Standardization protocol for *Aedes aegypti*-arbovirus infections.

Protocol version 1, based on infection of *Aedes aegypti* mosquitoes as drafted by partner IP.
Partner IP sent *Ae. aegypti* Paea to collaborators. **Protocols will be updated as reagents and data become available.**

Mosquito rearing.
Ae. aegypti Paea were first collected in 1994 in Paea, Tahiti, French Polynesia and since then, have been maintained in insectaries of partner IP. Eggs will be immersed in dechlorinated tap water for hatching. Larvae will be distributed in pans of 150-200 individuals and supplied with 1 yeast tablet dissolved in 1L of water every 48 hours. All immature stages to be maintained at 28°C ± 1°C. After emergence, adults are given free access to a 10% sucrose solution and maintained at 28°C ± 1°C with 70% relative humidity and a 16 : 8 light/dark cycle. A minimum of 4 generations are to be hatched by a receipient laboratory under their conditions before it is considered locally adapted.

Viruses for variability baseline work.
Three viral strains from EVAg were obtained by each partner from the Horizon 2020-funded European Virus Archive, EVAg:

- Zika virus (ZIKV) isolate Brazil/2016/INMI1 (isolated from a human case in Brazil Rio de Janeiro state Búzios on 28 January 2016): Genbank reference: KU991811, Ref-SKU: 009V-00880. Vero E6 cell produced.
- Chikungunya virus (CHIKV), strain UVE/CHIKV/2006/RE/LR2006_OPY1 (isolated from a human case on La Reunion island on 17 February 2006): Genbank reference: DQ443544, Ref-SKU: 001v-EVA83. Vero cell produced.
- Dengue 2 (DENV), strain UVE/DENV-2/2016/TH/8309 (isolated from a human case in France on 27 August 2016): Ref-SKU: 001V-02343. Vero E6 cell produced.

For the purpose of this experiment, the stocks obtained by EVAg were directly used for infection experimental infections as described below. This was to ensure that variability within mosquito colonies kept by each partner and feeding were the main range of variables affecting infection, dissemination and transmission; and not viral stock production.

***Mosquito infections and sampling*.**
Infectious blood meals to be offered to one-week-old females at a titer of 10^7 PFU/mL. Blood meals contained two parts washed rabbit erythrocytes and one part viral suspension supplemented with ATP at a final concentration of 5 mM. Engorged females are maintained in cardboard containers at 28°C with free access to 10% sucrose solution. When the number of engorged females is sufficient for further analysis, batches of 20-30 mosquitoes will be analysed at 3, 7, 14 days post-infection (dpi) to estimate three parameters describing vector competence: (i) the infection rate, corresponding to the percentage of mosquitoes with an infected body (including the midgut) among all mosquitoes analyzed (this parameter indicates whether the mosquito can be infected following infectious blood-meal; (ii) the disseminated infection rate, representing the percentage of mosquitoes allowing viral dissemination from the midgut into the mosquito hemocele for all mosquitoes with infected body (this measures the ability of the virus to cross the midgut barrier, penetrate the mosquito hemocoel and infect internal organs) ; and (iii) the transmission rate, giving the percentage of mosquitoes with infectious saliva among mosquitoes showing efficient viral dissemination.
For this, legs and wings are removed from each mosquito, followed by insertion of the proboscis into a 20 µL tip containing 5 µL FBS for 20 minutes. The saliva-containing FBS was expelled into 45 µL serum free L-15 media (Gibco), and stored at -80°C. Following salivation, mosquitoes are decapitated and head and body (thorax and abdomen) homogenized individually in 300 µL L-15 media

supplemented with 3% FBS using a homogenizer (such as Precellys, Bertin Technologies) followed by storage at -80°C. Samples are titrated by plaque assay in Vero E6 cells. Vero E6 cells indicated below are recommended by the Horizon 2020-funded Zikalliance consortium.

Virus quantification.

For head/body homogenates and saliva samples from ZIKV-infected mosquitoes, Vero E6 cell monolayers are to be inoculated with serial 10-fold dilutions of virus-containing samples and incubated for 1 hour at 37°C followed by an overlay consisting of DMEM 2X, 2% FBS, antibiotics and 1% agarose. At 7 dpi, overlay is removed and cells fixed with crystal violet (0.2% crystal violet, 10% formaldehyde, 20% ethanol) and positive/negative screening is performed for cytopathic effect (body/head homogenates) or plaques are enumerated (saliva samples ; 25 µL or 5 µL per well of a 6 well plate). Vero E6 cells (ATCC CRL- 1586) are maintained in DMEM (Gibco) supplemented with 10% FBS (Eurobio used at IP), penicillin and streptomycin, and 0.29 mg/mL l-glutamine. Titration of CHIKV and DENV is carried out by determination of focus-forming units (FFU) on C6/36 in 96 wells plates) for body and head homogenates and also saliva.

Infection readouts.

Batches of 24 mosquitoes to be analyzed at 3, 7, 14 days post-infection (dpi) to estimate:

(i) **IR**: infection rate corresponding to the percentage of mosquitoes with an infected body (including the midgut) among all mosquitoes analyzed;

(ii) **DIR**: disseminated infection rate representing the percentage of mosquitoes showing viral dissemination from the midgut into the mosquito hemocoel among all mosquitoes with infected body; it measures the ability of the virus to cross the midgut barrier, penetrate into the mosquito hemocoel and infect internal organs;

(iii) **TR**: transmission rate, giving the percentage of mosquitoes with infectious saliva among mosquitoes showing efficient viral dissemination.

Titer of the blood-meal used for infection with each virus: 10^7 PFU or PFU/mL.