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Re: Information Provided in Support of OX513A EUP and Section 3 Registration Applications

Dear Nancy,

Thank you for taking the time to speak with me and my colleague, Brad Shurdut, on Friday, June 29, regarding Oxitec's applications submitted under Section 5 and Section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for the OX513A mosquito. Oxitec has worked diligently with the Biopesticides and Pollution Prevention Division (BPPD) of the Office of Pesticide Programs (OPP) to address and satisfy all requirements under Section 5 of FIFRA for an Experimental Use Permit (EUP) to release the OX513A mosquito in the Florida Keys and Harris County, Texas, and for registration of the OX513A mosquito under Section 3 of FIFRA.

I would like to assure you that Oxitec has gone to great lengths to address all data requirements that BPPD has informed us are applicable to the OX513A mosquito, and that we are willing to expeditiously provide any additional data and information that may be required of us to support final approval of both the Section 5 EUP, and the Section 3 pesticide registration.

As you are aware, under the Pesticide Registration Improvement Act (PRIA) amendments to FIFRA, there are essentially 3 separate review periods relevant to experimental use permits and pesticide registrations. During the first review period – the 21-day content screen – EPA determines whether an application has all the components required for a particular type of application. The 21-day content screen is entirely non-substantive; it merely is a check to see if all the pieces of an application are present, all documents are properly signed and dated, etc. The second review period is a preliminary technical review. The purpose of this review period is for EPA to assess whether the applicant has submitted sufficient data and information for EPA to begin the substantive review of the application. Congress added the preliminary technical review period in the 2012 PRIA amendments to ensure that applications contained sufficient data and information for EPA to begin the substantive review of applications. The preliminary technical review was not intended by Congress to be a substantive review to assess whether a particular application completely satisfies the requirements for approval. If EPA determines that an application is substantively deficient, EPA notifies the applicant and identifies the deficiencies. The applicant then has 10 business days to satisfy the identified deficiencies. If the applicant submits sufficient data for EPA to begin the substantive review, the third review period begins. In the final substantive review of the application, the EPA is to assess whether the applicant presents sufficient data and information to satisfy the FIFRA standard of no unreasonable adverse effects.

The PRIA deadline for a decision on Oxitec's OX513A EUP application is July 25th. On March 22, 2018, BPPD informed Oxitec that it had determined that the OX513A Section 5 EUP application had specific deficiencies, and that it was necessary for Oxitec to address the identified deficiencies within 10 business days, i.e., by April 9, 2018. Oxitec met with BPPD, in person and via telephone, on March 28, to discuss the deficiencies identified by EPA and to ensure that Oxitec scientists were absolutely clear on the data and information that was required to satisfy these deficiencies. During

that meeting, BPPD staff and Oxitec scientists and management engaged in what Oxitec considered to be useful and informative discussion of the identified deficiencies. At the conclusion of the meeting, Oxitec had what it believed to be a clear understanding of the data and information that BPPD required to begin the substantive review of the OX513A EUP application. Oxitec resolved to make every effort to provide all information identified by BPPD as being required for initiation of the substantive review of the EUP application. Moreover, Oxitec has met weekly with EPA over this time and routinely inquired about any potential issues that still needed to be addressed.

Oxitec believes that it has submitted data and information that completely address all of the additional information requested by EPA. As part of this letter, we have provided a data matrix (Table 1) that was derived from the confidential appendix that BPPD included to the March 22 letter. This table provides the data deficiencies concerning allergenicity, assay uncertainties, human exposure to DsRed2 and tTAV proteins and the manufacturing process, and information submitted to address these deficiencies.

I'd like to also provide additional background on why Oxitec believes that the data and information submitted were completely responsive to BPPD's requests for additional data. The formal response to the March 22 letter is filed in OPP's central data repository as MRID 50560401, and it is accompanied by a number of subdocuments that each have their own separate MRID designations.

To begin with, I'd like to address the issue of the homology of the DsRed2 protein to a newly discovered protein that was claimed to be an allergen in a report by Japanese researchers. I note at the outset that it is not the DsRed2 protein that was listed as a putative occupational allergen. Rather, the Japanese researchers claimed that a Green Fluorescent Protein (GFP)-like protein had allergenic properties. This raised an issue with respect to DsRed2 because DsRed2 is a GFP, and the purported GFP-like protein has some sequence homology to DsRed2. The GFP-like protein, 'Akane' was originally listed on the AllergenOnline database in Feb 2018 and later withdrawn from the database on 23 March 2018, with a complete scientific rationale and explanation for the removal, written by all eight members of the AllergenOnline peer review panel. The panel's explanation for the removal of the GFP-like protein from the database is available on the AllergenOnline website: http://www.allergenonline.org/versionhistory.shtml (see also the linked pdf on that page).

In the deficiency letter dated 22 March 2018, BPPD requested that Oxitec address the listing of the Akane protein in the database and the 58% homology of DsRed2 to Akane. In MRID 50560401 (Appendix 1, p36-40, 9 April 2018), we provided the detailed scientific rationale for the removal of the Akane GFP-like protein from the AllergenOnline database on 23 March 2018, and for convenience, we have also provided a copy of this appendix (see pages 16-20). The scientific rationale that Oxitec included in MRID 50560401 provided a detailed scientific response by a panel of 8 independent allergen experts to the paper that claimed that the Akane protein was an occupational allergen. The response concluded that there was insufficient evidence to classify the Akane protein even as a putative allergen.

Subsequently, in a deficiency letter issued on 4 June 2018 in response to our Section 3 application, BPPD requested further "new data or expanded scientific rationales" for the removal of Akane from the AllergenOnline database. In a call with BPPD on 12 June 2018, BPPD indicated that the main data request was for an explanation regarding why the database initially included the protein as a putative allergen, but then later removed it, based on the same data (i.e., the paper published by the Japanese researchers). Oxitec responded to the 4 June Section 3 application deficiency letter on 19 June (MRID 50608001). In this response, Oxitec highlighted the procedures by which the AllergenOnline review panel reached their initial decision to include the Akane protein on the

database, and then the reasons for their_removal (MRID 50608001, p19-20 19 June 2018). Oxitec also requested that the chair of the AllergenOnline review panel, Prof Richard Goodman (University of Nebraska, Lincoln), provide in confidence to the BPPD reviewers, the minutes and emails relating to these decisions. However, it must be emphasized here that the main point is not whether the AllergenOnline review panel looked at any new data as part of the re-evaluation. Rather, what is important is how the existing data were assessed, and the sound bases and conclusions that the review panel relied upon in determining that the Akane protein should not be classified as an allergen.

It is also important to note that the chair of the peer review panel for a competing allergen database, COMPARE, was a signatory to the AllergenOnline database scientific rationale for the removal of Akane from the AllergenOnline database in March 2018. With the formal removal of the GFP-like protein from the Allergen Online database, and the ongoing re-evaluation of the COMPARE database listing, Oxitec believes that it is indisputable that the unconfirmed assertion by one group of researchers, that the GFP-like protein is an allergen, has no relevance to DsRed2. Moreover, we also noted in our response that homology screening is but one aspect considered as part of a weight of evidence assessment for allergenicity. DsRed2 also has a long history of safe use as a fluorescent marker used in pharmaceutical research, and is digested rapidly when challenged in vitro with simulated gastric fluids and with proteases involved in environmental degradation.

I'd also like to address the issue of protein quantitation in the OX513A mosquito, which is one of the pieces of data required to estimate likely human exposure to DsRed2 and tTAV proteins. This was one of the areas we discussed with BPPD scientists during a call on 28 March 2018. Based on the additional explanation provided by BPPD scientists, we thought we had a good understanding of the information requested and subsequently addressed the apparent data deficiencies highlighted by the reviewers.

In Oxitec's response (MRID 50560401, p17, 9 April 2018) to the EUP deficiency letter, BPPD scientists acknowledged the challenges of 'proving the negative' when OX513A did not express DsRed2 and tTAV at detectable levels. To help overcome this challenge, BPPD suggested providing reference journal articles that demonstrated that the antibodies used by Oxitec to quantify DsRed2 and tTAV proteins worked in a variety of eukaryotic species expressing DsRed2 and tTAV. Oxitec provided relevant peer-reviewed articles that demonstrate that the antibodies have been used to ascertain the presence of DsRed2 and tTAV in numerous eukaryote species including insects, which were requested by BPPD as part of the justification of the validity of these antibodies for the assays being conducted.

The BPPD reviewers had also raised a valid concern that the protein extraction methods used to extract DsRed2 and tTAV proteins from OX513A mosquitoes might not efficiently extract cytosolic proteins, and in response to this Oxitec repeated the experiments with new extraction methods to address this concern. In these repeated experiments we also provided positive controls in the form of purified DsRed2 and tTAV protein (from *E. coli*) mixed into wild-type *Aedes aegypti* cytosolic lysates (MRID 50560407, p7 and p11-40) which demonstrated that the antibodies were able to detect the proteins in the context of *Aedes aegypti* lysates (and that the proteins were stable in lysates, where protein degradation might be expected to occur). It appeared to us, from the discussion on 28 March 2018, that these two additional sets of data, together with the statistically robust assay limits of detection, would have been sufficient to address the concerns raised by BPPD.

In a call with BPPD on 12 June 2018, when we discussed the deficiency letter issued on 4 June 2018 in response to our application under Section 3 of FIFRA, at least one BPPD reviewer indicated that

they had not realised that positive controls including mosquito lysates had been provided, which seemed to indicate that they might not have reviewed the data fully. BPPD scientists seemed to indicate on that call that these positive controls would probably have been sufficient to address the apparent deficiency that was highlighted again on 4 June 2018.

However, as these concerns regarding positive controls were raised again in the Section 3 deficiency letter (4 June 2018), which prompted the call with BPPD on 12 June 2018, Oxitec carried out another set of additional experiments, provided in MRID 50608003 and MRID 50608001 on 19 June 2018, as outlined in points (c) and (d) below. These further controls corroborated and validated the data provided on 9 April 2018:

MRID 50608001, MRID 50608003 (19 June 2018):

a) Oxitec screened some of its other transgenic insect research strains (in a variety of insects, including mosquitoes and fruit flies), and found that a research strain of *Aedes aegypti* expressed tTAV and DsRed2 proteins at high levels, that were detectable using the same assay conditions as previously used for OX513A. Oxitec then used this research strain to demonstrate that all antibodies were able to detect DsRed2 and tTAV proteins endogenously expressed in *Aedes aegypti*, and hence the qWestern blot assays worked as expected (MRID 50608003, p11-25).

b) These positive controls should have addressed any potential remaining concerns from the BPPD reviewers concerning the validity of the exposure data for OX513A mosquito adults, mosquito larvae or female mosquito saliva.

c) Oxitec was also able to improve assay sensitivity by using slightly different gel matrices for these analyses, resulting in slightly lower LoDs, but with results corroborating those reported on 9 April 2018. The final set of exposure data were:

- **tTAV protein** cannot be detected in larval OX513A mosquitoes above the assay Limit of Detection (LoD) of 1.56 ng, and is detected in adult OX513A mosquitoes at *de minimis* amounts i.e., up to 1.34 ng/mosquito (assay LoD is 0.39 ng per adult mosquito) (MRID 50608003, p25).
- **DsRed2 protein** cannot be detected in larval or adult OX513A mosquitoes above the assay LoD of 25 and 15 ng per mosquito, respectively (MRID 50608003, p24).
- **tTAV and DsRed2 proteins** are not detectable in OX513A female saliva (from 5 female mosquitoes) above the assay LoD of 0.8 ng and 2.5-5.0 ng, respectively (MRID 50326404).

d) The BPPD review team also suggested on the call on 12 June 2018 that Oxitec attempt to quantify DsRed2 and tTAV by analysing pooled OX513A mosquito samples. Oxitec attempted to respond to this suggestion by carrying out the additional experiments suggested (MRID 50608001 p27-33), but the results demonstrated that this analytical method was not appropriate to address the experimental question.

In summary, the protein exposure data provided by Oxitec, both on April 9, 2018 and supplemented on June 19, 2018 with additional corroborating data, should be sufficient for BPPD to conduct an in-depth risk assessment for OX513A. In addition, **since human exposure to the OX513A proteins is only possible via a biting female's saliva, and there is no secretory signal that allows for the secretion of protein to the mosquito's saliva, human exposure is implausible. This is corroborated by the lack of detectable protein in our analysis of saliva samples.**

You also mentioned on our call that BPPD was seeking further information on quality control, containment measures, etc. as part of information on the manufacturing process. Some requests for further information were made in the 22 March 2018 EUP deficiency letter, and we discussed these

on a call with BPPD on 28 March 2018, indicating that we would provide summaries of all manufacturing SOPs to address the deficiency identified on 22 March 2018. No specific requests for information about quality control or containment measures were made in the EUP deficiency letter dated 22 March 2018. The SOP summaries were provided in MRID 50560402 (Confidential attachment, p16-20). BPPD made further specific requests for information regarding quality control and containment measures, but only in the Section 3 deficiency letter (4 June 2018). These were provided in full in MRID 50608001 (p14-18, a summary), and MRID 50608002 on 19 June 2018, and included the following:

a) 21 complete manufacturing and shipping SOPs (MRID 50608002, Attachments 2 and 5) which also included details of containment measures.
b) Images of mechanical sorting devices for separating larvae from pupae, and male from female pupae (MRID 50608002, Attachments 3 and 4).
c) Quality Control measures and required standards, for
    - fitness of lab-reared colonies
    - mating competitiveness
    - number of flying males
    - penetrance of OX513A trait
    - detection of resistance to self-limiting trait, and measures to mitigate
    - detection of establishment in the environment, and measures to mitigate

In summary, the manufacturing and quality control data provided by Oxitec, both on April 9, 2018 and supplemented on June 19, 2018 with additional details requested on 4 June 2018, should be sufficient for BPPD to conduct an in-depth assessment of our OX513A applications under FIFRA.

Nancy, I recognize that this is a rather voluminous response to our discussion last week. However, the team and I feel that it is important to ensure that you are fully aware of all of the data and information that Oxitec has provided to be responsive to the data requests by BPPD. Oxitec has diligently and promptly undertaken its very best efforts to address and satisfy every data request that it has received from BPPD. Moreover, as noted above, Oxitec has sought specific guidance from BPPD to ensure that there was complete understanding of what data and information were required, and that there was a mutual understanding of what data and information would satisfy those requests. Our team also conscientiously followed up with your staff weekly in the event there were questions or clarifications needed.

As we have discussed on numerous occasions, the OX513A mosquito has been demonstrated in numerous release scenarios worldwide to be a safe and effective way to significantly reduce populations of *Aedes aegypti*. Moreover, as Oxitec has demonstrated in numerous submissions for both the EUP and Section 3 applications, the active and inert proteins in OX513A, tTAV and DsRed2 have been utilized for many years by countless researchers in numerous different organisms with no concern that either of these proteins presents any risk of harm to any species, except in the context of transcriptional squelching that the tTAV protein is intended to cause. The potential for tTAV and DsRed2 proteins to pose risk to humans is only possible via a biting female's saliva. Based on the absence of detectable tTAV and DsRed2 proteins in OX513A female saliva ((MRID: 50326404), exposure to these proteins will be negligible. Furthermore, both tTAV and DsRed2 lack the secretory signals required for secretion of proteins from salivary glands into saliva (MRID: 50560401). **Hence exposure is not only *de mimimis* based on our assessment of salivary proteins, but also biologically implausible.**

Oxitec very much appreciates your time in addressing the issue of the OX513A FIFRA applications, and I look forward to speaking with you on Friday, July 13.

Best regards,

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Table 1: EPA's 90-Day Preliminary Technical Screening results, and Oxitec's Response				
	Oxitec's response			
EPA's 90-Day Preliminary Technical Screening Results, 22 Mar 2018	**MRID number**	**Page numbers**	**Summary of response**	**Data conclusions**
Allergenicity				
A search of the AllergenOnline database (Version 18A, February 01, 2018) found DsRed2 to have significant homology to a putative allergen (GFP-like protein; Kato et al., 2017). Thus, allergenicity of the modified DsRed2 cannot be excluded and human exposure to the protein must be addressed.	50560401 Appendix 1	36-40	The most recent version of AllergenOnline, Version 18b, 23 March 2018, no longer lists the GFP-like Akane proteins as allergens. Justification for this version update was provided and is summarised below. The Akane protein was removed because the initial paper identifying it as allergen failed to demonstrate IgE binding to the GFP-like protein. Conclusions were speculative based on very faint IgE blots that identified protein binding in both control and 'allergic' serum samples, and proteomic data identifying the sequence of the apparent allergen was ambiguous and showed poor protein coverage. Further detailed reasons for the removal of the Akane proteins from AllergenOnline (authored by the AllergenOnline review panel) are attached as an **Appendix 1** to this letter. **Note: Subsequently, in a deficiency letter issued on 4 June 2018 in response to our Section 3 application, BPPD requested further information regarding the removal of Akane from the AllergenOnline Oxitec responded to these requests on 19 June 2018, as outlined in Table 2 (below).**	• We have provided sufficient information for EPA to begin the substantive review of our applications. • Furthermore, we have provided data to show human exposure to the OX513A proteins via a biting female's saliva is not only biologically implausible for DsRed2, but also not detectable (see below).
Assay uncertainties: Quantitative Detection of DsRed2 and tTAV protein in OX513A female saliva				
Potential human exposure via female mosquito bites to tTAV and DsRed2 proteins	50560401	17	**1. BPPD requested we demonstrate that endogenously produced DsRed2 and tTAV can be recognised by VP16 and DsRed2 antibodies and indicated that the use *of E.coli***	• We have provided sufficient information for EPA to begin the substantive review of our applications.

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cannot be ruled out due to uncertainties in assay methodologies. Use of *E. coli* produced DsRed2 and tTAV proteins as positive controls are not sufficient to ensure the validity of the assays, particularly in light of a lack detection of mosquito/endogenously produced DsRed2 and tTAV proteins in whole mosquito larvae and adult assays.			**produced DsRed2 and tTAV proteins as positive controls were not sufficient, particularly in light of lack of detection in whole mosquito and adults assay.** **On a call with BPPD on 28 March 2018 , BPPD acknowledged the difficulties in 'proving a negative' if OX513A really did not express these proteins at a high enough level to be detected. To help address this question, Oxitec was requested to provide reference journal articles that demonstrated that the antibodies used worked in a variety of eukaryotic species expressing DsRed2 and tTAV as part of the justification of the validity of these antibodies for the assays conducted.** Oxitec provided relevant articles demonstrating that the antibodies worked in eukaryotes, including insects. Oxitec also provided *E. coli*-expressed proteins as positive controls, but 'spiked' these controls into wild-type mosquito lysates or wild-type mosquito saliva. Both the saliva protein detection study and the mosquito protein quantitation study were thus able to detect DsRed2 and tTAV proteins in the relevant analytes above the assay limits of detection. Hence, the antibodies used for protein detection were able to detect the relevant recombinant positive control samples. Further, the antibodies used have been validated in a wide variety of species. We also defined statistically robust limits of detection for each assay. Therefore, we did not expect that there would be	• We provided new data and information that supports the validity of the assay method and corroborates the assay results of negligible proteins

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			any difficulties in using these antibodies to detect these proteins expressed in mosquitoes, if they were present in quantities above the inherent limits of detection of the assays used, which they were not. Furthermore, we provided data that shows both tTAV and DsRed2 lack the secretory signals required for secretion of proteins from salivary glands into saliva (MRID: 50560401, 50608009) which further corroborated our analytical results and our contention that exposure via the dermal route is highly implausible.	
	50560401	18-20	**2. BPPD requested that we provide data on total protein extracted from saliva and how this relates to the protein amount expected to be secreted during normal blood feeding. BPPD remarked that the positive control Aegyptin is expected to be present in high concentrations in female saliva and the immunoblot may therefore not be sensitive enough to detect endogenously produced DsRed2 and tTAV.** We provided evidence to show that a) saliva collected from five female mosquitoes is comparable to the total amount of saliva present in an adult female's salivary glands, b) an Aedes adult female mosquito has approximately 3 µg of total salivary protein, and about half this amount is lost during the blood meal, c) mosquitoes reingest saliva while feeding, and about 25% of the salivary apyrase activity is recovered in the mosquito gut after a blood meal. In short, mosquitoes lose ~1.5 µg of salivary protein during the blood	• We have provided sufficient information for EPA to begin the substantive review of our applications • We provided new data and information that supports the validity of the assay method and corroborates the assay results of negligible proteins

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			meal, ~0.75 µg is reingested, and ~0.75 µg stays in the host. In the saliva Western blot study, we thus used induced saliva from 5 mosquitoes as the unit of detection. This corresponds to about one pair of mosquito salivary glands, and about 4 times the amount injected into a host during probing and feeding of the mosquito. The limits of detection for the recombinant proteins (TAV ~ 0.8 ng and DsRed2 ~ 2.5-5 ng) are in line with the lowest amounts of salivary proteins injected by a mosquito. Regarding the sensitivity of the Western blot assay: aegyptin is indeed present in high concentrations in mosquito saliva, which is why it was chosen as a control for efficient saliva extraction. However, blots were first probed for tTAV or DsRed2 and imaged, prior to stripping the blots and reprobing for aegyptin, which should rule out any concerns around aegyptin detection/overexposure affecting the sensitivity of the detection method chosen.	
Assay uncertainties: Quantitative Detection of DsRed2 and tTAV protein in whole body extracts of OX513A adults				
Mosquito-produced proteins could not be detected in whole mosquito body extracts, furthering uncertainties of the methodologies employed in the test of saliva. Validation controls for the whole mosquito larvae and	50560401	22	**1. BPPD requested we provide empirical data or information to show how the current protein extraction method utilized for adult mosquitoes will yield cytosolic proteins:** Oxitec acknowledged the concerns raised by EPA, and, following helpful discussions on 28 March 2018, conducted a new quantitative protein analysis on OX513A adults and larvae using a different extraction method that captures	• We have provided sufficient information for EPA to begin the substantive review of our applications. • We provided new data and information that supports the validity of the assay method and corroborates the assay results of negligible proteins.

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adults assays were poor and the assays displayed unexplained uncertainties. For example, DsRed2 can be detected in OX513A larvae by making use of its ability to fluoresce at certain wavelengths. These DsRed2 containing larvae can be seen under a microscope with the human eye. However, the assay data supplied by Oxitec shows that DsRed2 protein in fluorescing red larvae cannot be detected, rendering the whole mosquito larvae and adult protein assays questionable.			cytosolic proteins from mosquito adults and larvae. To confirm this, we also demonstrated using an additional Western blot control that we could detect the cytoplasmic protein Hsp70. Full details of the extraction methods used for adults and larvae, and the full analysis of tTAV and DsRed2 protein levels in these samples was submitted as MRID 50560407.	
	50560407	7, 11-40	**2. BPPD expressed assay uncertainties concerning the absence of positive controls and the two primary antibodies not being able to recognize endogenously produced proteins [** Following discussions with BPPD on 28 March 2018, Oxitec provided data to show that positive controls showing that *E. coli*-expressed DsRed2 and tTAV could be detected in wild-type *Aedes aegypti* cytosolic lysates (see discussion above for more details). **Note: In a call with BPPD on 12 June 2018, at least one of the BPPD reviewers indicated that they had not realised that positive controls including lysates had been provided. Hence BPPD scientists seemed to indicate on that call that these positive controls would probably have been sufficient to address the apparent deficiency. However, Oxitec responded further by providing additional controls as detailed in Table 2.**	
	50560401	22	**3. BPPD pointed out that DsRed2 can be detected in OX513A larvae by making use of its ability to fluoresce at certain wavelengths whilst the qWestern assay data supplied by Oxitec shows that DsRed2 protein in**	

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			fluorescing red larvae cannot be detected, and hence illustrated a concern with the assays: Oxitec provided sufficient data to explain this: DsRed2 protein is visible in larvae under a suitable fluorescence microscope but its distribution is limited to a number of small foci in a punctate pattern rather than being present in detectable quantities in every cell in the mosquito larva. Microscopy is inherently a much more sensitive detection technique than protein detection by Western blot. This explained the apparent discrepancy between the fluorescence microscopic detection of DsRed2, and the inability to detect DsRed2 by Western blot in crude larval extracts above the assay LoD of 50 ng.	
	50560401	23	**4. BPPD requested that we explain why LOD of the DsRed2 and tTAV proteins are less sensitive as in the saliva protein study (MRID 503264-04), and why two different primary antibodies were used for the detection of tTAV in the saliva and whole-body assays:** The repeated Western blot study (MRID 50560407) used the same antibodies as in the saliva protein study. The new LoDs for tTAV (1.6 ng in adults and 3 ng in larvae) and DsRed2 (50 ng) are consistently higher than the LoDs used in the saliva protein study (0.8 ng and 5.0 ng, respectively). Because of much higher protein background in the whole-mosquito lysates, the antibodies had to be used at lower concentrations than in the saliva study, which is most likely the reason for the reduced sensitivity relative to the saliva	

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			protein study.	
Human Exposure to DsRed2 and tTAV proteins				
Potential human exposure to tTAV and DsRed2 proteins via saliva from female mosquito bites cannot be ruled out due to uncertainties in assay methodologies.	50560401	19-20	As we have provided new data and information that supports the validity of the assay method, the assay results of undetectable tTAV and DsRed2 proteins in OX513A female saliva (from 5 female mosquitoes) remain valid. LoD for DsRed2 in saliva is 2.5-5.0 ng LoD for tTAV in saliva is 0.8 ng Furthermore, both tTAV and DsRed2 lack the secretory signals required for secretion of proteins from salivary glands into saliva (MRID: 50560401, 50608009). This data supports the assay results.	• We have provided sufficient information for EPA to begin the substantive review of our applications. • We provided new data and information that supports the validity of the assay and corroborates the assay results of undetectable proteins. • We provided data to show that both tTAV and DsRed2 lack the secretory signals required for secretion of proteins from salivary glands into. This data supports the assay results. Furthermore, this data also shows that human exposure to the OX513A proteins via a biting female's saliva is biologically implausible for DsRed2.
Manufacturing process				
Update your information to ensure that all standard manufacturing processes in all UK and US facilities are described.	50560402	Confidential Attachment	Updated information to address the deficiencies noted by EPA can be found in in the revised product characterization report MRID 50560402. A brief summary is also provided below.	• We have provided sufficient information for EPA to begin the substantive review of our applications **Further QC questions were received when EPA**

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Discrepancies between the rearing protocols, and MRID 503264-04 were noted. It appears that individual standard operating procedures (#00002-00011) depicted in the flow chart exist. Please specifically clarify the reason for the pupae sex sorting in step 00007. Also, provide other pertinent information, such as shipping conditions between the UK and the US. This information appears to have been previously submitted to FDA (MRID 504435-13).		15-20 2-3 13-14 15	Oxitec provided a summary description of each manufacturing SOP applicable to both UK and US facilities. There are 21 manufacturing SOPs in total. Discrepancy has been resolved. As mentioned in MRID 50560402, the reason for pupae sex sorting is for egg production, sex sorting is used to set up adult cages with controlled male:female ratios. As mentioned in MRID 50560402, OX513A Aedes aegypti eggs are packaged in triple containment and shipped with temperature-monitoring.	**sent the Section 3 deficiency letter on 4 June 2018 and these have been addressed in our response sent 19 June 2018, and, for convenience, are also summarised in Table 2.**

Table 2: 90-day Preliminary Technical Screening Results June 4, 2018 (Section 3), and Oxitec's Response			
Additional data provided (June 19, 2018)	**MRID number**	**Page numbers**	**Data conclusions**
Minutes/Emails from AllergenOnline documenting removal of Akane GFP-like protein from database	N/A	N/A	Minutes/emails were provided directly to BPPD by Prof Richard Goodman explaining the AllergenOnline process for inclusion and later removal of Akane from AllergenOnline.
Additional Western blots using Oxitec *Aedes aegypti* research strain expressing higher levels of DsRed2 and tTAV proteins	50608003	11-25	Antibodies used in 50326404 and 50560407 (9 April 2018) were re-validated and shown to be capable of detecting endogenously expressed DsRed2 and tTAV in an additional Oxitec *Aedes aegypti* research strain expressing DsRed2 and tTAV at higher levels than OX513A, and corroborating/validating the exposure data provided previously, showing no detectable DsRed2 in OX513A mosquitoes or in OX513A saliva, and tTAV only detectable in adult OX513A at 1.34 ng per mosquito, and undetectable in larvae or saliva. Oxitec was able to improve assay sensitivity by using slightly different gel matrices for these analysis, resulting in slightly lower LoDs, but with results corroborating those reported on 9 April 2018.
Additional Western blots using lysates from multiple mosquitoes (response to BPPD request in technical call on 12 June 2018)	50608001	27-33	Oxitec carried out these additional Western blots as suggested by BPPD scientists, and demonstrated that these methods had significant shortcomings which meant they were not appropriate to detect accurately the quantity of DsRed2 present in OX513A mosquitoes.
Complete manufacturing and shipping SOPS	50608002	Confidential Attachments 2 and 5	These supplement the summary of SOPs by providing full SOPs, as requested on 4 June 2018.
Images of Mechanical Sorting Devices for separating larvae from pupae and male from female pupae	50608002	Confidential Attachments 3 and 4	These diagrams show details of the mechanical sorting devices, as requested on 4 June 2018.
Quality control measures	50608002	25-29, and Confidential Attachments	Quality Control measures and required standards, including - fitness of lab-reared colonies - mating competitiveness - number of flying males - penetrance of OX513A trait - detection of resistance to self-limiting trait, and measures to mitigate - detection of establishment in the environment, and measures to mitigate