treated and untreated areas of the EUP will be chosen to be as similar as possible, including in terms of any known mosquito abatement activity. Details of any mosquito abatement activity in trial areas will be disclosed to EPA with data in the final report, at the time of a Section 3 application.
- The outer boundary of the trial area (denoted by the traps furthest from the central release point) will be greater than 500 m from commercial citrus growing areas and from sewage treatment plants.

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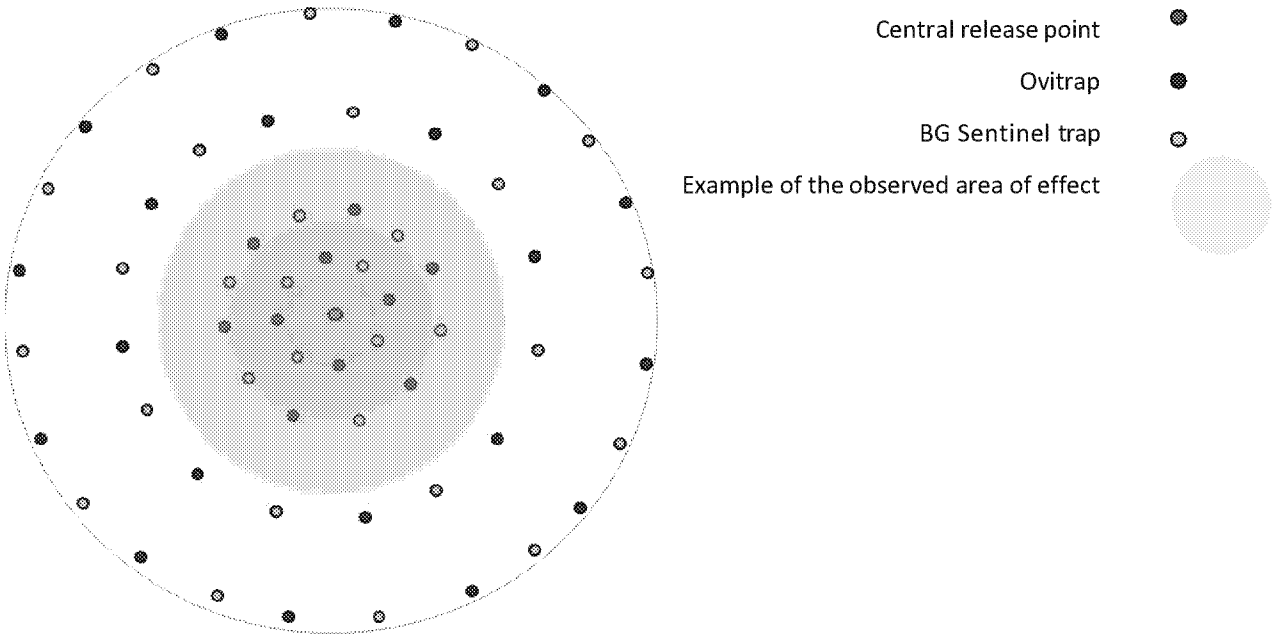

Figure 7. Schematic overview of a trial site for Trial A showing the central release point and a potential arrangement of 30 x egg traps (ovitraps) and 30 x adult traps (BG Sentinel® traps). Traps will typically be located between 25 and 400 metres from the central release point, although the maximum distance may be less dependent on the available landmass. Please note that traps may not be positioned precisely in concentric rings as shown.

Information on page 15 of this volume is confidential business information and, therefore, has been removed to a confidential attachment.

Cross-reference number 5

Table 5. No of sites, application rates (doses 1-3 i.e. lowest - highest), and replicates for Trial A, including the treated acreages and life-stages assessed.

Trial	Location*	Number of untreated areas (Required)	Number of treated areas (dose 1 - low) (Required)	Number of treated areas (dose 2 - medium) (Optional)	Number of treated areas (dose 3 - high) (Optional)	Maximum acreage per trial site	Maximum total treated acreage	Life stage assessed
Trial A	Florida - Monroe County	3	3	3	3	200	1800	Eggs or adults (one life-stage only)

**See Section 4.1 for discussion of trial locations.*

5.2.2. OX5034 transport

Known quantities of OX5034 eggs or adults will be delivered to release site(s) in triple layered containment. Ambient temperature within the vehicle should not exceed 86°F (30°C) during storage or transport. If the temperature is higher than 86°F (30°C), cooling aids (e.g. air-conditioning or ice packs) may be used, but minimum temperatures should not be lower than 15°C for eggs or 22°C for adults.

5.2.3. Application of OX5034 treatment

Two different release modes for OX5034 are envisaged for this EUP, viz. egg and adult release modes. The ultimate aim is to test the use of Mosquito Rearing Boxes, which enable the deployment of OX5034 mosquito eggs in specially designed Mosquito Rearing Boxes which facilitate egg-to-adult development in the field. We also may benefit from rearing OX5034 male adults for release (produced in a rearing facility without the use of tetracycline) to test dispersal and/or efficacy of OX5034 male adults in US field conditions. Full rearing and quality control protocols for each of these modes are provided in (Oxitec Ltd & MRID 50889424, 2019). Further details of each of these release modes are provided below. In all cases, the locations of each Mosquito Rearing Box/event will be given a unique identifier and georeferenced for accurate placement and mapping.

5.2.4. OX5034 Mosquito Rearing Boxes

At the prescribed release locations, in the case of egg releases prescribed amounts of water, mosquito food, and other additives will be added to Mosquito Rearing Boxes, as described in (Oxitec Ltd & MRID 50889401, 2019). A known quantity of OX5034 eggs will also be added to each Mosquito Rearing Box. Adult males of the

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desired quantity (anticipated 500-2,500 male adults per Mosquito Rearing Box[1]) will emerge within 22 days of the Mosquito Rearing Box setup and deployment.

5.2.4.1. Construction

- Component parts will be safe to handle, nontoxic to rearing process, robust and capable of withstanding local environmental conditions for at least 22 days.
- Protected from applications of insecticide, in particular of *Bti* (*Bacillus thuringiensis israelensis*), as far as is possible.
- Option to fix to typical urban landscape but can be used as a free-standing Mosquito Rearing Box.
- Easy and environmentally friendly disposal – safe and simple mess-free draining of water, with no parts left behind.
- The complete Mosquito Rearing Box will be weatherproof and also ensure environmental control of water temperature, condensation and light conditions to enable efficient mosquito development.
- For the purposes of the trial, Mosquito Rearing Boxes will be physically isolated from the public to prevent vandalism/tampering, or where that is not possible, located discretely and out of public view.
- Mosquito Rearing Box design will preclude the Mosquito Rearing Box becoming a breeding site for wild mosquitoes.

5.2.4.2. Mosquito Rearing Box setup and activation

Information on page 17 of this volume falls under FIFRA 10(d)(1)(A), and, therefore, has been removed to a confidential attachment.

Cross-reference number 6

5.2.5. OX5034 Adult Release

In the case of adult releases, known quantities of adults (already contained in pots or cages) will be allowed to acclimatize and rest for >10 minutes prior to release.

5.2.6. Field Monitoring Methods

5.2.6.1. Fluorescent Marker and PCR assessments

The fluorescent marker is readily visible in all life-stages apart from eggs and will be used for OX5034 identification (GL-SOP-00052) (Oxitec Ltd & MRID 50889401, 2019). Molecular analyses by PCR will be used to validate marker identifications in a minimum number of 40 fluorescent and 40 non-fluorescent screened individuals (QD-R-00109 or QD-R-00108). It is expected that this will be required only once, to ensure accurate

[1] Mosquito Rearing Boxes designed to produce other numbers of male mosquitoes, e.g. 500 males per box, 1,000 males per box, etc. may also be used. In all cases, the maximum number of males/acre/week indicated in the trial designs would not be exceeded.

identification by trial staff. In addition, all individuals will be taxonomically identified to genus and/or species level. Fluorescence screening will also be used to assess penetrance of the female-specific self-limiting gene (see Section 5.2.6.2 for ovitrap monitoring details and Section 5.3.3 for larval rearing methods as part of penetrance testing).

Please note that field-collected samples of *Aedes aegypti* will be taken and stored for subsequent analyses of genetic diversity and introgression of background genes.

5.2.6.2. Eggs

Ovitraps are a commonly used system for collecting *Ae. aegypti* mosquito eggs, mimicking natural breeding sites in which females lay eggs. Ovitraps consist of a pot containing water and a substrate (paper or wood) protruding above the water line on which eggs can be laid. The substrate used will be consistent across replicates and plots. Ovitraps will be positioned in sheltered locations, typically nearby residential, commercial, or utility premises. Appropriate consent for the placing and servicing of the traps will be obtained. Each trap will have a unique identifier and georeferenced for accurate placement and mapping.

A minimum of 30 ovitraps per plot for Trial A will be distributed across the trial area. Trapping intervals will typically be 7 days (maximum 9 days), at which time the water and oviposition substrate will be replaced, or the trap may be substituted for a new one. Oviposition substrates will be labelled and stored individually to prevent cross contamination during transport. Once at the laboratory they are dried at room temperature for a minimum of 2 days (maximum 14 days) to mature the eggs prior to hatching.

5.2.6.3. Ovitrap Density

The trapping density we recommend when used as surveillance tool is a minimum of 28 per site. This minimum number of traps per site (28) was calculated as the sample size required for multiple regression to detect a medium-sized effect (Cohen's $f^2 = 0.25$) with 80% statistical power when there are 4 predictor variables. This number was calculated using the computer program G*Power. A similar ovitrap density or higher has been used successfully previously (Harris et al., 2012; Gorman et al., 2015; Carvalho et al., 2015) and is not expected to confound or interfere with any measurements of efficacy. For Trial A, we propose using a minimum of 30 ovitraps per area through pre-release and treatment periods, increasing the number to a minimum of 48 per area during post-release monitoring periods, during which we will also increase the monitoring area by extending the distance from the centre to the perimeter by 100m in all directions.

5.2.6.4. Ovitrap Interval

The trapping interval we recommend for ovitraps when used as surveillance tool is weekly. This has been chosen as it permits constant trapping throughout the trial period yet strikes a balance between the maximum number of data points we could collect and a trapping interval that does not become a frustration to homeowners and offers operationally feasible surveillance. Oxitec has successfully used a weekly trapping interval for surveillance during *Aedes aegypti* product trials and published the results in peer-reviewed studies previously (Harris et al., 2012; Gorman et al., 2015; Carvalho et al., 2015).

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5.2.6.5. Adult female traps

BG-Sentinel® traps (Biogents, Germany) target both male and female adults of several Aedine species. They employ a combination of visual and olfactory (odours and/or CO_2) attractants to lure individuals towards a motorised fan and into a catch-bag. Power can be supplied by mains, battery or solar generated electricity. BG-Sentinel® traps will be positioned in sheltered locations, typically nearby residential, commercial, or utility premises. Appropriate consent for the placing and servicing of the traps will be obtained. Each trap will have a unique identifier and be georeferenced for accurate placement and mapping.

A minimum of 30 BG traps per plot for Trial A will be distributed across the study area. Locations of BG-Sentinel® trap units will be rotated to prevent bias between individual traps. The catch-bag in BG-Sentinel® traps can be changed daily, every few days or weekly. Trapping intervals will be the same across plots and will be a maximum of 9 days. Catch-bags will be labelled and stored individually to prevent cross contamination during transport. Once at the laboratory samples will be processed within 96 hours.

5.2.6.6. Adult Female Trapping Density

The trapping density we recommend when used as a surveillance tool evaluating changes in abundance is a minimum of 28 per area. However, for Trial A we propose using a minimum of 30 BG-Sentinel® traps per area. This will apply throughout the treatment period. During post-release monitoring periods only ovitraps will be used to detect disappearance of the transgene from the environment. This minimum number of BG traps per area (28) was calculated as the sample size required for multiple regression to detect a medium-sized effect (Cohen's $f^2 = 0.25$) with 80% statistical power when there are 4 predictor variables. This number was calculated using the computer program G*Power. Were too many of our released adults caught in BG-Sentinel® traps, this could reduce or interfere with the performance results obtained. Mark release recapture results to date in Brazil have shown that BG Sentinel® traps in direct line of site and in close proximity to the release point, when combined with point release of adults can catch a high proportion of released individuals. Therefore, care will be taken to ensure BG-Sentinel® traps are located appropriately and not in a direct line of site to release locations.

5.2.6.7. Adult Female Trapping Interval

The trapping interval we propose for BG-Sentinel® traps during Trial A is weekly, every few days, or daily. Weekly is sufficient for measurements of dispersal and daily allows the most accurate estimates of longevity.

5.2.6.8. Untreated comparator areas

For Trial A, untreated areas will be utilized to provide samples of larvae for mortality assessments that have not been exposed to any form of treatment. These areas will be of similar size and characteristics to treated areas; where possible, comparator and treatment areas will be randomly allocated. The number of untreated areas will be the same as the number of areas for each treatment rate. For Trial A this will be at least three. Untreated areas will be at least 500 m away from treatment areas.

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5.3. Objective of the Program

5.3.1. Trial A

The objectives of the Field Trial A will be to quantify from a single release point:

- Efficacy of the active ingredient (% mortality observed in fluorescent female progeny compared with untreated, i.e. non-fluorescent females).
- The adult over-flooding ratio achieved i.e. Oxitec males:wild male ratio (using BG traps).
- The proportion of treated i.e. fluorescent individuals within each ovitraps (mating fraction).
- Dispersal distance of released adult male OX5034 mosquitoes (maximum and mean flight distances from the central release point observed by BG trap catches).
- Dissemination distance of the transgene (maximum distance that fluorescent individuals are found from the central release point observed by ovitrap catches).
- Duration and scale of residual activity (time until disappearance of adult males and fluorescent larvae, and the rate of disappearance in the environment measured until no individuals have been found for a minimum of 10 consecutive weeks i.e. a period sufficient for at least two discrete generations.

5.3.2. Application

It is anticipated that for eggs or adults the interval between applications would be <22 days. The longest interval anticipated between releases/deployments of Mosquito Rearing Boxes will be evaluated. Target application rates will be fixed for the duration of the releases. In some cases the effects of a single box deployed may be assessed and in some cases a series of consecutive releases will be assessed. For efficacy metrics, consecutive weekly releases will be used.

In either case, the maximum weekly release rates will be 20,000 males per acre for Trial A (maximum of 20,000 males total per area per week as Trial A is a single release point). We anticipate that Trial A would be completed at a minimum of one application rate (with 3 replicates) not including untreated comparator sites.

5.3.3. Mortality assessments

Eggs from each ovitrap will be induced to hatch (to synchronise hatching) then screened for fluorescence and counted within 24 hours. Larvae will be reared under laboratory conditions at 27°C [+/- 2°C], 70% [+/- 10%] relative humidity, 12h: 12h light: dark cycle and fed *ad libitum*. Once pupated, remaining individuals will be placed into cages for adult emergence. Post-emergence, all adults will be taxonomically identified to species level, screened for fluorescence, and sexed. Note, as outlined in section 5.2.6.1, a minimum number of 40 fluorescent and 40 non-fluorescent *Ae. aegypti* will undergo molecular identification by quantitative PCR to complete a one-time validation of the fluorescence screening by confirming their genotype as either OX5034 or wild *Aedes aegypti* (QD-R-00109 or QD-R-00108). This may require a repeat procedure should this molecular assay fail for any reason.

5.3.4. Persistence Measurements

OX5034 mosquitoes possess a self-limiting gene and a fluorescent marker gene. The self-limiting gene, when passed onto offspring, prevents female progeny from surviving to functional adulthood in the absence of

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tetracycline. By design, male progeny survive and can develop through to adulthood and potentially mate with wild females. OX5034 genes are therefore passed down as a single copy from male parents only, and as they are subject to normal Mendelian inheritance patterns, are not expected to establish at the proposed trial site but decline predictably following the cessation of releases over the course of <10 generations (Oxitec Ltd & MRID 50889416, 2019).

Ovitrap data will be used to quantify the presence (anticipated decline) of the fluorescence gene over time. Monitoring will continue until at least 10 consecutive weeks without the presence in ovitraps of OX5034 fluorescent mosquitoes. Given that the generation time for *Aedes aegypti* is usually 4 weeks or less, this period should be sufficient to allow two discrete generation times without fluorescence. Note, as outlined in section 5.2.6.1, a minimum number of 40 fluorescent and 40 non-fluorescent *Ae. aegypti* will undergo molecular identification by quantitative PCR to complete a one-time validation of the fluorescence screening by confirming their genotype as either OX5034 or wild *Aedes aegypti* (QD-R-00109 or QD-R-00108). This may require a repeat procedure should this molecular assay fail for any reason.

5.4. Male Dispersal Measurements

The dispersal distance of adult male OX5034 will be assessed within Trial A.

- Each OX5034 Mosquito Rearing Box (containing eggs) or release pot (adults) will have contained a known quantity of individuals prior to release and samples will be inspected post-release to estimate the actual number of released males.
- OX5034 adult males are inherently marked by the fluorescent protein, but to distinguish between released homozygous males and hemizygous males of subsequent generations, if required release cohorts will be marked using fluorescent powders. In the case of adults, this will be done by agitating the insects in a closed container that has been coated on the inside with fluorescent powder prior to release. In the case of eggs, the inside surfaces of the release device will be coated in the fluorescent powder, enabling the adults to contact the powder prior to emergence from the box. The males (whether released as eggs or adults) will be released from a single point source, either as a single release (adults) or in a sustained manner over a period of several weeks (eggs).
- To monitor dispersal a network of BG Sentinel® traps (minimum of 30) will be positioned to a distance of up to 400m depending on available land area. Catch bags will be collected and replaced between daily (maximum frequency) or weekly (minimum frequency). Trapped mosquitoes will be screened for fluorescence and identified as marked males (OX5034), unmarked males (WT) and unmarked females (WT). The trapping period extends from the time of the first release until three consecutive days without recaptures of powder-marked males. The mean and maximum dispersal distances of OX5034 adult males will be calculated. If dispersal data are not normally distributed, median and maximum values, and interquartile range will be reported.

5.5. Data Analysis Methods

Efficacy data analyses are subject to ongoing discussions with EPA. Statistical methods, as outlined below, will be used to analyse data, and additional data analyses may also be carried out in support of product registration under FIFRA Section 3.

5.5.1. Efficacy

The evaluations of each replicate will yield survival data, i.e., number of females surviving (reaching adulthood). Therefore, the recommended[2] calculation to account for survival rates in untreated replicates is an adaption of Mulla's formula.[3] The output of this formula is the control adjusted percentage mortality (efficacy):

E=100*((C-T)/C) or E=100*(1-T/C)

where:

E = percentage efficacy in individual ovitraps from the treated area.

C = percentage of untreated females surviving across all ovitraps in control areas.

T = percentage of treated, i.e. fluorescent females surviving in individual ovitraps.

5.5.2. Trial A replication

We anticipate that in each climate zone utilised, Trial A would evaluate a minimum of one application rate and untreated controls with each being replicated at least three times (minimum of six sites in total).

5.5.3. Test Acceptance Criteria

Each of the efficacy studies (i.e. data from any single plot) will be considered valid providing:

- The total number of larvae (sum of fluorescent and non-fluorescent) collected from all ovitraps over the course of the study is equal to or greater than 100.

The adult male OX5034 dispersal experiment will be determined as valid providing:

- The total number of marked (i.e. released) OX5034 fluorescent male adults that are recovered over the course of the study, is greater than 20.

5.6. Statistical Analysis

5.6.1. Efficacy

We will assess differences in the numbers of females surviving between release and non-release treatments, as well as how survival is affected by the following variables:

- Release rate (the number of male adults released/exiting a single mosquito rearing box)
- Trap distance (how far the trap is from the point of release)

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[2] *Guidelines for laboratory and field testing of mosquito larvicides. Editors: Dr M. Zaim/WHOPES, 39 p., Publication date: June 2005. WHO reference number: WHO/CDS/WHOPES/GCDPP/2005.13*

[3] *Mulla MS, Darwazeh HA. Activity and longevity of insect growth regulators against mosquitoes. Journal of Economic Entomology, 1975, 68:791–794.*

- Site (which site replicate is being measured)

We will assess the difference in female survival between release and non-release sites using general linear mixed-effects models (GLMM) with the random effect of site, a crossed random effect of trap distance, and the covariates life stage and release rate. The model will use a quasibinomial distribution and a logit link function since the response variable is recorded as a percentage. Percent efficacy and 95% confidence intervals will be calculated from the results of the GLMM. The efficacy (E) of OX5034 to kill female mosquitoes is calculated using an adaption of Mulla's formula:

$$E=100*((C-T)/C) \textit{ or } E=100*(1-T/C)$$

E = percentage efficacy in individual ovitraps from the treated area.

C = percentage of untreated females surviving across all ovitraps in control areas.

T = percentage of treated, i.e. fluorescent females surviving in individual ovitraps.

5.6.2. Persistence Monitoring

The Kaplan-Meier estimator will be used to characterise the persistence of the OX5034 gene in release sites. Estimated median values, 95% confidence intervals, interquartile ranges and maxima will be reported.

5.7. The amount of active ingredient required for the projected acres in Trial A is based on the proposed application rates and formulation:

A minimum of one trial will be performed. The precise location(s) of the trial site(s) and related plots are yet to be determined and will be reported to the EPA before the protocol is initiated. Trial A treatment and control sites will be selected from a total of 12 locations in Florida as shown in Table 6.

1. Maximum number of **treated** trial sites: 9
2. Maximum number of **treated** acres: 1,800/year
3. Maximum quantity applied per acre: 20,000 males per acre per week, or 0.082 milligrams of active ingredient (tTAV-OX5034) per acre per week.
4. Maximum quantity applied per treated site (single release point only): 20,000 males per week, or 0.082 milligrams of active ingredient (tTAV-OX5034) per week.
5. Maximum quantity applied for program (9 sites maximum): 180,000 males or 0.738 milligrams of active ingredient (tTAV-OX5034) per week.

The maximum quantity applied per treated site in Trial A is fixed at 20,000 males per site per week, from a single release location.

The maximum quantity applied across all sites during Trial A deployments is calculated by multiplying the maximum quantity applied per site (20,000 males per site per week) by the total number of treated sites (9 sites across Monroe County, FL).

20,000 males x 9 sites = **180,000 males per week for all Trial A sites taken together.** See Table 4 for dose rates per year per state.

5.8. Trial B

5.8.1. Trial B Site selection criteria

The objectives of the Field Trial B will be to quantify various parameters across multiple release points. All sites (both treated and untreated controls) will comply with the following criteria:

- Total trial area: minimum of 10 and maximum of 80 acres (FL) or 40 acres (TX). The Trial B defined areas are smaller than for Trial A, because the release point spacing will be based on the dispersal/coverage determined in Trial A or in previous trials in other locations, e.g. Brazil.
- Confirmed presence of *Aedes aegypti* (based on surveillance data).
- Available documentation of mosquito abatement (other than experimental treatment) during the period of study. Treated and untreated areas of the EUP will be chosen to be as similar as possible, including in terms of any known mosquito abatement activity. Details of any mosquito abatement activity in trial areas will be disclosed to EPA with data in the final report, at the time of a Section 3 application.
- The distance between trial locations (i.e., control and treated locations) will be at least 500 m. Dispersal distances measured from egg releases from the Mosquito Rearing Boxes will be used to support dispersal distances for Trial B.
- The outer boundary of the trial area (denoted by the traps furthest from the central release point) will be greater than 500 m from commercial citrus growing areas or sewage treatment plants.

Final site selections will aim to provide sites that are similar in terms of mosquito pest pressure (abundance), based on mean number of larvae per trap per week.

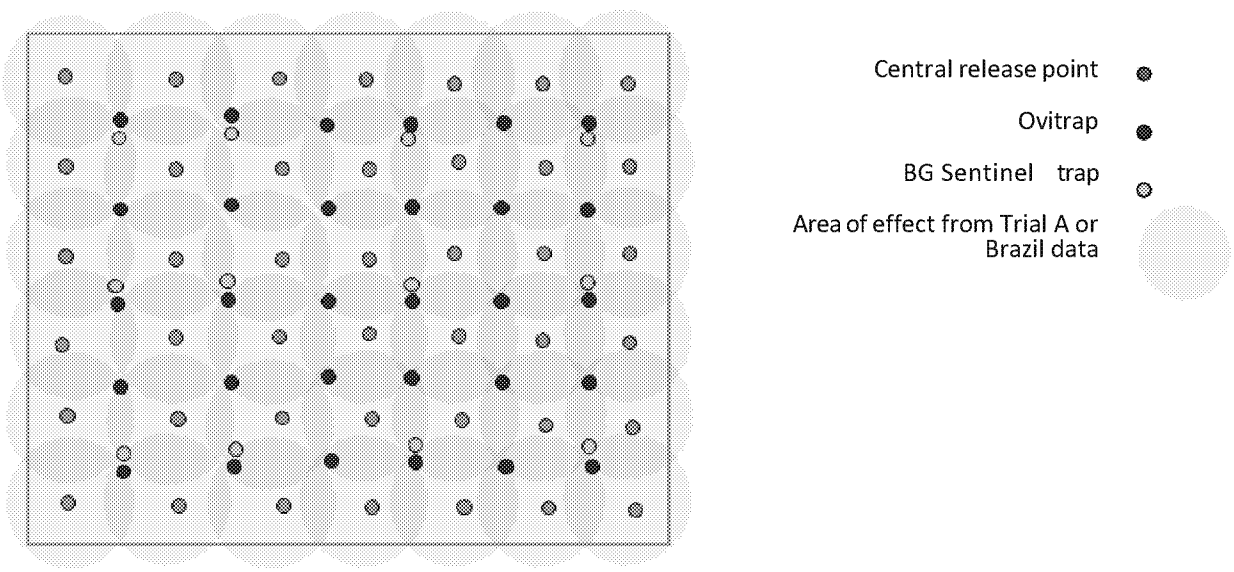

Figure 9. Schematic overview of a trial site for Trial B showing the multiple release points and a potential arrangement of egg traps (ovitraps, in red) and adult traps (BG Sentinel® traps, in green). The observed area of effect from Trial A and/or data generated from trials in Brazil will be used to inform the release point locations for Trial B. The number of release points shown (36) is for illustrative purposes only.

Table 6. No of sites, application rates (doses 1-3 i.e. lowest - highest), and replicates for Trial B, including the treated acreages and life-stages assessed. Please note that both locations, or only one location (FL or TX) may be used.

Trial	Location*	Number of untreated areas (Required)	Number of treated areas (dose 1 - low) (Required)	Number of treated areas (dose 2 - medium) (Optional)	Number of treated areas (dose 3 - high) (Optional)	Maximum acreage per trial site	Maximum total treated acreage per year	Life stage assessed
Trial B	Florida - Monroe County	3	3	3	N/A	80	480	Eggs only
	Texas - Harris County	3	3	3	N/A	40	240	Eggs only

**One (either FL or TX) or both locations may be used. See Section 4.1 for discussion of trial locations.*

5.9. General Experimental Methods

5.9.1. OX5034 transport

Known quantities of OX5034 eggs will be delivered to release site(s) in triple layered containment. Ambient temperature within the vehicle should be between 59°F (15°C) and 86°F (30°C) during storage or transport of eggs. If the temperature is higher than 86°F (30°C), cooling aids (e.g. air-conditioning or ice packs) may be used.

5.9.2. Application of OX5034 treatment

The aim for Trial B is to test the use of Mosquito Rearing Boxes, which enable the deployment of OX5034 mosquito eggs in specially designed Mosquito Rearing Boxes which facilitate egg-to-adult development in the field. In all cases, the locations of each Mosquito Rearing Box/event will be given a unique identifier and georeferenced for accurate placement and mapping.

5.9.3. OX5034 Mosquito Rearing Boxes

At the prescribed release locations, in the case of egg releases prescribed amounts of water, mosquito food, and other additives will be added to Mosquito Rearing Boxes, as described in (Oxitec Ltd & MRID 50889401, 2019). A known quantity of OX5034 eggs will also be added to each Mosquito Rearing Box. Adult males of the

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desired quantity (anticipated 2,500 male adults per Mosquito Rearing Box[4]) will emerge within 22 days of the Mosquito Rearing Box setup and deployment.

5.9.3.1. Construction

- Component parts will be safe to handle, nontoxic to rearing process, robust and capable of withstanding local environmental conditions for at least 22 days.
- Protected from applications of insecticide, in particular of *Bti* (*Bacillus thuringiensis israelensis*), as far as is possible.
- Option to fix to typical urban landscape but can be used as a free-standing Mosquito Rearing Box.
- Easy and environmentally friendly disposal – safe and simple mess-free draining of water, with no parts left behind.
- The complete Mosquito Rearing Box will be weatherproof and also ensure environmental control of water temperature, condensation and light conditions to enable efficient mosquito development.
- For the purposes of the trial, Mosquito Rearing Boxes will be physically isolated from the public to prevent vandalism/tampering, or where that is not possible, located discretely and out of public view.
- Mosquito Rearing Box design will preclude the Mosquito Rearing Box becoming a breeding site for wild mosquitoes.

5.9.3.2. Mosquito Rearing Box setup and activation

Information on page 26 of this volume falls under FIFRA 10(d)(1)(A), and, therefore, has been removed to a confidential attachment.

Cross-reference number 7

5.9.4. Field Monitoring Methods

5.9.4.1. Fluorescent Marker and PCR assessments

The fluorescent marker is readily visible in all life-stages apart from eggs and will be used for OX5034 identification (GL-SOP-00052) (Oxitec Ltd & MRID 50889401, 2019). Note, as outlined in section 5.2.6.1, a minimum number of 40 fluorescent and 40 non-fluorescent *Ae. aegypti* will undergo molecular identification by quantitative PCR to complete a one-time validation of the fluorescence screening by confirming their genotype as either OX5034 or wild *Aedes aegypti* (QD-R-00109 or QD-R-00108).In addition, all individuals will be taxonomically identified to genus and/or species level. Fluorescence screening will also be used to assess penetrance of the female-specific self-limiting gene (see Section 5.9.4.2 for ovitrap monitoring details and Section 5.11.2 for larval rearing methods as part of penetrance testing).

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[4] Mosquito Rearing Boxes designed to produce other numbers of male mosquitoes, e.g. 500 males per box, 1,000 males per box, etc. may also be used. In all cases, the maximum number of males/acre/week indicated in the trial designs would not be exceeded.

Please note that field-collected samples of *Aedes aegypti* will be taken and stored for subsequent analyses of genetic diversity and introgression of background genes.

5.9.4.2. Eggs

Ovitraps are a commonly used system for collecting *Ae. aegypti* mosquito eggs, mimicking natural breeding sites in which females lay eggs. Ovitraps consist of a pot containing water and a substrate (paper or wood) protruding above the water line on which eggs can be laid. The substrate used will be consistent across trial sites. Ovitraps will be positioned in sheltered locations, typically nearby residential, commercial, or utility premises. Appropriate consent for the placing and servicing of the traps will be obtained. Each trap will have a unique identifier and georeferenced for accurate placement and mapping.

A minimum of 30 ovitraps per plot for Trial B will be distributed across the trial area. Trapping intervals will typically be 7 days (maximum 9 days), at which time the water and oviposition substrate will be replaced, or the trap may be substituted for a new one. Oviposition substrates will be labelled and stored individually to prevent cross contamination during transport. Once at the laboratory they are dried at room temperature for a minimum of 2 days (maximum 14 days) to mature the eggs prior to hatching.

5.9.4.3. Ovitrap Density

The trapping density we recommend when used as surveillance tool is a minimum of 28 per site. This minimum number of traps per site (28) was calculated as the sample size required for multiple regression to detect a medium-sized effect (Cohen's $f^2 = 0.25$) with 80% statistical power when there are 4 predictor variables. This number was calculated using the computer program G*Power. A similar ovitrap density or higher has been used successfully previously (Harris et al., 2012; Gorman et al., 2015; Carvalho et al., 2015) and is not expected to confound or interfere with any measurements of efficacy. For Trial B we propose using a minimum of 30 ovitraps per area through pre-release and treatment periods, increasing the number to a minimum of 48 per area during post-release monitoring periods, during which we will also increase the monitoring area by extending the distance from the centre to the perimeter by 100m in all directions.

5.9.4.4. Ovitrap Interval

The trapping interval we recommend for ovitraps when used as surveillance tool is weekly. This has been chosen as it permits constant trapping throughout the trial period yet strikes a balance between the maximum number of data points we could collect and a trapping interval that does not become a frustration to homeowners and offers operationally feasible surveillance. Oxitec has successfully used a weekly trapping interval for surveillance during *Aedes aegypti* product trials and published the results in peer-reviewed studies previously (Harris et al., 2012; Gorman et al., 2015; Carvalho et al., 2015).

5.9.4.5. Adult female traps

BG-Sentinel® traps (Biogents, Germany) target both male and female adults of several Aedine species. They employ a combination of visual and olfactory (odours and/or CO_2) attractants to lure individuals towards a motorised fan and into a catch-bag. Power can be supplied by mains, battery or solar generated electricity. BG-Sentinel® traps will be positioned in sheltered locations, typically nearby residential, commercial, or utility premises. Appropriate consent for the placing and servicing of the traps will be obtained. Each trap will have a unique identifier and be georeferenced for accurate placement and mapping.

A minimum of 5 BG traps for Trial B will be distributed across the study area. Locations of BG-Sentinel® trap units will be rotated to prevent bias between individual traps. The catch-bag in BG-Sentinel® traps can be changed daily, every few days or weekly. Trapping intervals will be the same across plots and will be a maximum of 9 days. Catch-bags will be labelled and stored individually to prevent cross contamination during transport. Once at the laboratory samples will be processed within 96 hours.

5.9.4.6. Adult Female Trapping Density

The trapping density we recommend when used as a surveillance tool evaluating changes in abundance is a minimum of 28 per area. For Trial B they will not be used to evaluate changes in abundance and will only be used to evaluate overflooding (OX5034 male:wild male) and/or sex (male:female) ratios. These two metrics do not specifically relate to any product label claims but allow a preliminary assessment of the dosage applied, therefore a minimum of 5 per area is proposed. This will apply throughout the treatment period. During post-release monitoring periods only ovitraps will be used to detect disappearance of the transgene from the environment. Mark release recapture results to date in Brazil have shown that BG Sentinel® traps in direct line of site and in close proximity to release points, when combined with point release of adults can catch a high proportion of released individuals. Therefore, care will be taken to ensure BG-Sentinel® traps are located appropriately and not in a direct line of site to release locations.

5.9.4.7. Adult Female Trapping Interval

For Trial B we will service BG-Sentinel® traps weekly (maximum 9 days), as the primary metric will be assessing overflooding (OX5034 male:wild male) and/or sex (male:female) ratios. These two metrics do not specifically relate to any product label claims but allow a preliminary assessment of the dosage applied. This weekly interval has been chosen as it permits a less variable ratio to be obtained and strikes a balance between the maximum number of data points we could collect and a trapping interval that does not become a frustration to homeowners and offers operationally feasible surveillance.

5.9.4.8. Untreated comparator areas

For Trial B, untreated areas will be utilized to provide samples of larvae for mortality assessments that have not been exposed to any form of treatment. These areas will be of similar size and characteristics to treated areas; where possible, comparator and treatment areas will be randomly allocated. The number of untreated areas will be the same as the number of areas for each treatment rate. For Trial B this will be at least three.

5.10. Objective of the Program

The objectives of the Field Trial B will be to quantify across multiple release points:

- Efficacy of the active ingredient (% mortality observed in fluorescent female progeny compared with untreated, i.e. non-fluorescent females).
- The adult over-flooding ratio achieved i.e. Oxitec males:wild male ratio in each BG trap.
- The proportion of treated i.e. fluorescent individuals within each ovitrap (mating fraction).
- Duration and scale of residual activity (time until disappearance of fluorescent larvae, and the rate of disappearance in the environment measured until no individuals have been found for a minimum of 10 consecutive weeks i.e. a period sufficient for at least two discrete generations.

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- The presence of fluorescent larvae in natural *Aedes aegypti* breeding sites, including those published as relevant in the US such as septic tanks, disused tires, flowerpots, planters, trivets (Hribar et al., 2004) and plastic buckets, trash cans, and discarded plastic containers (Hribar et al., 2001). **Note that these data will not count towards any calculations of efficacy.** See Section 5.11.3 for details.

In total a minimum of one trial (with 3 replicates at each application rate) will be performed. The precise location(s) of the trial site(s) and related plots are yet to be determined and will be reported to the EPA before the protocol is initiated.

5.11. Efficacy Measurements: Trial B

5.11.1. Application

It is anticipated that for eggs or adults the interval between applications would be <22 days. The longest interval anticipated between releases/deployments of Mosquito Rearing Boxes will be evaluated. Target application rates will be fixed for the duration of the releases. The maximum weekly release rates will be 20,000 males per acre for Trial B (maximum of 1,600,000 males total per area per week as Trial B is limited to 80 acres in FL, and a maximum of 800,000 males total per area per week as Trial B is limited to 40 acres in TX). We anticipate that in each climate zone utilized, Trial B would be completed at a minimum of one application rate (with 3 replicates) not including untreated comparator sites.

5.11.2. Mortality assessments

Eggs from each ovitrap will be induced to hatch (to synchronise hatching) then screened for fluorescence and counted within 24 hours. Larvae will be reared under laboratory conditions at 27°C [+/- 2°C], 70% [+/- 10%] relative humidity, 12h: 12h light: dark cycle and fed *ad libitum*. Once pupated, remaining individuals will be placed into cages for adult emergence. Post-emergence, all adults will be taxonomically identified to species level, screened for fluorescence, and sexed. Note, as outlined in section 5.2.6.1, a minimum number of 40 fluorescent and 40 non-fluorescent *Ae. aegypti* will undergo molecular identification by quantitative PCR to complete a one-time validation of the fluorescence screening by confirming their genotype as either OX5034 or wild *Aedes aegypti* (QD-R-00109 or QD-R-00108). This may require a repeat procedure should this molecular assay fail for any reason.

5.11.3. Cryptic Breeding Sites

At least 6 natural breeding sites per area for Trial B will be identified and checked on a weekly cycle (maximum 9 days) for the presence of *Ae. aegypti*. No egg-laying substrate will be used as the intention is to examine 'natural' breeding sites and so collections will consist of larvae only. If found, larval samples of *Ae. aegypti* will be taken to the laboratory for screening of fluorescence to establish if they were fathered by OX5034 males. It is anticipated that any fluorescent larvae collected would therefore themselves be males, as fluorescent females are expected to die at early larval stages. These collections would not contribute to efficacy or other measurements. Descriptions of the types of natural breeding site, locations, and larval counts will be provided.

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5.11.4. Persistence Measurements

OX5034 mosquitoes possess a self-limiting gene and a fluorescent marker gene. The self-limiting gene, when passed onto offspring, prevents female progeny from surviving to functional adulthood in the absence of tetracycline. By design, male progeny survive and can develop through to adulthood and potentially mate with wild females. OX5034 genes are therefore passed down as a single copy from male parents only, and as they are subject to normal Mendelian inheritance patterns, are not expected to establish at the proposed trial site but decline predictably following the cessation of releases over the course of <10 generations (Oxitec Ltd & MRID 50889416, 2019).

Ovitrap data will be used to quantify the presence (anticipated decline) of the fluorescence gene over time. Monitoring will continue until at least 10 consecutive weeks without the presence in ovitraps of OX5034 fluorescent mosquitoes. Given that the generation time for Aedes aegypti is usually 4 weeks or less, this period should be sufficient to allow two discrete generation times without fluorescence. Note, as outlined in section 5.2.6.1, a minimum number of 40 fluorescent and 40 non-fluorescent *Ae. aegypti* will undergo molecular identification by quantitative PCR to complete a one-time validation of the fluorescence screening by confirming their genotype as either OX5034 or wild *Aedes aegypti* (QD-R-00109 or QD-R-00108). This may require a repeat procedure should this molecular assay fail for any reason.

5.12. Data Analysis Methods

Efficacy data analyses are subject to ongoing discussions with EPA. Statistical methods, as outlined below, will be used to analyse data, and additional data analyses may also be carried out in support of product registration under FIFRA Section 3.

5.12.1. Efficacy

The evaluations of each replicate will yield survival data, i.e., number of females surviving (reaching adulthood). Therefore, the recommended[5] calculation to account for survival rates in untreated replicates is an adaption of Mulla's formula.[6] The output of this formula is the control adjusted percentage mortality (efficacy):

E=100*((C-T)/C) or E=100*(1-T/C)

where:

E = percentage efficacy in individual ovitraps from the treated area.

C = percentage of untreated females surviving across all ovitraps in control areas.

T = percentage of treated, i.e. fluorescent females surviving in individual ovitraps.

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[5] *Guidelines for laboratory and field testing of mosquito larvicides. Editors: Dr M. Zaim/WHOPES, 39 p., Publication date: June 2005. WHO reference number: WHO/CDS/WHOPES/GCDPP/2005.13*

[6] *Mulla MS, Darwazeh HA. Activity and longevity of insect growth regulators against mosquitoes. Journal of Economic Entomology, 1975, 68:791–794.*

5.12.2. Trial B replication

We anticipate that in each climate zone utilized, Trial B would evaluate a minimum of one application rate and untreated controls with each being replicated at least three times (minimum of six sites in total).

5.12.3. Test Acceptance Criteria

For each of the efficacy studies (i.e. data from any single plot) will be considered valid providing:

- The total number of larvae (sum of fluorescent and non-fluorescent) collected from all ovitraps over the course of the study is equal to or greater than 100.

5.13. Statistical Analysis

5.13.1. Efficacy

We will assess the difference in number of the percentage of females surviving between release and non-release treatments, as well as how survival is affected by the following variables:

- Release rate (the number of male adults released/exiting a single mosquito rearing box)
- Trap distance (how far the trap is from the point of release)
- Site (which site replicate is being measured)

We will assess the difference in female survival between release and non-release sites using general linear mixed-effects models (GLMM) with the random effect of site, a crossed random effect of trap distance, and the covariates life stage and release rate. The model will use a quasibinomial distribution and a logit link function since the response variable is recorded as a percentage. Percent efficacy and 95% confidence intervals will be calculated from the results of the GLMM. The efficacy (E) of OX5034 to kill female mosquitoes is calculated using an adaption of Mulla's formula:

$$E=100*((C-T)/C) \textit{ or } E=100*(1-T/C)$$

E = percentage efficacy in individual ovitraps from the treated area.

C = percentage of untreated females surviving across all ovitraps in control areas.

T = percentage of treated, i.e. fluorescent females surviving in individual ovitraps.

5.13.2. Persistence Monitoring

The Kaplan-Meier estimator will be used to characterise the persistence of the OX5034 gene in release sites. Estimated median values, 95% confidence intervals, interquartile ranges and maxima will be reported.

5.14. Long-range testing plans

It is anticipated that trial(s) will be initiated in 2020, pending regulatory approval, and that OX5034 trials will be completed within 24 calendar months of initiation.

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5.15. The amount of active ingredient required for the projected acres in Trial B is based on the proposed application rates and formulation:

A minimum of one trial will be performed. The precise location(s) of the trial site(s) and related plots are yet to be determined and will be reported to the EPA before the protocol is initiated. Trial sites and control sites will be selected from a total of 18 locations in two states as shown in Table 7.

1. Maximum number of **treated** trial sites (all states): 12
2. Maximum number of **treated** acres (all states): 1,800 (see Table 5).
3. Maximum quantity applied per acre: 20,000 males per acre per week, or 0.082 milligrams of active ingredient (tTAV-OX5034) per acre per week.

Table 7. No of sites, application rates, and replicates for Trial B, including the treated acreages and treatment rates. Please note that both locations, or only one location (FL or TX) may be used.

Trial	Location	Number of treated areas (dose 1 - low) (Required)	Number of treated areas (dose 2 - medium) (Optional)	Maximum acreage per trial site	Maximum total treated acreage per year	Maximum quantity of mosquitoes per treated site per week	Maximum quantity of active ingredient per site per week
Trial B	Florida - Monroe County	3	3	80	480	1,600,000	6.56 mg
	Texas - Harris County	3	3	40	240	800,000	3.28 mg

The maximum quantity applied per treated site is calculated by multiplying the maximum quantity applied per acre (20,000 males per acre per week) by the number of acres per trial site (80 acres per Trial B site in FL, 40 acres per Trial B site in TX) (Table 8). See Table 4 for dose rates per year per state.

6. Anticipated Test Dates and Duration

It is anticipated that the trial(s) will be initiated in 2020, pending regulatory approval, and that OX5034 treatments will be completed within 24 calendar months of initiation.

The EUP trial design is divided into two study designs (Trial A and Trial B). These may take place simultaneously or sequentially. At each study site, either or both of the two study designs may be deployed. The details of which study design will be undertaken at which site will be reported to the EPA before initiation.

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7. Method of Disposition:

Any OX5034 *Aedes aegypti* not utilized in the program will be killed by freezing and then disposed of in general waste. In addition, any fluorescent larvae hatched from ovitraps, or fluorescent adults from BG traps, once transported to the lab for identification, will also be disposed of by freezing at <15 °C for 12 hours or longer, and then disposed of in general waste.

8. Deviations

Any deviations to this protocol are identified in writing and reported to the Head of Field Operations for review. Deviations will be investigated, tracked through to closure and reported in the final study report.

9. List of Acronyms, Abbreviations and Technical Terms

WT = wild type

tTAV = tetracycline-repressible transactivator variant protein

N/A = not applicable

10.References

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