Infravec-2 Horizon 2020

Guidelines for the design and operation of containment level 2 and 3 insectaries

Version 1, 2018

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1. Foreword

The containment of certain classes of arthropods (for example those which are non-native to an area, those which are **genetically modified (GM)** and those which are infected with notifiable pathogens) is an essential prerequisite for safe work and manipulation in scientific studies. This document describes guidelines for the design of insectary infection facilities to be operated at **containment levels (CLs)** 2 and 3 for work with infected and/or genetically modified mosquitoes. It could also form the basis for studies on other arthropods, for example ticks or sandflies, with some modification to reflect their special requirements.

This document has been developed with input from experts based in France, Germany, Spain, Switzerland and the UK and reflects current good practice across laboratories with experience of safely handing mosquitoes at these containment levels. It provides experience-based advice to assess and manage:

1) the risks to individuals working with pathogens,
2) the risk of escape of arthropods, including infected arthropods, into the environment, and
3) the risk of establishment of arthropod populations, particularly those capable of transmitting disease, in new regions.

2. Definition of hazard groups and containment levels

Generally, national and international bodies classify biological agents into **hazard groups (HGs)**, also sometimes called **risk groups (RGs)**, to reflect the risks they represent to laboratory workers and the environment and whether prophylaxis/treatments are available. In the EU, guidelines on the **HG/RG** classification of microorganisms are given in Article 2(d) of Council Directive 90/679/EEC[1], and containment levels are defined in Annexe V of the same directive. The **CL** required for work with **GM** organisms is similarly defined within the EU by Article 4(3) of Council Directive 2009/41/EC[2]. These definitions are typically further refined by national bodies such as the Advisory Committee for Dangerous Pathogens (ACDP) in the UK, the Comisión Nacional de Bioseguridad in Spain, Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM) in France, Zentrale Kommission für die Biologische Sicherheit (ZKBS) in Germany and Bundesamt für Gesundheit (BAG) in Switzerland. Similar classification systems may be used to represent risks to animal health, as for example defined by the UK's Specified Animal Pathogens Order[3].

Hazard Groups range from HG1 to HG4 and determine, in general terms, the CL required for safe handling and manipulation of micro-organisms. Containment Levels reflect a set of containment principles and precautions to isolate dangerous pathogens in an enclosed laboratory. They range from the lowest level (CL1) to the highest (CL4). They are also named

[1] Council Directive 90/679/EEC of 26 November 1990 on the protection of workers from risks related to exposure to biological agents at work, Official Journal L 374, p1

[2] Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms; Official Journal 125 (21st May 2009) pp75-97.

[3] The Specified Animal Pathogens Order 2008 (statutory instrument number 944); http://www.legislation.gov.uk/uksi/2008/944/pdfs/uksi_20080944_en.pdf

biosafety levels (BSL-1 to BSL-4) or pathogen or protection level (P1 to P4) depending on country, and this needs to be verified locally.

The containment level and safety procedures required for safe laboratory work with biological agents or genetically modified organisms generally reflect not only the intrinsic risk of the agent (as indicated by its HG rating) but also the nature of the work, and additional safety procedures are required to work safely with infected arthropods. The consequences of genetic modification of biological agents, or of arthropods, also need to be taken into account when assessing the safety of planned procedures, as well as any relevant legislation.

HG, CL and GM regulations need to be carefully evaluated when designing insectaries and planning work within. The provision of a safe working environment and the assessment and management of risk resulting from work is typically a legal requirement for employers. The guidelines provided here meant to support decision-makers in the process of ensuring that their workplace offers adequate protection to workers, the general public and the wider environment when working with biological agents within arthropods (specifically mosquitoes).

A general overview of the four different HGs and CLs is provided in Table 1 below. In practice, most GM work and most laboratory work involving infected mosquitoes will occur at CL2 or CL3, but some tick-borne arboviruses are classified as HG4. Generally speaking CL2 is for medium risk biological agents and genetically modified agents and mosquitoes, and CL3 for high risk biological agents and genetically modified agents and mosquitoes. Risk assessments must take into account the biology and ecology of arthropod species. For example, work with autochthonous species that could harbor/transmit a given pathogen; exotic or novel species may require studies to be carried out at higher biosafety level.

Table 1: Summary of Hazard Groups (HG) and Containment Levels (CL) for the purpose of this document.

HG	Risk to individual	Risk to community	Minimum CL	Laboratory practice	Safety equipment
HG1	None or very low	None or very low	CL1	Standard microbiological procedures	- Bench work - Personal protective equipment (PPE): lab coats and gloves, eye and face protection if needed
HG 2	Moderate	Low	CL2	CL1 plus: - Limited access - Biohazard signs - Sharps bins Precautions - Hand washing sink near to exit - Risk assess waste and/or medical surveillance	CL1 plus: - Microbiological Safety Cabinets (MSC) when potential aerosols/splashes - Autoclave available in the building
HG 3	High	Low	CL3	CL2 plus: - Controlled access - Decontamination of waste - Clothing change before entry and protective clothing	CL2 plus: - MSC/HEPA-filtered isolators for all work - Alternatively, respiratory protection as needed (full face masks or masks/protective glasses). - Physical separation of the laboratory - Double-door access and selfclosing, airlock entry - Negative air flow - No recirculation of air - Shower facility (or air shower). - Autoclave available in the laboratory - HEPA Filtration at all exhaust air. - Pressure monitoring - Safety systems for lone working; monitoring/CCTV for laboratory spaces.

HG 4	Very high	Very high	CL4	CL3 plus: - Identifiable clothing change before entry - Chemical shower on exit - All material decontaminated before exit	CL3 plus: - Class III MSC or class I/II with air-supplied positive pressure suits, - Double ended autoclave (through wall), effluent system - HEPA Filtered air at the entry and the exhaust. - Separate or isolated zone - Monitoring/CCTV and safety systems for all aspects of work; safe storage of pathogens and biological material.

The containment requirements for specific biological agents will depend on the HG classification in the country of use, which may vary. Similarly, the requirements for arthropod containment and the classification of work involving infected arthropods may vary by country. As such, any locally applicable rules such as licensing, validation procedures, risk assessments, and specific structural requirements for buildings where work is to be conducted in containment take precedence over the recommendations in this document.

The following areas need to be considered in assessing the overall risk of an activity involving infected mosquitoes or other arthropods:

1) The risk of mosquito escapes to the environment leading to the establishment and indefinite persistence of a population of genetically modified and/or non-native mosquitoes. This should include not only the likelihood of escape but also the feasibility of establishment given the local environmental conditions and their impact on the potential for the survival and reproduction of the species in question.
2) The risk posed by the mosquito to laboratory workers: for instance, the potential for transmission of biological agents inadvertently present in mosquitoes collected at field sites where other pathogens are circulating.
3) The public health, animal health or environmental risk posed by the biological agents infecting the mosquitoes to laboratory workers and the environment.
4) The risk to materials and resources posed by the pathogen to arthropod colonies (for example contamination of non-infected cultures or affecting fitness of the arthropod colonies).
5) The risk of pathogen transmission between individuals, also specifically from a facility worker to community.
6) The availability of treatments and preventive measures.

When working with mosquitoes and pathogens that are genetically modified, the CL required may need to be increased depending on the planned activities and on the nature of the transgenic modifications.

3. Organisms and pathogens used by contributors

This guidance document is informed by the experience of the contributors, and as such particularly reflects requirements for work with *Aedes*, *Culex* and *Anopheles* mosquitoes, which can be endemic, imported or exotic species. Moreover, these species can be genetically modified and/or be transinfected with endosymbionts, *e.g. Wolbachia*. Among Infravec-2 participants, pathogens studied are mainly parasites such as *Plasmodium*, *Leishmania*, and

arboviruses (or in some cases insect-specific viruses) of the *Bunyavirales* order or *Togaviridae, Reoviridae* and *Flaviviridae* families.

4. Insectary layout and equipment

In the context of design proposals, we will focus on CL2 and CL3 insectaries. Specifics are as described below.

4.1 The CL2 insectary

4.1.1 General considerations

1) Adequate information, instructions, training and supervision of all workers and visitors.
2) Access restricted to users (PIN keypad or physical key).
3) Access to autoclave in building, and incinerator or tissue digester if required (for example animal carcasses), plus validated inactivation and waste disposal procedures.
4) Protocols for disinfection in place; waste streams according to national regulations.
5) Vector control measures such as traps to prevent intrusion/escape of arthropods are recommended throughout insectary rooms (light traps, attractor tape, fly catcher [electrical or mechanical]).
6) Display (in the laboratory) of key **standard operating procedures (SOPs)** and emergency procedures.
7) Equipment for the safe storage of biological material (e.g. locking freezers).
8) If genetically modified organisms are to be used, other specific regulations such as Biohazard signs on access doors/rooms may be required.
9) Record keeping for risk assessments, standard operating procedures and other relevant records.
10) Monitoring of activities to ensure implementation and effectivity of risk assessments, controls and standard operating procedures.

4.1.2 Layout

Generally, any potential exit routes from the insectary need to be controlled; for example, through curtains to stop escapes from reaching outside/anterooms or mesh for drainage/air vent systems. Barriers such as mesh need to be an appropriate size to contain the relevant arthropod: adult *Culicoides* biting midges, for example, may escape through mesh hole sizes that are effective for containing adult mosquitoes. The same principle applies when considering mesh sizes needed for blocking drains to prevent the accidental release of eggs.

A. Access and insectary rooms

Insectaries should generally consist of an antechamber or preparation room for activities such as material preparation and storage which also should contain a sink for hand washing and water supply, as well as rearing rooms for housing and working with arthropods (see Figure 1). A freezer for killing adult mosquitoes within their primary containers may also be useful. In case of work with GM and/or infected mosquitoes, a lobby with double doors before the antechamber may add an extra layer of containment. Cooling the lobby (to 4°C) may be effective at limiting the movement of certain species, particularly tropical species, but for most CL2 applications these will present too high a cost.

Two design approaches are recommended to rear and work with arthropods: a temperature/humidity-controlled room or environmental chambers.

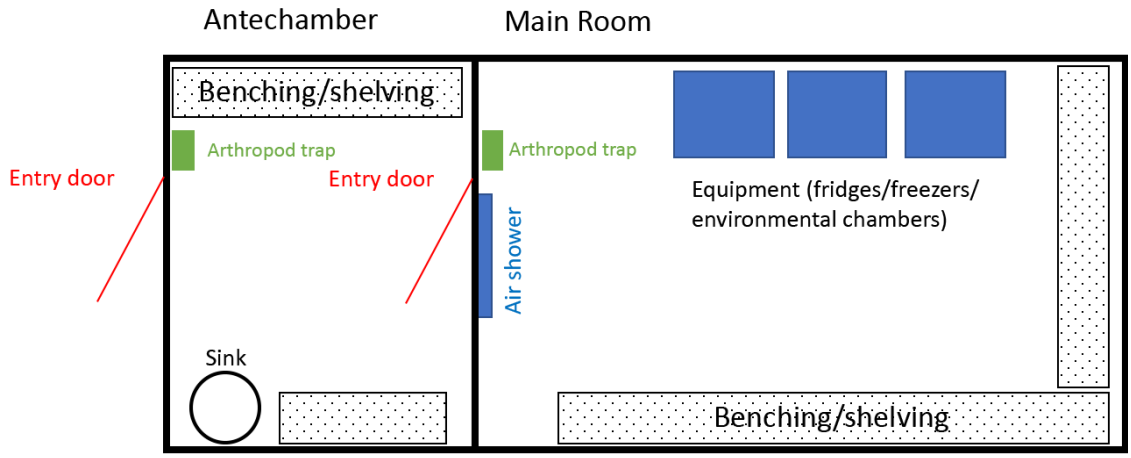


Figure 1: Typical layout for a CL2 insectary. The main room is either humidity or temperature controlled (in this case, electric equipment must be placed outside the humidity room, in the antechamber or in a room close by) or equipped with environmental chambers. Air showers/curtains can be useful upon exit to avoid escapes. Benching and shelving can be stacked up to gain storage space.

Option 1: the temperature/humidity-controlled room

This approach, sometimes called controlled-temperature rooms (CT rooms), is preferable if large numbers of mosquitoes are handled and uses space more efficiently (see Figure 2) but there is a high risk of fungal or bacterial contamination if the room is operated at high humidity. This risk may be reduced by using appropriate benching material, wall coatings or materials and specialist paint, although such choices will be associated with a cost.

Surfaces such as benches need to be impervious to water and resistant to alcohol or solvents/cleaning products to allow thorough decontamination. Care should be taken that mosquito survival is not impeded by the chemicals used, for example through chemical traces left in breeding pans or released into the room. This solution requires dedicated engineering oversight and maintenance. Lighting is crucial and windows, if any, should be covered to ensure a controlled environment; in some cases, light timers are useful to provide regular day/night cycles. It is critical that any equipment in such an environment which is sensitive to heat or humidity is adequately protected; if this is not feasible it should be stored separately when not in use.

Figure 2: Stacked shelves housing breeding pans, cages and storage space.

Option 2: environmental chambers

Environmental chambers (Figure 3) provide additional containment and generally allow environmental conditions (temperature, humidity, light) to be controlled more reliably and cheaply than an entire controlled-temperature room. However, it represents a relatively inefficient use of space and limits the volume of work that can be done at one time. It also requires regular maintenance by users. This normally includes cleaning, and for controlled-humidty environmental chambers may also include topping up with sterile water and removing waste water, although for an additional cost chambers can be directly connected to a water supply and draining system to decrease the maintenance. A secondary internal glass door makes it easier to identity any breaches of primary containment without breaching secondary containment.

Climatic chambers

Figure 3: Example of an environmental/climatic chamber with humidity, temperature and light control. As depicted on the left panel, the chamber is connected to a water container for water supply and to a waste container to collect the waste water.

B. Manipulation room

A separate room is normally used for manipulation, dissection and injection of mosquitoes, as well as for any other procedures requiring stereomicroscopes and other non-portable equipment (such as freezers killing arthropods, refrigerators or CO_2 supplies for anaesthesia, and freezers for storage of material). An example of such a room is shown in Figure 4. If a humidity/temperature-controlled room is used, electric material should be placed in a separate room to protect equipment.

Dissection room

Figure 4: Room with stereomicroscopes and material for mosquito manipulation, such as dissection and injection

4.1.3 Containment and manipulation of non-infected mosquitoes or other arthropods

The container which directly contains the arthropods is defined as the primary containment. For mosquito larvae, this is normally a rearing pan with a mesh or rigid transparent cover. If covered by a net or transparent cover, adult mosquitoes can be hoovered up after an emergency and transferred into a cage. Mosquito pupae should be picked up and transferred into a small cup further placed inside a cage for emergence of adults. Containers such as mesh

cages can be used for large numbers of adult mosquitoes or smaller containers with mesh top for example used for blood feeding (such as those shown in Figure 5) for smaller numbers of adult mosquitoes. Relevant information should be written on primary containers (species and other details for example origin, treatment etc.).

Breeding pans Cage design Meshed pot

Figure 5: Breeding pans with larvae; typical cage design allowing access and with information on mosquitoes; meshed pot (here with feeder on top) containing a smaller number of mosquitoes.

For adult arthropod manipulation, insects can be anesthetised in their primary container in a fridge for 10-30 minutes and then transferred on a Petri dish placed on an ice bucket or freezer for 5 minutes at -20°C. Alternatively, they can be hoovered from a cage with a small electric hand-held vacuum with a mesh cover on the inlet and transferred into a plastic container resting in water ice to anesthetise them. Note that a mouth pooter should not be used (trigger user allergy to mosquito scales in the long term, infectious agents may pose risks) though if necessary, for very fragile arthropods this needs to be risk assessed. Chill tables or CO_2 anesthesia tables are a good alternative for working with large numbers of mosquitoes. Note that CO_2 is the only effective option to anesthetise mosquito species originating from cold and temperate regions, as these will be tolerant of low temperatures and may remain active. Incubation times and methods described above have to be assessed and adapted for individual mosquito species and will depend on the facilities available.

4.1.4 Containment and manipulation of HG2-infected arthropods or for quarantine measures

Pathogen-specific risks should be considered, including the volume of pathogen and the potential for transmission (via aerosol, etc.) involved in a procedure. This requires careful consideration of relevant biosafety and biosafety legislation for handling of pathogens; for example, whether feeding on the bench is acceptable or whether additional measures are necessary.

Some arthropods such as infected mosquitoes should be ideally kept inside a climatic chamber as it provides a good secondary containment. Ideally, infected mosquitoes or mosquitoes in quarantine should not be reared in the same room than non-infected mosquitoes. It is important to establish that procedures used for infected mosquitoes are effective against escapes.

Gloveboxes (Figure 6) are generally a good preventive measure to contain mosquitoes or other flying arthropods, and control escapes during procedures that require manipulation of these outside of primary containment when mosquitoes are infected or presumed infected.

For manipulations such as dissection, where there is a higher risk of worker exposure to infected material, respirator masks and safety glasses should be considered. However, for small arthropods these may not permit necessary adequate handling.

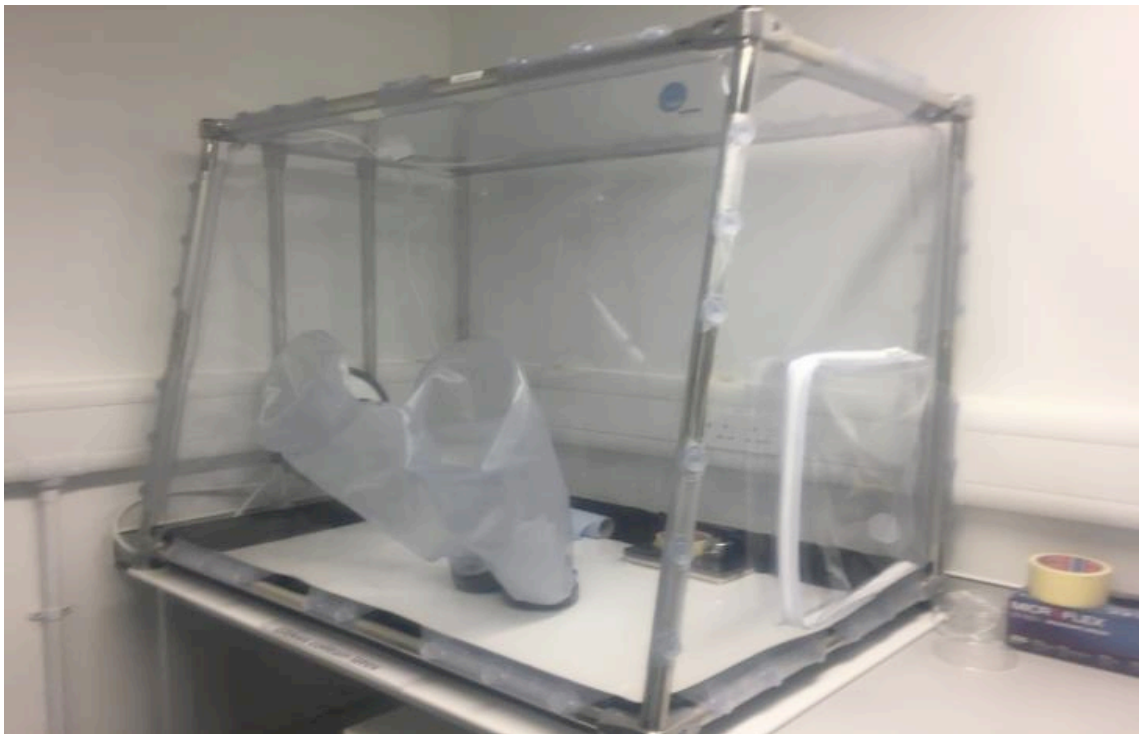


Figure 6: Simple glove box design, as used to manipulate infected mosquitoes

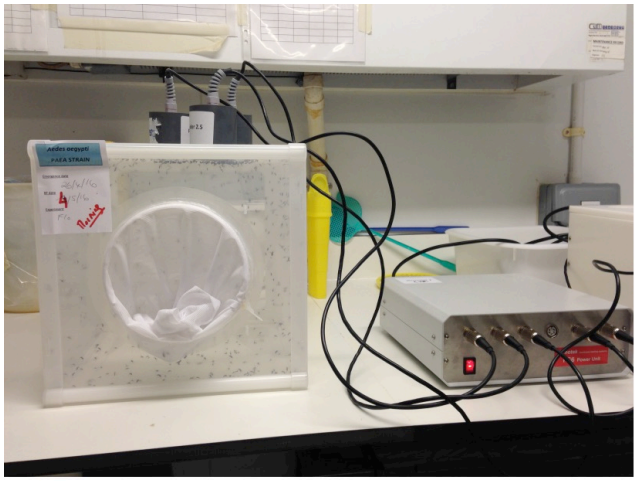
Breeding may be carried out as a quarantine measure for mosquito strains and species in which case breeding pans need to be housed in CL2 containment; glove boxes as described above or larger meshed containment to house pans can provide a layer of security when for example mosquitoes develop to flying stages and manipulation (*e.g.* opening cages to place egg laying pot) is required.

4.1.5 Feeding devices

Blood feeding is frequently carried out on bench spaces, especially when the blood meal does not present a credible risk of infection via the most likely routes such as aerosol transmission. Laboratories use bloodfeeding devices such as Figure 7 (left panel) or similar to feed arthropods which facilitate preparation/assembly of the blood meal with temperature control.

Artificial feeding membranes or parafilm can be problematic, though animal skin (eg pork intestine, chick skin) or membranes can be used. This may be either purchased commercially or produced by the research site. The effectiveness of each feeding method needs to be assessed for each species as it may be affected by local conditions.

For routine daily feeding of male and female mosquitoes, cotton imbibed with for example 10% sugar solution can be placed inside cages or on top of smaller containers (Figure 7, right panel).



Bloodfeeding device on cage

Sugar solution in cage

Figure 7: Feeding devices for blood feeding (left panel) including infectious blood meal or sucrose feeding during colony maintenance (right panel), here with sugar solution in a bottle placed inside the cage. Sugar pads placed on top of smaller primary containment is also an option

Other feeding methods may be more appropriate for other arthropods. For example, for *Culicoides* midges one method is to aspirate live *Culicoides* with a mouth aspirator and transfer them inside a plastic "feeding chamber" (Figure 8, left panel) which has a netted lid and a membrane (as described above) on the bottom side. This primary container will be inserted in a secondary container, the "infectious blood reservoir chamber" (Figure 8, middle panel) containing infectious blood and a magnet to allow constant mixing of pathogen and blood. These two containers are sat on a large petri dish containing water pre-warmed water to 25-30°C. All these three containers are allocated on a heating magnetic stirrer pre-warmed to 25-30°C. Temperature is checked by a probe inserted in the water bath during the whole feeding (Figure 8 middle panel).

Figure 8: Enclosed feeding device system for Culicoides; primary container "feeding chamber" (left panel) where live Culicoides will be kept during blood meal; secondary container, "infectious blood reservoir chamber" (middle panel) in a water bath and magnetic stirrer with temperature probe, and a transport box (right panel) for movement of the containers (feeding chamber and blood meal container) between rooms

4.1.6 Insectary waste

Waste disposal is generally regulated by national and/or local biosafety regulations. It is recommended that all solid waste from mosquitoes should be discarded into autoclave bags, left for one night in a freezer (to kill all mosquitoes and avoid potential escapes before autoclaving), autoclaved and discarded. This includes all material including gloves, mosquito debris, egg laying papers, wet cottons, cottons rolls, larval food waste, plastic pipettes, feeding

membranes, hand paper towels with blood or mosquito debris, single usage cardboard boxes, plastic petri dishes, tubes, plastic cups, and so on.

Water from larval rearing should be filtered (for example through 50 µm Nitex cloth mounted on a hoop fishing net) to remove any embryos/larvae/adult escapees before it enters the normal waste water system. The larval containers can also be scalded with or immersed in boiling water to kill any eggs/larvae, or materials can be frozen at -20°C overnight. Filter mesh sizes, freezing times and the effectiveness of boiling water depend on the species involved and trial runs may be necessary, or data from other laboratories may have to be obtained.

For infectious solid and liquid waste, additional controls such as inactivation by spraying or immersion with disinfectants agents such as Virkon appropriately validated against the target biological agent might be considered before freezing/autoclaving and disposal, though generally double-bagging of waste followed by autoclaving is sufficient. Freezing for twelve hours alone is sufficient to inactivate some arthropod waste such as that of sand flies, though this need verified for species used.

4.1.7 Personal safety measures

As PPE, regular laboratory protective clothing such as lab coat and gloves are required. Safety spectacles, puncture resistant gloves (and masks in some settings) may be recommended specifically for pathogen work as determined by risk assessment. Risk assessments for pregnant and/or breastfeeding women should be carried out.

4.1.8 Emergency measures

A key concern in the insectary is the escape of arthropods (especially if infected). Individual escapes or low numbers can be dealt with easily by direct killing (handheld zappers, or fly catchers are useful). Larger numbers are more difficult to deal with and may necessitate the use of insecticide (or even formaldehyde-based fumigation or Vapour Hydrogen Peroxide (VHP) decontamination by fumigation) but this would kill all arthropods in the insectary and thus negatively affect operations for long periods. As mosquitoes require daily sugar feeding for hydration, larger escapes may be more effectively dealt with by sealing the affected room or environmental chamber and waiting for mosquitoes to die (typically within 1-2 weeks post escape) before re-entering and cleaning the insectary, but this depends on environmental conditions such as humidity and needs to be assessed for each species. To have confidence in the effectiveness of such methods they must be validated using trial runs before any infectious work begins.

Spills with pathogen-containing material need to be considered and dealt with according to local/national safety practices, for example decontamination with inactivating agents and guidelines vary and may also have to be validated in some instances.

4.2 The CL3 insectary

The CL3 insectary operates a higher safety level to CL2 and this requires specific, additional safety measures that inform layout and operation of such structures. These will be explained below.

4.2.1 General considerations:

1) Adequate information, instructions, training and supervision of all workers and visitors. In case of external visitors, direct and continuous supervision should be implemented.
2) User access restrictions required (pin and/or swipe card and/or fingerprint electronic readers among others) and dedicated protective clothing/PPE.
3) The laboratory must be protected with an intruder alarm in some cases according to the pathogens used and the national regulations.

4) Sealed laboratory (secondary containment or barrier) to prevent any accidental release of pathogen and HEPA filtration of air outflow.
5) Negative pressure environment with a gradient (for example from -20 to -80 Pa depending on number of rooms) towards the insectary and cell culture rooms to be in place maintaining as well as a flow of air into the room. However, this can be detrimental to survival of some arthropods such as sand flies.
6) Access to autoclave within the CL3 suite or double-ended; and if required (for example animal carcasses), access to incinerator or tissue digester; validated inactivation and waste disposal procedures must be in place.
7) Protocols for disinfection in place; waste streams according to national regulations.
8) Vector control measures such as traps to prevent intrusion/escape of arthropods are recommended throughout insectary rooms. These can include light traps, attractor tape, fly catcher [electric or mechanic], mesh (according to arthropod size; on vents, for example); if sinks/pipes are present liquid traps/barriers should be considered.
9) Display or acces to (in the laboratory) key standard operating procedures [SOPs] and emergency contacts and procedures.
10) Safe storage of biological material must be ensured. Pathogens falling under HG3 regulations can only be used within such a facility and must be securely stored (by key or password) in dedicated spaces.
11) Biohazard signs on access doors may be required.
12) Record keeping for training, risk assessments, standard operating procedures and other relevant records.
13) Monitoring of activities to ensure implementation and effectivity of risk assessments, controls and standard operating procedures. Personnel should ensure enough provisions in their laboratory (disinfectant, PPE, etc.) and all streams for bringing in and taking out material should be described, and risk assessed.
14) Vision panels and/or web cams/CCTV to allow supervision of workerswithin the CL3 laboratory.
15) Transport measures (containers etc.) for movement of samples between rooms and in/out of the facility. The exit of inactivated samples from CL3 to CL2 should be done by pass-through or hatches with double door and integrated and validated disinfection/inactivation system.

4.2.2 Layout:

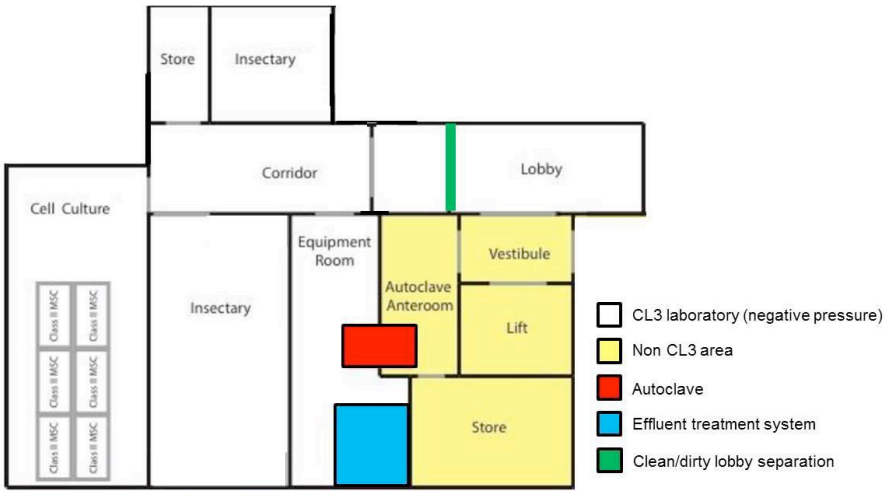


Figure 9: Typical layout of a CL3 insectary. Access to the CL3 area is via a lobby divided (by a door or simply by a mark on the floor) into a clean (entrance side) and a dirty (CL3 corridor side) area. After the lobby, a corridor can give access to insectary room(s) and a cell culture room which can be used to prepare and store viruses. Mechanisms to avoid simultaneous opening of doors should be put on place. Store and equipment rooms are optional.

A. Access

The CL3 facility must be separated from any other activities in the same building. Normally a CL3 suite will be within other laboratory space (ideally within a CL2 laboratory) or in an area segregated from other areas. It is generally suggested that a CL3 insectary possesses a lobby to put on/off personal protection and which also provides another level of segregation as shown in Figure 8. Access needs to be controlled; depending on design access to the insectary may require access permission. The lobby should be separated (by a door or simply a mark on the floor) in two areas: clean (entrance side) and CL3 area (CL3 corridor side). The clean area can be used for personal belongings and clean PPE but also contains a handwashing sink. The CL3 area is used to store PPE (*e.g.* protection glasses, suit) in use and should contain an autoclave bin to discard any used PPE. Socks, shoe covers or CL3 specific shoes should be worn when entering the Cl3 area and, removed before leaving to the clean area.

When entering and leaving a CL3 insectary, a logbook must be completed with the worker's name, the pathogen(s) being used, the work to be performed, the time of entry and exit and the entrance door pressure gauge reading which must be a negative value before entry or system indicating pressure gradient is adequate (red/green light warning systems etc.). Working out of hours might be subjected to certain rules such as presence of a "buddy" in the building. In case of lone work, a lone alert device, remotely connected with safety personnel/control rooms and capable of summoning assistance in the case of an accident (with connectivity throughout the suite) should be kept attached to the worker for the entire duration of work.

To prevent any aerosolised virus exiting the CL3 insectary, an interlocking system should be used that prevents simultaneous opening of the outer (lobby) and inner (corridor) doors. Another way to reduce risk for some situations and mosquitoes (for example tropical species) is to cool the lobby to 4°C to limit the risk of escapes.

B. Insectary rooms

After the lobby, the main corridor gives entry to the insectary rooms (with an air shower a useful addition, to be activated upon exiting to remove any arthropods hiding on clothing). We recommend a layout with climatic chambers (see Figure 3) as in addition to allowing variable conditions (temperature, humidity, light) these are easier to maintain, can be replaced easily and provide additional containment. However, these require regular maintenance by users such as cleaning, filling and collecting waste water. A waste draining system is a critical issue in this setting as it necessitates decontamination before removal from the CL3 insectary, either by autoclaving or by a specialised effluent treatment system. A fridge may be present in each insectary room to allow anesthesia of mosquitoes in their primary containment before sampling/manipulating them (see below) but users may have other requirments for these procedures and this needs individually assessed. Depending on space, the room can accommodate climatic chambers and bench space for manipulation of mosquitoes such as blood feeding and dissection (in an HEPA-filtered isolator or not, see below). If more than one room is available, activities can be split either by type of experiment (blood feeding versus dissection) or by pathogen used (aerosol transmitted versus non-aerosol transmitted; for the former HEPA filtration should be put in place).

C. Other rooms and equipment:

Besides insectary rooms, a cell culture room with incubators and class II MSCs is useful to grow and manipulate/prepare pathogens *(i.e.* parasites, viruses). A class II microbiological safety cabinets (MSC) will be required to prepare infectious blood to infect mosquitoes. An equipment room can be used to place freezers (-80°C to store viruses for example) but also autoclave (within the equipment room or doubleended with exit in an anteroom located out of the CL3 laboratory) as well as an effluent treatment system, if required. All equipment such as freezers, centrifuges etc. should be planned into layout during the early design stages, for example power consumption and heat generation may have to be taken into account. Generally, material and consumables should be stored outside the CL3 insectary to avoid overcrowding and easy detection of any potential escapees. Given running costs and size considerations, room usage should be maximised. A store room inside the CL3 laboratory can be used to stock in use consumables. Also, to facilitate disinfection of the whole suite, less material and consumables as possible should be stored inside the CL3 laboratory. A storage space outside and close to the CL3 laboratory is recommended for long term storage and access to required consumables.

4.2.3 Containment and manipulation of infected mosquitoes:

A. Containment of infected mosquitoes

Generally, uninfected mosquitoes are reared outside the CL3 insectary and a limited number of adult mosquitoes is brought in, in secured primary containment, before the infectious blood meal. For the primary containment of mosquitoes, we recommend not using cages but secured pots (strong enough to withstand for example drops) which can be easily transferred, for example to a fridge for anaesthesia of mosquitoes (before sampling them). Importantly, the number of arthropods per container should be relatively small and fixed (*e.g.* 20 to 50 per box) and recounted to confirm that no escapes have occurred before and after each manipulation step. Mosquito boxes should be marked with information such as the number of mosquitoes, the species of mosquito, the responsible person, any pathogens present, and the date that the experiment began. Environmental chambers provide secondary containment and should have a glass door allowing vison of any escape before opening the chamber. If a humidity/temperature-controlled room is used, mosquito boxes can be placed into meshed cages providing secondary arthropod containment.

Breeding of infected mosquitoes to the next generation might be required for certain types of experiments, for example studies of vertical transmission. In this case, larvae can be reared in small containers tightly covered by secured mesh and small pots placed in larger boxes covered as well by a secured net. For transport, it might be safer to close the larger box with a lid to avoid the risk of spillage of the water in the rearing container. Pupae should then be transferred into a small cup or flask inside a primary adult containment for emergence.

B. Manipulation of infected mosquitoes

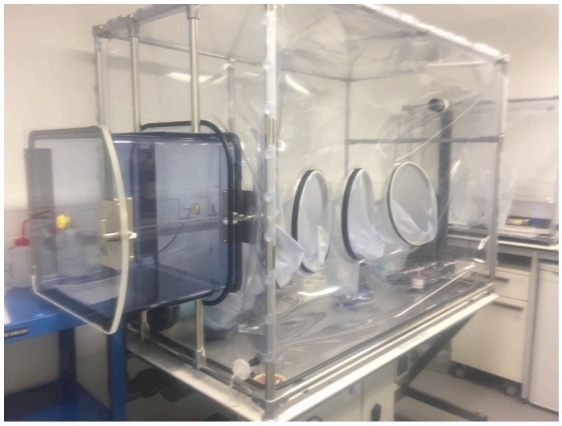
Portable vacuums should not be used for collecting infected adults due to the risk of escape. Instead, mosquito containers may be be chilled, as described previously (page 9), and then transferred into a HEPA-filtered isolator/glovevebox (see below) and opened (for example over a chilled Petri dish place on an ice bucket) and mosquitoes transferred onto the Petri dish until further manipulation. For experiments involving infectious blood meals and mosquito sampling or manipulation, HEPA filtered-isolators/gloveboxes working under negative pressure can be useful; these can also be designed to include stereomicroscopes for dissection of arthropods (Figure 10). These designs provide a good containment (for mosquitoes and for the pathogen) but render fine manipulations (dissections, injections) more difficult to handle and slow the work which can be problematic when large number of mosquitoes is required for an experiment. Importantly, ease of mosquito manipulation during manipulations such as dissection with sharps may improve safety of experiments and this should be taken into account when choosing the design. Gloveboxes without air filtration are also used for such purposes but in that case PPE measures need to be risk-assessed; a “glove bag” type device that can be suspended in a class II MSC may also be considered.

Other options can include MSCs -class II or III- though again air flow may prevent efficient feeding and increase escape risk due to potential sweep away of mosquitoes. Also, this would prevent the use of a stereomicroscope though a camera and side screen can be set up, but this set up is not ideal for dissections.

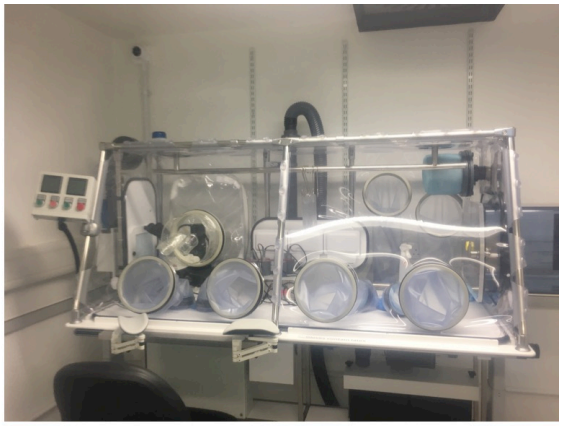
In case HEPA-filtered isolators cannot be used and to facilitate experiments and manipulation of arthropods, respirator masks such as filtering facepieces (FFP) 3 masks (shown in Figure 11) or the use of Purifying Air Powered Respirators (PAPR) should be assessed. Nevertheless, all steps involving opening mosquito containers should ideally be performed into a glovebox to limit escape of infected mosquitoes into the CL3 insectary.

Individual risk assessments need to be carried out for each setting and planned work. Pathogen-associated risks need to be considered individually, with pathogen transmissibility (aerosol transmission etc.) a relevant factor.

Again, local/in-house biosafety and biosafety rules for handling of pathogens may influence whether feeding and manipulating mosquitoes on the bench/glovebox with a mask is acceptable or whether more complex structures are necessary.



HEPA filter-glove box design for CL3



HEPA-filter glove box incorporating microscope

Figure 10: HEPA-filtered isolator design working under negative pressure for manipulation of infectious material such as HG3 pathogens (left panel), with hatch for entering material while work is ongoing; similar isolator with incorporated microscope stand and adjustable height for procedures such as dissection (right panel).

Figure 11: Filtering face piece (FFP) 3 masks that can be used in CL3.

4.2.4 Feeding devices

As described above, blood feeding can be carried out using devices such as Hemotek (Figure 7, left panel) which allow contained and temperature-controlled delivery of an infectious blood meal. These can be placed into glove boxes or isolators (or a MSC, if disturbances by airflow are not considered an issue) and integration of power supply and space is an important factor to consider in design; for routine, daily feeding of male and female mosquitoes, cotton imbibed with for example 10% sugar solution can be placed on top of primary containers in environmental chambers. Importantly, cotton should be considered as infected if using viruses which can be transmitted through mosquito saliva.

Bloodmeal-engorged arthropods are collected and transferred inside a new primary container (normally a cardboard box to avoid increased humidity and steam during further incubation); this primary container is then transferred inside a secondary container (see Figure 12). Only at this stage will the two containers be removed from the glovebox and transferred into an incubator which will serve as third layer of containment. Feeding solution such as 10% sugar solution can be provided directly onto the primary container net to avoid the opening of the box. If concerned about humidity and ventilation, the lid of the secondary container can be opened but this results in loss of the secondary containment function.

Figure 12: Example of primary container (left) and secondary container (right) with primary containers inside.

Alternatively, the primary container can be placed into a meshed cage which ensure humidity and ventilation inside the environmental chamber (see Figure 13).

Figure 13: Containers with mosquitoes (sealed with mesh) in meshed cage inside environmental chamber.

4.2.5 Insectary waste

Waste disposal in CL3 facilities is generally regulated by national and local/or local biosafety regulations. It is recommended that all solid waste from mosquitoes or otherwise infectious should be sprayed with or immersed in a validated inactivating agent (such as Virkon), autoclaved and/or incinerated. If waste is not disposed of straight into the autoclave and to avoid any potential escape of intact insects such as mosquitoes, solid waste can be stored in a CL3 insectary freezer for at least one night before being transferred to the autoclave bins. Non-infectious material will be routinely autoclaved, or heat treated with validated methods before exiting the contained structure.

All liquid waste must be decontaminated chemically using an inactivating agent, which must be validated, or by autoclaving (small volumes) or by chemical or thermal effluent treatment via an effluent treatment plant (higher volumes).
Reusable material which cannot be autoclaved can be immerged overnight or sprayed copiously with an inactivating agent before being removed from the CL3, preferably by airlock or pass-through systems (which must avoid exposure of surrounding areas to air from inside the CL3 facility)

4.2.6 Movement of samples from CL3 to CL2 laboratories

Samples can be transferred from CL3 to CL2 facilities for further analysis, for example specific analyses (e.g. RT-PCR, bioimaging microscope, Western blot etc.) that are not available in the CL3 insectary. Prior to movement of material, disinfection and inactivation of samples are required. For this, the outer surfaces of containment/containers must be wiped with disinfectant or dipped in disinfectant according to local (or in house) biosafety regulations. Infectious samples can be inactivated by their storage into lysing buffer, boiling, extraction, fixation with formaldehyde etc but this needs individually risk assessed. Proper pass-through

(such as liquid disinfectant-containing) or airlock systems may be useful, but care must be taken that these do not allow air from inside CL3 to exit into surrounding rooms/areas.

4.2.7 Personal safety measures

Laboratory protective clothing including full body suits (head covered), safety spectacles, overshoes and two pairs of gloves are required. Puncture resistant gloves, and masks in some settings, may be recommended specifically for pathogen work and as determined by risk assessment and planned procedure.

Working at CL3 presents a potentially serious risk to all personnel, therefore to minimise hazards everyone must maintain a high level of good microbiological practice to contain and minimise any aerosol formation in case of virus manipulation.

All glass should be kept to a minimum and where plastic alternatives are available, they should be used.

Sharps (forceps, scissors, syringes and capillaries, glass slides and coverslips, dissection cups) should be enclosed in their dedicated sealable container in the CL3 Insectaries and labelled as sharps. When in use, the sharps are placed such as that they will not come into contact with the users' hands. When manipulating forceps/sharps, hands are never crossed to avoid injuries. Puncture resistant gloves should be worn when handling sharps, such as the capillary needles used for microinjection systems (such as Nanoject).

As previously, pregnant or breast-feeding personnel need to have risk assessments carried out before any work in the CL3 insectaries.

4.2.8. Disinfection of the CL3 insectaries

For emergency disinfections following a spillage, escape of a large number of mosquitoes, routine disinfection to allow servicing (once a year is generally enough), change of micro-organisms, etc, and commission/decommissioning the laboratory, the CL3 should be fumigated (either with formaldehyde, vaporised hydrogen peroxide [VHP] or chlorine dioxide). Sealability of the CL3 is therefore also required to avoid any leakage of these gases, especially for formaldehyde. Importantly, the surfaces need to be thoroughly cleaned after fumigation, (usually not required for VHP although it is a good practice,) to avoid any negative influence on mosquitoes taken to CL3 afterwards. It is important that pipes/vents etc. are considered when sealability is assessed.

4.2.9 Emergency measures

Key concern in the CL3 insectary is escapes of arthropods (especially if infected) and exposure to pathogens of workers and community. Individual escapes or low numbers can be dealt with easily by direct killing (handheld zappers, or fly catchers are useful) however this must be followed by decontamination procedures if arthropods were potentially infectious. This can be classed as exposure if escapes take place in the insectary room rather than contained environment such as an isolator. If the escapes cannot be crushed or if larger numbers of insects escape, it may necessitate fumigation of the facility, or wait for the arthropods to die (ideally lock room; shifting temperature to higher or lower temperatures may be considered) before re-entering the CL3 insectaries. However, survival must be tested before in each new environment and disinfection may be necessary to eliminate risks associated with the pathogen. In any case, clear labelling of rooms or equipment (isolator, climatic chamber etc.) in which there would have been an escape to establish a perimeter of caution and prevent any risk to other users of the facility. Spills with pathogen-containing material such as blood need to be considered and dealt with according to local/national safety practices, for example decontamination with inactivating agents and which outside of the contained environment could require fumigation of the facility.

An emergency plan, explaining actions for different incidents or undesirable events (power failure, autoclave failure, no operation of effluent decontamination system, etc.) should be written and implemented.

5. General considerations

5.1 Review and maintenance

Procedures and structures are critical to safe and efficient running of insectaries. **Standard operating procedures (SOPs)** should, regardless of containment level, be reviewed at least once a year, and at least when new techniques, arthropods or pathogens are introduced or following incidents and accidents. It is likely that procedures especially in new insectaries need to be adapted and tests are recommended before SOPs are finalised. **Structures** encompass the physical envelope of the insectary (walls/windows/doors) but also heating, water, electricity, light and equipment (environmental chambers, blood feeding devices, devices for proper waste treatment, etc.). It is recommended that maintenance contracts are agreed to assess the integrity of structures (universities frequently have specialised staff) and equipment verified in regularly as requested by the manufacturer / warranty conditions to ensure good functioning. In addition, quality certifications or accreditations will impose specific deadlines regarding such verification or calibration operatives.

5.2 Training

Following theoretical training and lectures on specific aspects of biosafety related to the activities to be carried out (risk assessments, SOPs etc.).

CL2: Demonstration of procedures and then at least once under supervision or until competent with procedures.

CL3: Experience at CL2 level should be a requirement. Training should be provided in specific techniques by defined numbers of sessions, first by shadowing experienced staff and then under supervision. Competence is verified under all circumstances by senior staff.

Refresher training should be offered for staff who have not been working in the facility following initial training (local requirments may vary but for example no active work for 6 months could be used as cut off). This should be discussed with facility supervisors to define needs. Training records should be kept according to local or national legislation.

5.3 Vaccinations and travel restrictions

Where possible, vaccination should be offered, either for work with the pathogens or before travel to affected areas. Moreover, blood sources (human etc.) may make vaccination mandatory. It is recommended not to work in insectaries with anopheline mosquitoes for 6 weeks if returning from areas where malaria may be present.

For arboviruses, it is recommended to not enter insectaries for a week following return from areas where arboviruses that can be transmitted by mosquitoes in the facility are endemic or currently being transmitted. If no symptoms are present after this point, the workers may re-enter the facility. Self-monitoring is essential, and potentially medical investigation if infection is suspected (for example after bites, length of stay in affected area etc). When working with animal pathogens (*e.g.* Rift Valley fever phlebovirus), it may be recommended not to visit facilities with potentially susceptible animals following experiments for a duration that should be decided on a virus-specific risk assessment basis. Such assessments should be carried for the pathogens and insect species to be studied in a given location on a case by case basis.