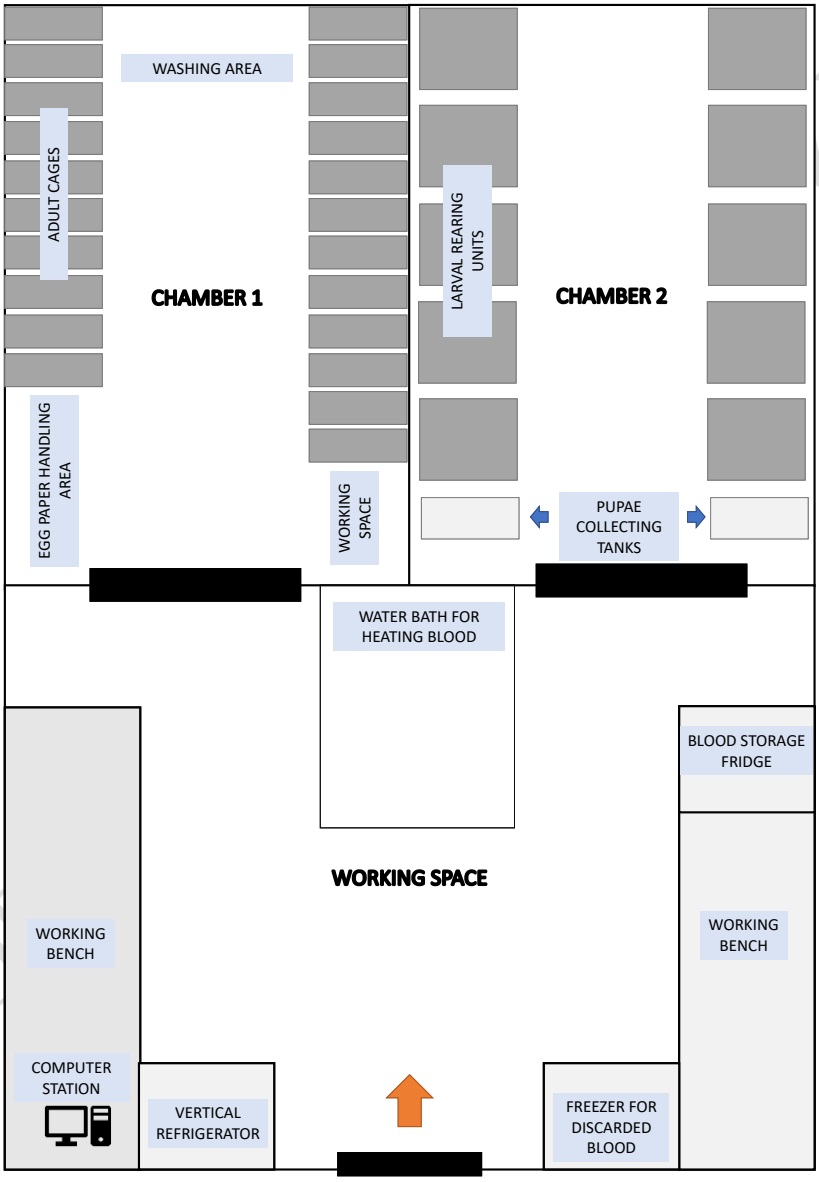
Purpose

As part of the SIT industrialization process for the control of *Aedes albopictus*, it is necessary to develop methodologies aimed at the automation and standardization of the various phases that lead to the production of sterile males.
This SOP intends to define all the procedures necessary for the production of sterile males, which can be subjected to improving changes.

1. MOSQUITO MASS PRODUCTION FACILITY PLAN



CLIMATIC CHAMBER 1: maintenance of mosquito colonies (28±2°C, 92±3% RH, 14:10 L:D).
CLIMATIC CHAMBER 2: larval rearing (30±2°C).
WORKING SPACE: activities related to the sterile males production (20°C).
Area of the SIT module: 85 sqm.

2. SIT PRODUCTION FLOW

3. MOSQUITO MASS REARING

3.1 Adult colony maintenance

Each adult cage is labeled with an ID code and has a data sheet which is filled with the data related to the cage.
The cages are loaded with about 3000 male pupae and 10000 female pupae in order to have a sex ratio M/F=1/3 to optimize egg productivity. For each rearing cycle, 4 blood meals and two egg collections are made. The first blood meal is scheduled 6-7 days after inserting the female pupae. At the end of each rearing cycle, the data collected on the description sheet will be recorded in the DB for analysis and quality control.

Blood supply and management

The blood is collected every week at Macellazione Trasformazione Carni Valpa in San Felice sul Panaro (MO) in via Grande, or at Azienda Rubizzani in Camposanto (MO) in via Prato Grasso. The swine or bovine blood (10-15 L) is collected and transported in dedicated plastic containers. The transport document must be filled and signed by the supplier following the procedure for using animal by-products.
The blood must be defibrinated immediately upon arrival using a mixer and placed in the fridge dedicated to blood storage. The residual blood must be disposed following the procedure for the use of animal by-products.

3.2 Eggs management

Once removed from the cage, egg papers are left to dry for 24 hours. The egg papers are then placed in a closed box for at least 7 days before hatching. The eggs are brushed off each paper and weighed. The brushed eggs can be stored at 25-28°C and 80-85% RH for several days before use. The hatching rate of *Ae. albopictus* eggs is progressively reduced within 6 months.
Eggs and diet are directly incorporated into each rearing tray using T 0 vegetable and transparent capsule (GALENO srl). Each capsule contains 110 mg eggs and 225 mg diet, for a total weight

of 335 mg. Since hatching, a progressively increasing quantity of larval diet is provided daily until pupation.

3.3 Larval rearing and sexing

Each rearing unit is labeled with an ID code and has a data sheet which is filled with the data related to the unit.
The trays are filled with water the day before placing first instars to allow water temperature to stabilize. Larvae are fed for 5 days with a mix of Premium Growth Substrate (INNOVAFEED) and KOI fish food (50:50).
The day after pupation onset, pupae are collected and sexed by means of manual separators (Fay-Morlan) or automatic sex sorter.
At the end of each rearing cycle, the data collected on the description sheet will be recorded in the DB for analysis and quality control.

4. IRRADIATION

Male pupae aged 2-48 hrs are irradiated inside a carbon canister of 1.8 litre capacity, using the RADGIL X-ray irradiator (20 rotations/min and delivered dose ratio of 4.5 Gy/min). A dose of 40 Gy was selected to irradiate batches of 30,000 males in 250 ml water.

5. PACKAGING and SHIPMENT

In order to keep to a minimum the size of the package for the shipment of adult mosquitoes thus reducing also the cost of shipment and standardize the conditions of transport, the following procedures are adopted.
After irradiation, the male pupae are left to emerge inside grey plastic emergence tubes (11 cm diameter, 30 cm length) with netted top and bottom, placed vertically inside a plastic tray filled with water to a depth of about 2 cm in climatic controlled condition (28±1°C, 80±5% RH, 14:10 L:D). Each tube contains around 2200 pupae and provided a vertical resting surface area of about 1.3 sqcm per adult (supplementary resting surface fixed inside each tube). To assure adult nourishment, 10% sugar solution is sprayed along the surface of each tube and cotton pads imbedded in it are placed on the top of the netted lid. The tubes are maintained for two/three days to allow complete adult emergence and then carefully removed from water to dry and stacked horizontally in a refrigerated cabinet (8±1°C) for about 15 minutes before packaging.
The cold shock anaesthetized sterile males are then transferred into white or transparent cups by carefully shaking each tube, with the help of a plastic funnel to ease the pouring of the males inside each cup, and then the males are divided into two cups in order to have around 1000 males per cup. These cups are then stacked on top of each other and inserted in groups of up to 20 cups inside a cardboard cylinder (120 mm diameter, 180 mm height, cardboard thickness 3 mm); a maximum of four cylinders could be vertically stored in each package (80,000 males per box). The polystyrene box (outside dimension 35 x 35 x 48 h cm, wall thickness 5 cm, 25 l capacity; CB-30 Dryce) is placed inside a cardboard box with the appropriate labels requested by the express courier (Figure 1).

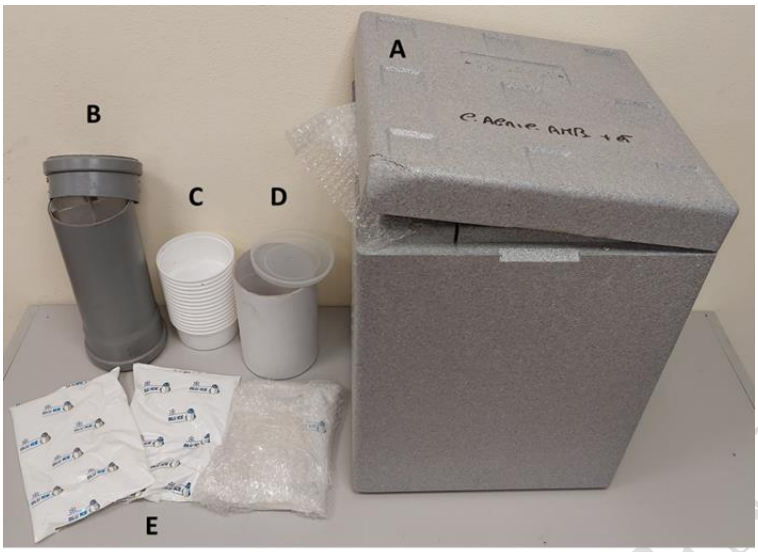


Fig. 1 – A) Polystyrene container; B) emerging tube (max. 2200 pupae); C) plastic cups for the adults; D) cardboard cylinder to hold cups; E) PCM gel packs (Blue Ice and Green Ice to maintain cooling during the transport of Aedes albopictus males.

Usually, the mosquitoes are delivered within 24 hours from CAA facility to the release areas. The temperature and humidity are recorded by means of a data logger placed inside the package (Figure 2).

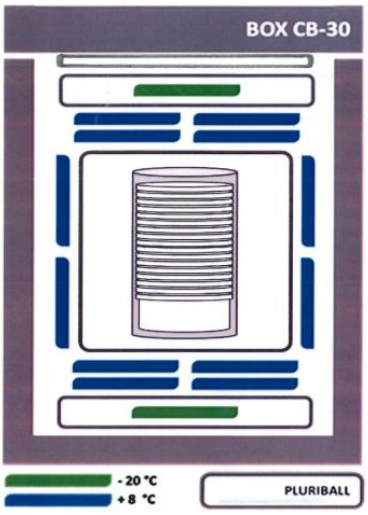


Figure 2. Scheme of the adult mosquito shipment packaging.

The label "UN3373 BIOLOGICAL SUBSTANCES CATEGORY B" is attached to the cardboard box (Figure 3).

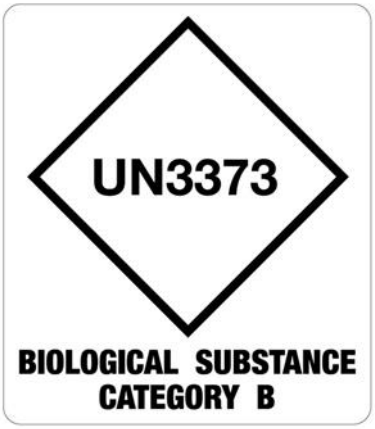


Fig. 3. Label to be attached to the package containing the sterile males to be shipped.

In the case of releases without the need for an express courier, once the emergence of mosquitoes is complete, the tubes are placed in polystyrene containers and transported in the field in the predefined release area.

6. QUALITY CONTROL

Sex ratio control
For each batch of male pupae, a sample of at least 300 pupae is taken and sexed under a stereomicroscope. The sex ratio should be ≤ 1.00%. In case of sex ratio > 1.00%, a new separation cycle is carried out.

Flight capacity
The flight ability of the males is periodically checked using both flight tubes and aspirator tubes.

7. FIELD RELEASE

For the mosquito releases, the actions to follow are:
• Preparation of boxes or tubes for shipment and transport to the release area;
• Preparation of shipping packages with boxes;
• Preparation of the release area: release on foot, by car, by bike, by drone;
• Definition and georeferencing of the release points or the release path;
• DHL management for shipments (if required).

8. LIST OF EQUIPMENT

The SIT module is provided with the following equipment:
• n. 80 mass rearing cages;
• n. 10 larval rearing units;
• n. 1 stereomicroscope;
• n. 1 automatic sex sorter;
• n. 3 manual separator (Fay-Morlan);
• n. 2 laboratory scales;
• n. 1 water bath;
• n. 1 mill to mince the larval diet;
• n. 1 mixer to defibrinate blood;
• n. 100 emergence tubes;

• n. 1 vertical fridge;
• n. 1 fridge dedicated to fresh blood storage;
• n. 1 freezer dedicated to discarded blood.

9. WORKING PERSONNEL SAFETY

In order to reduce the risks associated with the activities described in this SOP, the personnel in charge should use the personal safety protection devices (SPD) as indicated in the risk assessment document for the tasks of the laboratory technician (gloves and lab coat).

10. ETHICS STATEMENT AND SANITARY REGULTAION

Research carried out on invertebrates such as mosquitoes do not require a specific permit according to the directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.
The blood used for blood-feeding of mosquitoes is collected during routine slaughtering of pigs or cows in national authorized abattoirs at the highest possible standards strictly following EU laws and regulations.
CAA is authorized by the Emilia-Romagna Public Health Department to use animal by-products under the Regulation (EC) No. 1069/2009 that indicates the sanitary rules for the use of animal by-products and derived products not intended for human consumption.
The request for the authorization of the use of insect food as animal by-products is in progress.