Additional information requested in RIVM communication of October 24th 2016

Administrative

Q1. To facilitate the evaluation of the submission, you are requested to supply a table of contents in which all documents and supplementary material (e.g reports, SOPs) are indicated, including titles and page numbers.

A. aegypti 0X513A

Q2. In the submission is stated that published evidence is available to indicate that the piggyBac #OX513A construct would be refractory to movement, even if exposed to exogenous transposases. Please supply evidence to substantiate this claim for A. aegypti OX513A.

Q3. Estimation of numbers of A. aegypti OX513A during monitoring will be primary based on the fluorescence trait, based on the expression of the dsRED2 gene. Please provide results that confirm the fluorescence trait to be expressed under all tested field conditions, for example by comparison of results obtained by fluorescence with data obtained with insert-specific PCR.

Q4. The bioinformatic analyses of the newly expressed proteins in strain OX513A with respect to their potential allergenicity and toxicity are performed in 2013. Since databases are regularly updated, please supply new analyses using up-to-date databases.

Q5. The vertical transmission of dengue (serotypes 1-4) and chikungunya viruses in A. aegypti OX513A females, and a wild type comparator strain have been evaluated to assess whether OX513A females were more competent for vertical transmission of dengue and chikungunya viruses than females from a wild type strain. Please indicate of similar studies have been performed with respect to the zika virus, as A.aegypti is also known to be a vector of zika.

Release on Saba

Q6. In the submission is mentioning of several variants of the *A. aegypti* OX513A strain, e.g. with an Asian-derived genetic background or a Latin American genetic background. Please indicate which variant will be used for the release in Saba.

Q7. It is not clear who will be responsible for containment measures and quality control during all steps of the release of A. aegypti OX513A in Saba, e.g. import of eggs, rearing of mosquitoes, release of mosquitoes, monitoring and analyses of eggs and mosquitoes in traps. Please indicate who is responsible for all steps and how it is guaranteed that all SOPs are followed during all phases of the release.

Q8. Please indicate how the waste water containing tetracycline derived from the rearing facility will be treated before it is disposed of?

Q9. In the submission it is indicated that the abundance of A. aegypti on Saba is relatively low. Given this relatively low abundance of A. aegypti and the number of inhabitants on Saba, what is the estimated number of GM mosquitoes that will be released?

Q10. A. aegypti can be dispersed over longer distances by passive transport on boats, trains and modes of long distance transport. The submission mentions that International Sanitary Regulations require ports and airports to be clear of A. aegypti for 400m (WHO, 2005). However, release of A. aegypti OX513A is also foreseen in and around the harbor and airport of Saba. Please indicate whether the measures taken in Saba with respect to these International Sanitary Regulations are sufficient to prevent dispersal of A. aegypti OX513A by boat or airplane?

1. To facilitate the evaluation of the submission, you are requested to supply a table of contents in which all documents and supplementary material (e.g reports, SOPs) are indicated, including titles and page numbers.

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba.

Supplemental information- Page numbers assigned in compiled pdf file *CONTAINS CBI- RIVM OX513A SABA- Technical Dossier and ERA- -September 2016 v.1- SECURED*

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Appendices 514

2. In the submission is stated that published evidence is available to indicate that the piggyBac #OX513A construct would be refractory to movement, even if exposed to exogenous transposases. Please supply evidence to substantiate this claim for *A. aegypti* OX513A.

Programmatic use of OX513A depends on trait stability under intensive mass rearing conditions over successive generations. It is thus critical to the success of the OX513A program that the strain remains stable in order to perform consistently as operational programs expand.

The use of the non-autonomous *piggyBac* transposon for germ-line transformation of insects has been well described (Handler, 2002; O'Brochta, 2014), and molecular characterisation of OX513A confirms a single copy insertion event at the expected TTAA *piggyBac* target site, and that OX513A does not contain vector backbone sequences from the plasmid used for transformation or the helper plasmid. Additionally, the trait follows expected Mendelian inheritance patterns for a single copy insertion. Data is described in the submission in the following sections:

- *OX513A Technical Dossier Part A- Section 3 - Vector used in the transformation of OX513A*
- *OX513A Technical Dossier Part A- Section 5.8 - Stability of the insert in OX513A*; which includes reference to *Appendix 1 Molecular Characterisation*, providing experimental evidence that the insertion event has taken place as intended at a TTAA *piggyBac* insertion site in a single copy number

As referenced in *OX513A Technical Dossier Part C- Section 2.2 Horizontal gene transfer* subheading *Remobilisation of the transposon* (page 36 of 99), published literature has been reviewed for evidence that the *piggyBac* construct used in the development of OX513A is refractory to movement even in the presence of exogenous transposases. Section A below provides a summary of this published evidence, and Section B references evidence that the insert has remained stable in the breeding population for over 118 generations.

A. Published evidence that integrated non-autonomous piggyBac vectors are highly stable in the Aedes genome when exposed to exogenous transposase under a wide variety of conditions.

Gene vector and transposable element behavior in mosquitoes has been reviewed (O'Brochta et al., 2003) and evidence presented based on experimental crosses between an *Aedes aegypti* line containing a reporter gene, and line containing a transposase source. In that study 3000 progeny were examined, and a subset of 100 were suspect of having a transposition event based on the expression pattern of the reporter gene. Molecular characterization of the suspect progeny revealed no evidence that *piggyBac* could be efficiently remobilized in *Aedes aegypti.* While the need to understand the post integration behavior of *piggyBac* and other vectors had been acknowledged, it was concluded that the stability of the vectors tested in the germ line of *Aedes aegypti* indicated that the stability of the transgenics would be very high even in the presence of homologous functional transposase.

In the case where unstable integration events of *piggyBac* helper plasmids along with the intact vector sequence has been observed in *Aedes aegypti* (Adelman et al., 2004), despite the presence of a transposase source and many intact donor elements, no conservative (cut and paste) transposition of *piggyBac* was observed in those lines, further supporting the body of evidence that *piggyBac* constructs in *Aedes aegypti* would be refractory to movement, even in the presence of a transposase source.

Comprehensive studies have since been undertaken on post integration characteristics of *piggyBac* vectors in *Aedes aegypti.* Sethuraman et al (2007) experimentally examined the post-integration activity of *piggyBac* transposable element gene vectors in *Aedes aegypti* using two different approaches. In one approach, the embryos from five independent transgenic lines of *Aedes aegypti*, each with a single integrated non-autonomous *piggyBac* transposable element gene vector, were injected with plasmids containing the *piggyBac* transposase open-reading frame under the regulatory control of the hsp70 promoter. No evidence for remobilization was found in the adult *Aedes aegypti*, whereas remobilization was readily detected in similar lines of *D. melanogaster*. The second approach taken by Sethuraman et al (2007) built on the observations of O'Brochta et al (2003) and examined heterozygous progeny of crosses between five *Aedes aegypti* lines containing stable non-autonomous *piggyBac* insertions (reporter-line) and five *Aedes aegypti* lines expressing functional transposase (helper line). Over 20,000 progeny from eight different combinations were recovered, and 150 individuals suspect for transposition events were selected based on expression patterns of reporter genes. In addition, over 11,000 progeny of a cross between reporter lines, and three lines containing non-functional transposase were examined. In this case 361 individuals exhibited gene expression patterns which warranted further analysis, and 100 were analysed for *piggyBac* remobilization. In either experimental approach, no evidence of the *piggyBac* transposon remobilization was observed.

In *D. melanogaster*, and other model organisms, two small RNA pathways have been implicated in the regulation of transposons, and the Piwi-interacting RNA pathway in particular (Pi-RNAs), which has been shown to be involved in the silencing of transposons, has been further investigated in *Aedes aegypti* (Arensburger et al., 2011). Based on sequence analysis of piRNAs in *Aedes aegypti,* in comparison to *D. melanogaster,* marked differences were observed, suggesting that aspects of the piRNA system differed between the two organisms, and that the observed stability of transposons in *Aedes aegypti*, may be attributable to these differences. The molecular mechanisms through which transposon silencing may be effected in *Aedes aegypti* is discussed in Arensburger et al (2011), but the broad conclusion is that these small RNA pathways are implicated in maintaining the integrity of the *Aedes aegypti* genome in the presence of numerous transposons and viruses.

Building on the observations that *piggyBac* cannot be mobilized once integrated into the genome of *Aedes aegypti*, yet can be readily mobilized in other species, Palavesam et al (2013) undertook studies to understand whether the positional context of the insertion played a role in the post integration silencing of *piggyBac* in *Aedes aegypti.* Evidence revealed that in *Aedes aegypti* cell lines in culture, *piggyBac* elements integrated into the genome were transpositionally silent in the presence of functional transposase, whereas plasmid borne

piggyBac elements could be remobilized under the same conditions. As the immediate flanking DNA sequence had been previously reported to account for almost all variance in *piggyBac* mobility in *D. melanogaster*, the potential that local sequence played a role in silencing mobilization of genomically integrated *piggyBac* elements in *Aedes aegypti* cell was examined. Integrated *piggyBac* elements, refractory to movement were excised from *Aedes aegypti* cell lines along with approximately 1 kb of flanking DNA, and were integrated into the same positions in the *D. melanogaster* genome where they exhibited high levels of activity. It was suggested from these studies that in *Aedes aegypti* there are higher scale genetic or epigenetic factors involved in the transpositional silencing of *piggyBac.*

B. Since its development, ongoing molecular genotyping has been undertaken via the Oxitec quality management system (QMS) to verify the insert has remained stable as intended.

The Quality Management System in place at Oxitec establishes a Quality Control (QC) programme for *Aedes aegypti* OX513A requiring routine genotyping every four generation equivalents. Via quantitative PCR per standard operating procedure QD-SOP-00071 "*OX513A QC Procedure for Colony Genotyping by SYBR Real-Time PCR using MX3005P*", the presence of the OX513A transgene at the defined insertion site is confirmed. In addition, the lack of presence of wild-type alleles and/or other transgenic mosquito strains is confirmed. In each assay, a minimum of 920 individuals are tested after rearing under standard insectary conditions for OX513A. The procedure also serves as the standard method for genotyping of samples collected from traps in *Ae. aegypti* OX513A field release sites as required.

The OX513A strain has been reared under insectary conditions since 2002 for over 118 generational equivalents as of November 2016,. A generational equivalent is considered a generation based on time rather than discrete generations as cage populations are contiguous.

Routine QC genotyping has been performed since 2014 and has confirmed insert stability in OX513A.

Therefore the weight of published evidence on the lack of movement of the *piggyBac* vector in *Aedes aegypti* along with the evidence of genetic stability in OX513A, even under mass-rearing conditions provides a weight of evidence that the **#OX513A construct would be refractory to movement even if exposed to exogenous transposases.** Furthermore no evidence of genetic instability has been detected in the progeny when OX513A has been used in field trials.

References

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3. Estimation of numbers of *Aedes aegypti* OX513A during monitoring will be primary based on the fluorescence trait, based on the expression of the dsRED2 gene. Please provide results that confirm the fluorescence trait to be expressed under all tested field conditions, for example by comparison of results obtained by fluorescence with data obtained with insert-specific PCR.

The DsRed2 marker gene is visible in larvae and pupae and not in the eggs and adults of *Aedes aegypti* OX513A. OX513A eggs are therefore required to be hatched in controlled laboratory conditions for visualisation of DsRed2 marker gene expression under the relevant excitation light wavelength, and adults require molecular analysis by PCR to confirm the presence of the marker gene.

The stability of expression of the DsRed2 trait in *Aedes aegypti* OX513A is critical to strain performance, and early in strain development the stability and expected Mendelian inheritance pattern was first characterised in OX513A and a subset confirmed by PCR validation as described in *OX513A Technical Dossier Part A - Appendix 1 Section 3.4 Mendelian inheritance ratios*.

The DsRed2 fluorescent trait is used operationally as a rapid and convenient way to assess the presence of the OX513A transgene construct without the need for molecular analysis such as PCR. It should be noted that visualisation of DsRed2 expression is not undertaken in the field, rather eggs are recovered from field sampling in ovitraps, which represent a sample of the field population, and are hatched and scored under laboratory conditions to visualise the DsRed2 fluorescent marker. The DsRed2 marker is best visualised in the first instar larvae, although it is present in later stages and adults, however scales on the adult body make visualisation more challenging. The eggs may be exposed to variations in environmental conditions at different field sites globally, but this does not impact DsRed2 trait expression for field monitoring purposes as this activity is always done under controlled laboratory conditions in conformance with Standard Operating Procedures (SOPs) after field collected eggs have been reared under insectary conditions. The SOPs used at the monitoring stage of the OX513A program are designed to ensure that eggs from ovitraps are consistently hatched to yield larvae suitable for screening, and to ensure that larvae hatched from field-trapped eggs are consistently and accurately scored for the DsRed2 marker. Controlled conditions for the expression of the DsRed2 trait and scoring of fluorescence in the evaluation of eggs collected from ovitraps in the field are described in *SOP 00030_01 OX513A Hatching Larvae from Ovitrapped Eggs,* and *SOP 00031_01 OX513A Fluorescence Screening of Larvae.*

The most critical parameters for ensuring the DsRed2 trait can reliably inform program decisions are assessed routinely through the OX513A quality management system (QMS). The capacity for the OX513A males to pass on the DsRed2 marker gene to the wild *Aedes aegypti* population as intended is a key parameter which has been assessed in the laboratory in *Aedes aegypti* of several different genetic backgrounds as presented in *OX513A Technical Dossier Part A Section 5.1.3 Mating competitiveness.* To ensure that released OX513A males perform consistently in passing on the OX513A construct to offspring, the QMS incorporates routine testing every 12 generations to evaluate if there have been any changes regarding mating competitiveness in the rearing colony used to supply OX513A eggs to field operations. Additionally, OX513A colony genotyping is performed on 920 samples every 4 generations which uses molecular characterisation to confirm the stability of the insert. Relevant Standard

Operating Procedures for the above noted activities are referred to in *OX513A Technical Dossier Part A section 5.8.1 OX513A quality control.*

In field operations, metrics used to inform the adaptive management of OX513A releases depend on the accurate measurement of DsRed2 fluorescence in the lab from field caught samples as described above. The DsRed2 marker has been used to monitor OX513A releases under a variety of environmental conditions typical to the urban/peri urban *Aedes aegypti* habitat, representing a variety of infestation levels, human population, housing densities, immigration rates from untreated areas, and seasonality. The DsRed2 marker has consistently performed as expected in informing the adaptive management of OX513A programs across sites described in *OX513A Technical Dossier Section 7.1 Previous Aedes aegypti vector control projects using OX513A.* Notably, longevity and dispersal studies were undertaken in an atypical *Aedes aegypti* habitat in Malaysia (Lacroix et al., 2012) whereby OX513A were released into a forested area and monitoring was conducted using both adult and ovitrapping methods. As part of this study ovitraps were brought back to the laboratory after one week and eggs hatched under controlled conditions. First and second instar larvae were scored for fluorescence and all larvae were allowed to develop to adult and then genotyped by PCR. No anomalies were reported regarding the correlation between fluorescence and PCR results. Adult trapping is generally done via BG-Sentinel traps which incorporate a chemical lure to draw adults to the trap and a fan which draws the adults into a mesh bag once they are near. The adult traps are used principally to look at total numbers of adults of various species, and PCR has been used to genotype adults captured from BG-Sentinel traps in certain instances (Lacroix et al 2012).

Under the OX513A adaptive management model, based on initial measures of the *Aedes aegypti* population and range finding releases as described in *OX513A Technical Dossier Part B - 1.3 Determination of release rates- phased approach*, a mating fraction of >0.5 is targeted and is measured through the expression of the DsRed2 trait in larvae hatched under controlled conditions from field collected eggs. Based on the estimations of the existing wild *Aedes aegypti* population at any time during the OX513A program, and as a function of the released cohort of OX513A males, trends in the mating fraction can be anticipated and depend on the reliable expression of DsRed2. If the target mating fraction was not achieved in consideration of the known population parameters, this observation by the Oxitec local Field Operations Manager would trigger investigation. If expression of the DsRed2 trait was suspect a follow up investigation by the quality management team would likely ensue. To date, over 180 million OX513A male mosquitoes have been released in suppression programs and trials globally and the DsRed2 Trait has performed consistently.

Supplemental information - Stability of the DsRed2 trait expressed in insects recovered from field released Oxitec pink bollworm

Although the OX513A program does not utilize a DsRed2 screening protocol in life stages of OX513A directly recovered from the field, and relies on expression of the trait under controlled conditions, the pink bollworm (*Pectinophora gossypiella*) strain OX1138B depends on DsRed2 expression and monitoring in field caught life stages. OX1138B was developed exclusively as an alternative to fluorescent powders to accurately mark sterile irradiated male bollworm, a pest of cotton, in irradiation based sterile insect technique (SIT) programs. OX1138B is thus not a self-limiting strain, but only contains the DSRed2 marker. (Simmons et

al., 2011) report over a three year period, ranging from caged releases to an operational demonstration in Arizona USA whereby over 15 million OX1138B were released in 2008, that DsRed2 screening was highly reliable. Validation of trapping results by a PCR spanning the junction of the insert was undertaken on a small subset of the captured moths expressing the DsRed2 trait with no reported anomalies (Simmons *et al,* 2011).

References:

Lacroix, R., McKemey, A.R., Raduan, N., Kwee Wee, L., Hong Ming, W., Guat Ney, T., Rahidah, A.A.S., Salman, S., Subramaniam, S., Nordin, O*., et al.* (2012). Open field release of genetically engineered sterile male Aedes aegypti in Malaysia. PLoS One *7*, e42771.

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4. The bioinformatic analyses of the newly expressed proteins in strain OX513A with respect to their potential allergenicity and toxicity are performed in 2013. Since databases are regularly updated, please supply new analyses using up-to-date databases.

A request for an updated bioinformatics analysis has been made and delivery of the report is pending.

5. The vertical transmission of dengue (serotypes 1-4) and chikungunya viruses in A. aegypti OX513A females, and a wild type comparator strain have been evaluated to assess whether OX513A females were more competent for vertical transmission of dengue and chikungunya viruses than females from a wild type strain. Please indicate of similar studies have been performed with respect to the zika virus, as A.aegypti is also known to be a vector of zika.

Vertical transmission (VT) studies as described in *OX513A Technical Dossier Section 5.7 Vertical transmission of Dengue and Chikungunya viruses in OX513A* specifically for Zika virus (ZIKV) have not been carried out in OX513A as dengue virus (DENV) and chikungunya virus (CHIKV) are representative of the two principle genera of arboviruses of public health concern (i.e. *Alphaviruses* and *Flaviviruses*) and it has been suggested (Rosen, 1988) that the mechanism of vertical transmission of different *Flaviviruses* viruses in *Aedes* occurs in the same manner during oviposition. There is no evidence to suggest that the capacity for vertical transmission of Zika virus (ZIKV) should differ from that of other *Flaviviruses* (i.e. DENV). Additional rationale are provided below.

While there are many arboviruses of concern to both human and animal health (Go et al., 2014), those associated with recent large outbreaks of public health concern (DENV, CHIKV, and ZIKV) belong to two taxonomic families (Mota et al., 2016). The *Togaviridae* (CHIKV) and the *Flaviviridae* (DENV and ZIKV) families are both single stranded positive sense RNA viruses. The other principle arbovirus families *Bunyaviridae, Rhabdoviridae,* and *Orthomyxoviridae* are single stranded negative sense RNA viruses, while the *Reoviridae* have a double stranded RNA genome.

CHIKV is of the *Alphavirus* genus belonging to the *Togaviridae* family (see Lo Presti et al., 2016 for an updated review of the molecular epidemiology, evolution and phylogeny of CHIKV). Structurally, *Alphaviruses* are described as enveloped spherical virions, 60 to 70 nm in diameter with a positive-sense, monopartite, single-stranded RNA genome of approximately 11.7 kilobases. The lipid-containing envelope has two (rarely three) surface glycoproteins that mediate attachment, fusion, and penetration. The icosohedral nucleocapsid contains capsid protein and RNA and virions mature by budding through the plasma membrane. Both DENV and ZIKV are of the *Flavivirus* genus belonging to the *Flaviviridae* family. Structurally *Flavivirus* virions are spherical and 40-50 nm in diameter with a positive-sense, non-segmented, single-stranded RNA genome of approximately 10.9 kilobases. The lipid-containing envelope has one surface glycoprotein that mediates attachment, fusion, and penetration, and an internal matrix protein. The nucleocapsid contains capsid protein and RNA and virions mature at intracytoplasmic membranes. Both DENV and ZIKV genomes encode a single polyprotein post-translationally cleaved into three structural proteins: capsid, pre-membrane/membrane and envelope; as well as seven non-structural proteins. See Schmaljohn and McClain, 1996, and Mota et al., 2016 for generic descriptions of *Alphaviruses* and *Flaviviruses*.

A mechanism of vertical transmission of DENV by mosquitoes has been proposed (Rosen, 1987) where-by transmission takes place when the fully formed egg, enclosed in the chorion, is oviposited. Based on the further experimental demonstration of the infection of intact eggs, for the three flaviviruses Dengue, Japaneese encephalitis, and St Louis encephalitis (Rosen, 1988) it was suggested that the same mechanism of vertical transmission prevails for

all mosquito-borne flaviviruses. Determinants of arbovirus VT in mosquitoes has been extensively reviewed (Lequime et al., 2016) whereby over a century of published primary literature on natural and experimental VT was assembled to identify biological factors associated with the efficiency of arbovirus VT in mosquito vectors. It was highlighted that while there are more than 530 described arboviruses, about a hundred are pathogenic to humans and mainly belong to four distinct genera of RNA viruses: *Alphavirus* (e.g., CHIKV), *Flavivirus* (e.g., ZIKV and DENV), *Orthobunyavirus* and *Phlebovirus*. A robust statistical framework was employed to propose environmental, taxonomic, and physiological predictors of arbovirus VT. The lowest taxonomic classification consistently recognised in drawing conclusions on VT of Arboviruses is at the genus level (e.g. *Alphavirus, Flavivrus*), and the *Aedes–Flavivirus* pair was the most represented vector–virus combination, with DENV the most represented viruses as reviewed by Lequime et al (2016). Additionally, the vertical transmission of ZIKV in *Aedes aegypti* has been evaluated (Thangamani et al., 2016), and the transmission of ZIKV by infected *Aedes aegypti* females to some F_1 offspring was observed. These results were acknowledged as expected given of results of other studies of VT with *Flavivirus* pathogens in their mosquito vectors.

Given that CHIKV is the predominant *Alphavirus* of public health concern within the Arboviruses, and DENV the most common representative *Flavivirus,* the use of DENV, and CHIKV in the vertical transmission studies for OX513A (Mansor et al., 2016) provides representation of the two principle genera of arbovirus of public health concern, and further studies on the VT of ZIKV in OX513A specifically, have thus not been undertaken.

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6. In the submission is mentioning of several variants of the *A. aegypti* OX513A strain, e.g. with an Asian-derived genetic background or a Latin American genetic background. Please indicate which variant will be used for the release in Saba.

The OX513A strain now used in all projects globally, and which would be used in the proposed program in Saba is the Latin American background strain. OX513A was generated through the transformation of the Rockefeller strain of *Aedes aegypti* as described in (Phuc et al., 2007) and subsequently out-crossed into *Aedes aegypti* of a Latin American genetic background provided by Instituto Nacional de Salud Pública (Mexico). It is our lead strain subject to regular quality assurance testing as described in the Standard Operating Procedures (SOPs) referenced in *OX513A Technical Dossier Part A Section 5.8.1* and is used in field programs around the world. As such there is a high degree of confidence of the genetic integrity of the insert in the background strain and its mating competitiveness.

Aedes aegypti is characterised as living and breeding in and around human habitation in the urban environment and is considered to have become "domesticated" throughout its global range as the result of association with the movement of humans. Molecular analysis has been undertaken (Brown et al., 2011) on *Aedes aegypti* individuals from 24 locations spanning 13 countries and five continents to understand the genetic relatedness of *Aedes aegypti* populations globally. Two distinct genetic clusters were identified which comprise domestic populations outside of Africa, and domestic and forest populations within Africa. Molecular based evidence also supports Africa as the ancestral origin of the species, as well as the existence of the 2 sub-species *Aedes aegypti aegypti* and *Aedes aegypti formosus.* This recent work with molecular markers (microsatellites) is also consistent with earlier studies on genetic variation conducted using isoenzyme variation (Failloux et al., 2002) which characterised the existence of a common domestic form of *Aedes aegypti* present in both Southeast Asia and South America. The global spread and invasiveness of the domestic cluster *Aedes aegypti aegypti* has been relatively recent in evolutionary time (i.e associated with the slave trade, global shipping, World War II), and the results of the mating competitiveness studies described in *OX513A Technical Dossier Section 5.1.3.1 Mating competitiveness in the laboratory* show no significant differences in the capacity of OX513A to mate with wild-type *Aedes aegypti* from various global sources, irrespective of its background strain.

OX513A in the Latin American background has thus been adopted as the strain for programmatic use globally, attributing the fact that it has originated from the genetic cluster associated with the global domestication of *Aedes aegypti.*

References:

Brown, J.E., McBride, C.S., Johnson, P., Ritchie, S., Paupy, C., Bossin, H., Lutomiah, J., Fernandez-Salas, I., Ponlawat, A., Cornel, A.J.*, et al.* (2011). Worldwide patterns of genetic differentiation imply multiple 'domestications' of Aedes aegypti, a major vector of human diseases. Proc Biol Sci *278*, 2446-2454.

Failloux, A.-B., Vazeille, M., and Rodhain, F. (2002). Geographic Genetic Variation in Populations of the Dengue Virus Vector Aedes aegypti. J Mol Evol *55*, 653-663.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A*., et al.* (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC Biol *5*, 11.

7. It is not clear who will be responsible for containment measures and quality control during all steps of the release of A. aegypti OX513A in Saba, e.g. import of eggs, rearing of mosquitoes, release of mosquitoes, monitoring and analyses of eggs and mosquitoes in traps. Please indicate who is responsible for all steps and how it is guaranteed that all SOPs are followed during all phases of the release.

Oxitec will send an accountable local project manager to reside in Saba and oversee implementation of the project throughout the initial community engagement and local set-up stage, plus the entire duration of mosquito releases (12 months anticipated). Oxitec will also have a presence in the 12 months of maintenance which immediately follows, if deemed necessary. Any non-conformance with SOP's will be recorded and reported to UK central operations, and corrective actions taken under the direction of the Oxitec Quality Assurance Manager.

Activities require local staff to be supplied by the Saba Department of Agriculture, Hygiene & Vector Control. Four local staff will be required during the first year during the preparation and intervention phases, and two staff in the following year for maintenance and monitoring. Full training and supervision will be given by Oxitec who will work in close partnership with the Saba Department of Agriculture, Hygiene & Vector Control, to ensure all participants are trained in Standard Operating Procedures relevant to their function. Training records will be retained on file as part of standard procedure.

After the initial proposed two year project has finished (set-up plus one year elimination plus one year maintenance), activities to maintain the local *Aedes aegypti* population under control such as ongoing supply of OX513A eggs, and technical support visits will be available subject to contractual agreements.

8. Please indicate how the waste water containing tetracycline derived from the rearing facility will be treated before it is disposed of?

Consistent with OX513A *Aedes* aegypti control projects and release trials to date under different regulatory regimes globally, wastewater from the rearing facility would be disposed of consistent with the manner of local waste water disposal without any additional treatment. In Saba this is currently via cesspits.

The chlortetracycline estimated to be present in the wastewater generated from the rearing facility is approximately 1.7 grams/week based on a maximum production of 300 males/person/week for a population of 2000 people. The rationale for this calculation and conclusion is presented in *OX513A Technical Dossier Part B Section 1.5*. For a relative comparison, 1.7 grams/week is less than, or in the range of, a single individual receiving therapeutic treatment for acne of 500mg twice per day for a 2-week period (i.e. 7 grams per week) whereby only 30% of chlortetracycline, or 60-80% dose of tetracycline is absorbed (see *OX513A Technical Dossier Part C Section 2.6* referenced below for additional information).

The assessment of potential risks associated with tetracycline disposal in the environment in the context of the current proposal are addressed in *OX513A Technical Dossier Part C* under 2 different risk areas as outlined below:

- *2.1 Persistence and invasiveness, including vertical gene transfer*: Dose response to tetracycline and its analogues page 26 of 99 of Part C
- *2.6 Environmental impacts of the specific techniques used for the management of OX513A* (Step 1)- Potential release of tetracycline into the environment page 69 of 99 of Part C

9. In the submission it is indicated that the abundance of A. aegypti on Saba is relatively low. Given this relatively low abundance of A. aegypti and the number of inhabitants on Saba, what is the estimated number of GM mosquitoes that will be released?

Estimates for release numbers in the context of the proposed Saba project can be made with a reasonably high degree of confidence given the relatively low abundance of *Aedes aegypti* reported, and based on experience in suppression projects to date. Estimates below are maximums based on the assumption that the *Aedes aegypti* population on Saba at the time of project implementation would be consistent with past estimates. The transition from intervention to maintenance phases would be gradual and take place based on the adaptive management of the releases in response to ongoing suppression data.

- Considering the adaptive management model may require variable release rates over the entire island at different times, taken on average over the first 12 months, releases are anticipated to average 160 OX513A males/person/week. For a population of approximately 1800 this would mean that up to approximately 15 million OX513A males would be released during the initial 12-month period.
- The program is anticipated to move to a maintenance phase after the first 12 months, or earlier if suppression targets are met, requiring lower level releases to ensure residual *Aedes aegypti* in the environment are targeted (e.g. unhatched desiccated eggs in the environment referred to as the egg bank). In this phase, averaged over the whole island, the release rates are not expected to exceed on average 50 OX513A males/person/week. This would equate to up to approximately 4.7 million OX513A males during the subsequent 12 months.
- As a matter of perspective, the 2016 permit from the Cayman Islands Department of the Environment for a 12-month project covering a population of approximately 2000 people was granted for the release of up to 22 million OX513A. *Aedes aegypti* is much more abundant in Grand Cayman than in Saba, thus release numbers are projected to be lower.

10. A. aegypti can be dispersed over longer distances by passive transport on boats, trains and modes of long distance transport. The submission mentions that International Sanitary Regulations require ports and airports to be clear of A. aegypti for 400m (WHO, 2005). However, release of A. aegypti OX513A is also foreseen in and around the harbor and airport of Saba. Please indicate whether the measures taken in Saba with respect to these International Sanitary Regulations are sufficient to prevent dispersal of A. aegypti OX513A by boat or airplane?

Currently around the ports of entry in Saba, the harbour and airport, methods used in vector control efforts for *Aedes aegypti* are consistent with that used island wide. In addition to regular surveillance and breeding site control, this includes the use of lethal ovitraps (http://www.in2care.org/), and biological control approaches such as the use of larvivorous fish in breeding sites, and the application of larvicidal products based on *Bacillus thuringiensis*. The World Health Organisation (WHO) has issued the document *Vector Surveillance and Control at Ports, Airports, and Ground Crossings* (WHO, 2016a) which provides guidance on the design of appropriate monitoring and control measures at ports of entry which serve to help identify the local characteristics and critical issues regarding the risk of import and/or export of vectors. WHO recommends data collection to inform vector control activities should focus on:

- the description of the environment (natural and urban) of the port of entry and surrounding 400-metre perimeter or wider;
- local entomological situation;
- epidemiological context (endemic or potential health risks associated with invasive vectors).

The approach in Saba is thus consistent with WHO guidance, as programmes of surveillance and vector control are expected to vary considerably and be proportionate and adapted to the local context of each port of entry. In this case due to the relatively low *Aedes aegypti* population on Saba, insecticidal fogging for adult *Aedes aegypti* has not been deemed necessary. No spraying inside airplanes or ferries, or fogging for adult *Aedes aegypti* currently takes place under the direction of the Saba Department of Agriculture, Hygiene & Vector Control. The Saba public health authority has indicated that insecticidal spraying of boats and planes leaving Saba may be considered in the future if warranted.

The recently issued *Report of the WHO Ad-hoc Advisory Group on aircraft disinsection for controlling the international spread of vector-borne diseases* (WHO, 2016b) notes current specifications for aircraft disinsection products have been established by the WHO Pesticide Evaluation Scheme (WHOPES), and consist of synthetic pyrethroids commonly used in mosquito control programs. The susceptibility of the OX513A strain to five commonly used insecticides including those from the pyrethroid family has been evaluated, and additionally OX513A has been screened for the presence of specific mutations which are associated with resistance to pyrethroids and DDT. These data are presented in the *OX513A Technical Dossier Part A Section 5.2.3 Susceptibility to chemical insecticides* and demonstrate that there is no evidence to indicate that OX513A is less susceptible to treatment than the comparator.

Beyond the susceptibility of OX513A to current insecticide controls, the likelihood of establishment if dispersed beyond Saba because of passive transport by boat or airplane would be consistent with the conclusions in the *OX513A Technical Dossier Part C Section 2.1 Persistence and invasiveness, including vertical gene transfer* which considers principally:

- OX513A does not have the potential to persist or invade in the receiving environment
- OX513A is not able to reproduce successfully with insects of a different species in the receiving environment.
- OX513A homozygous adults and OX513A hemizygous offspring do not have increased fitness that could allow it to persist or invade more than wild *Aedes aegypti*.
- OX513A does not have introduced traits likely to alter the habitat and/or geographic range of the OX513A mosquito, or hybrid populations.

Therefore, although current vector control methods on Saba do not use adulticides on planes or boats, even if an Oxitec mosquito were to leave the island it would be very unlikely to establish in other locations as it does not live for more than a few days, it is susceptible to currently used insecticides and there is a selective disadvantage conferred by the trait, i.e. the trait will cause increased mortality of the insects.

References:

WHO (2016a). Vector Surveillance and Control at Ports, Airports, and Ground Crossings. World Health Organization.

WHO (2016b). Report of the WHO Ad-hoc Advisory Group on aircraft disinsection for controlling the international spread of vector- borne diseases. World Health Organization *WHO/HSE/GCR/2016.12*.