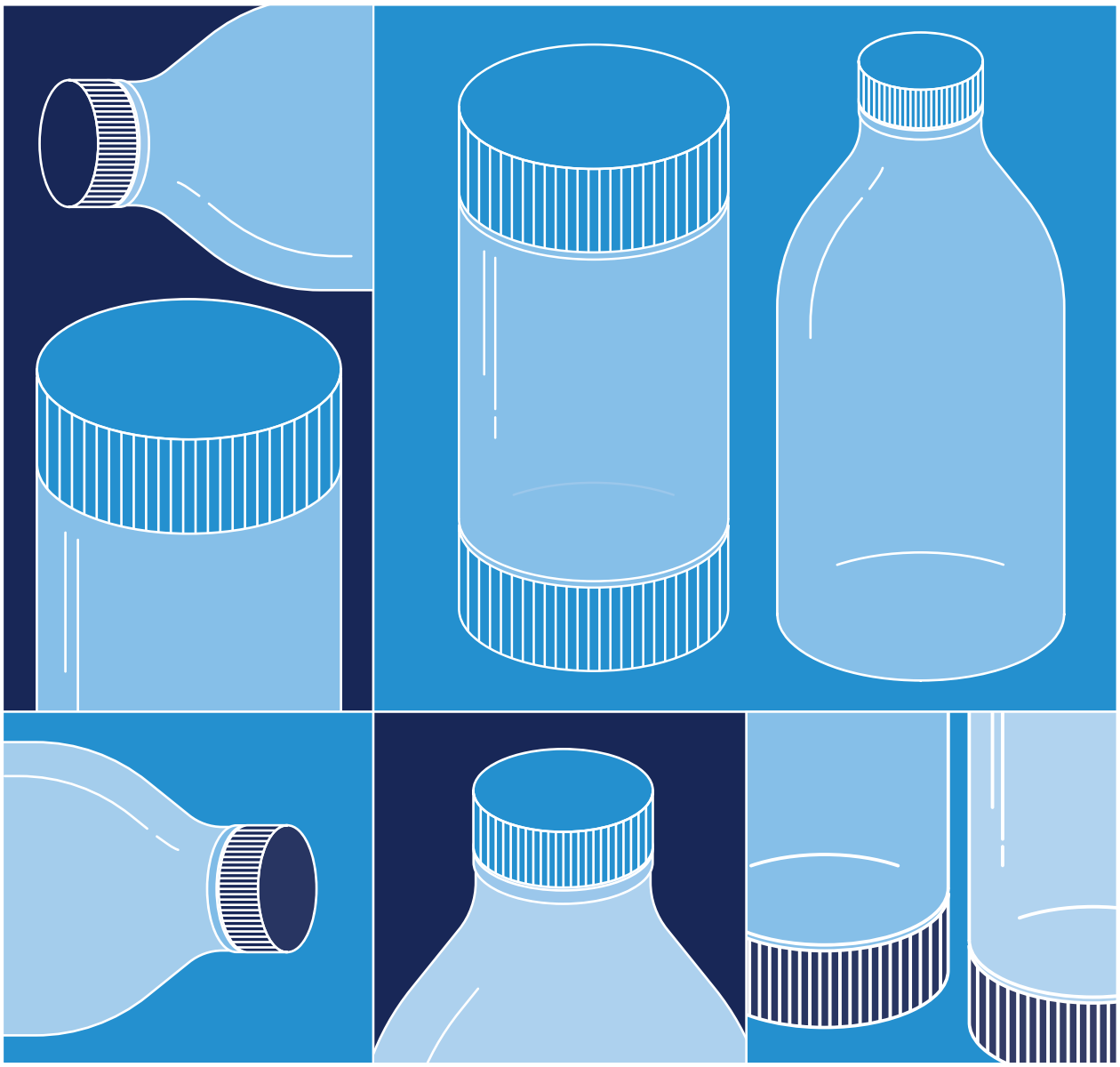
World Health Organization

Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions



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ISBN 978-92-4-005108-9 (electronic version)
ISBN 978-92-4-005109-6 (print version)

Suggested citation. Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO.

Cataloguing-in-Publication (CIP) data. CIP data are available at http://apps.who.int/iris.

Contents

Acknowledgements

The production of this document was led by Lucia Fernandez Montoya, World Health Organization (WHO) Global Malaria Programme; and Rajpal S Yadav, WHO Department of Control of Neglected Tropical Diseases. Contributions were provided by WHO staff members Samira Al-Eryani, Lauren Carrington, Emmanuel Chanda, Dennis Navarro Costa, Tessa Knox, Jan Kolaczinski, Prabhjot Singh, Jennifer Stevenson and Raman Velayudhan. The Global Malaria Programme and the Department of Control of Neglected Tropical Diseases would like to thank the following external individuals for reviewing the present document (ordered alphabetically by country and then by institution name): Charles Wondji (Centre of Research in Infectious Diseases, Yaoundé, Cameroon and Liverpool School of Tropical Medicine, Liverpool, United Kingdom of Great Britain and Northern Ireland), Ahmad Ali Enayati (Mazandaran University of Medical Sciences, Sari, Islamic Republic of Iran), Vincent Corbel (Institut de Recherche pour le Développement, Montpellier, France and Worldwide Insecticide resistance Network (WIN)), Basil Brooke (Vector Control Reference Laboratory, National Institute for Communicable Diseases, Johannesburg, South Africa), Thomas Churcher (Imperial College, London, United Kingdom), Geraldine Foster, Rosemary Lees, Philip McCall and David Weetman (Liverpool School of Tropical Medicine, Liverpool, United Kingdom), Ben Lambert (University of Exeter, Exeter, United Kingdom), Catherine Moyes (University of Oxford, Oxford, United Kingdom), Silvie Huijben (Arizona State University, United States of America), John Gimnig and Audrey Lenhart (Centers for Disease Control and Prevention, Atlanta, United States), Seth Irish (U.S. President's Malaria Initiative, Centers for Disease Control and Prevention, Atlanta, United States) and Jennifer Armistead (U.S. President's Malaria Initiative, USAID, Washington D.C., United States).

Important note

This document supersedes the WHO *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, second edition*, and the instructions regarding resistance monitoring provided in *Monitoring and managing insecticide resistance in Aedes mosquito populations: interim guidance for entomologists.* From the date of publication of this document, insecticide resistance in adult mosquito vectors should be monitored following the guidance provided here.

Abbreviations

CDC United States Centers for Disease Control and Prevention

DC discriminating concentration

DDT dichlorodiphenyltrichloroethane

DHIS2 District Health Information System 2

h hour

IRS indoor residual spraying

ITN insecticide-treated mosquito net

kdr knockdown resistance

MERO rapeseed oil methyl ester

min minute

PBO piperonyl butoxide

SOP standard operating procedure

USM Universiti Sains Malaysia

WHO World Health Organization

Glossary

ace-1	The target site gene for carbamate and organophosphate insecticides. Resistance (formerly known as insensitive acetylcholinesterase) is caused by a mutation, G119S, in the *ace-1* gene. The level of resistance depends on the number of copies of this gene.
behavioural resistance	Modification in mosquito behaviour that leads to avoidance of, or reduced contact with, insecticides.
bioassay	In applied entomology, experimental testing of the biological effectiveness of a treatment (e.g. insecticide) by deliberately exposing insects to it.
cuticular resistance	Reduction of a mosquito's absorption of insecticide due to a thickening or alteration in composition of its cuticle.
F1 mosquito progeny	The first generation of mosquitoes reared in the laboratory from the eggs of wild-caught females. They are one of the two generations of mosquitoes that can be used in standard resistance monitoring bioassays.
F0 mosquitoes	Wild-caught adult mosquitoes or adult mosquitoes emerging from wild larvae collected in the field. They are one of the two generations of mosquitoes that can be used in standard resistance monitoring bioassays.
indoor residual spraying	Operational procedure and strategy for vector control involving spraying interior surfaces of dwellings with a residual insecticide to kill or repel endophilic mosquitoes.
insecticide	Chemical product (natural or synthetic) that kills insects. Ovicides kill eggs; larvicides kill larvae; pupacides kill pupae; adulticides kill adult mosquitoes. Residual insecticides remain active for an extended period. *Note:* Insecticides used for vector control are approved by the WHO Vector Control Products Assessment team (https://extranet.who.int/pqweb/vector-control-products).
insecticide, cross-resistance	Resistance to one insecticide by a mechanism that also confers resistance to another insecticide, even when the insect population has not been selected by exposure to the latter.

insecticide diagnostic dose	The amount of insecticide active ingredient that mosquitoes are expected to absorb while being exposed to a discriminating concentration of an insecticide for a fixed period, and which, after mosquitoes absorb it, reliably kills all susceptible mosquitoes so that any survivors may be assumed to be resistant.
insecticide discriminating concentration	The concentration of an insecticide that, when a sample of mosquitoes is exposed to a surface treated with it for a standard period of time, reliably kills susceptible mosquitoes so that any survivors may be assumed to be resistant.
insecticide mode of action	The process of how an insecticide works on an insect at a molecular level. Mode of action refers to a functional change at the molecular level of a specific target site resulting from exposure of insects to an insecticide active ingredient.
insecticide resistance	Property of mosquitoes to survive exposure to a standard dose of insecticide; may be the result of physiological or behavioural adaptation. *Note:* The emergence of insecticide resistance in a vector population is an evolutionary phenomenon due to either behavioural avoidance (e.g. exophily instead of endophily) or physiological factors, whereby the insecticide is metabolized, not potentiated, or absorbed less than by susceptible mosquitoes.
knockdown resistance (*kdr*) mutation)	Knockdown resistance is caused by one or more mutations in the sodium channel receptor, the target site of pyrethroids and organochlorine compounds (e.g. dichlorodiphenyltrichloroethane – DDT), that prevents mosquitoes from being "knocked down" and killed by exposure to these insecticides.
juvenile hormone mimics	Chemicals that disrupt and prevent metamorphosis in pre-metamorphic mosquito immature instars, and reduce fecundity and fertility of adult mosquitoes. *Note:* Pyriproxyfen is a juvenile hormone mimic.
larvicide	Substance used to kill mosquito larvae. *Note:* Larvicides are applied in the form of oils (to asphyxiate larvae and pupae), emulsions or small pellets or granules of inert carrier impregnated with insecticide, which is released gradually when they are placed in water.
larval source management	Management of aquatic habitats (water bodies) that are potential habitats for mosquito larvae, in order to prevent completion of development of the immature stages.

long-lasting insecticidal net	A factory-treated mosquito net made of material into which insecticide is incorporated or bound around the fibres. The net must retain its effective biological activity for at least 20 WHO standard washes under laboratory conditions and 3 years of recommended use under field conditions.
metabolic insecticide resistance	Changes in the amount or specificity of a metabolic enzyme so that it detoxifies an insecticide before it reaches the target site.
net, insecticide-treated	Mosquito net that repels, disables or kills mosquitoes that come into contact with the insecticide on the netting material. Insecticide treated nets (ITNs) include those that require treatment and retreatment (often referred to as conventional nets) and those that are "long-lasting" (see definition of long-lasting insecticidal net). *Note:* Untreated mosquito nets can also provide substantial protection against mosquito bites, but they have less effect against vectorial capacity and transmission rates.
oviposition inhibition	Reduction or complete blocking of female mosquito egg laying as a result of exposure to a juvenile hormone mimic.
phenotypic resistance	Development of an ability in a strain of insects to tolerate doses of toxicants that would prove lethal to the majority of insects in a normal population of the same species.
public health value	A product has public health value if it has proven protective efficacy to reduce or prevent infection and/or disease in humans, at the individual level, community level or both.
resistance intensity	Strength of resistance in mosquitoes to insecticides, resulting from the level of expression of resistance phenotype(s). Resistance intensity is measured by testing the ability of mosquitoes to survive exposure to 5× and 10× a standard discriminating concentration of insecticide. *Note:* Resistance intensity may be higher where mosquitoes express multiple resistance mechanisms. Insecticide-based vector control is more likely to fail in areas of high resistance intensity, and therefore an urgent response may be required, ideally after investigating operational significance.
space spraying	Application of a cold or thermal aerosol (fog) with droplets of <30 µm diameter that have low terminal velocity and thus remain airborne for as long as possible to provide rapid knockdown and mortality of flying insects, leaving little or no residual effect. *Note:* WHO does not recommend the use of this intervention for malaria control.

susceptibility bioassay	A bioassay in which a sample of mosquitoes from a mosquito population is exposed to a discriminating concentration of an insecticide for a fixed period to distinguish susceptible from resistant mosquitoes and determine their relative proportions.
susceptible population	A mosquito population is considered to be susceptible to an insecticide when 98% or more of the mosquitoes in a sample taken from the population die after being exposed to a discriminating concentration of the insecticide. *Note:* A vector population can be susceptible to one insecticide class but resistant to another class with an unrelated mode of action.
synergist	A substance that does not itself have insecticidal properties, but that, when mixed and applied with insecticides of a particular class, considerably enhances their potency by inhibiting an enzyme that normally acts to detoxify the insecticide in the insect.
target site resistance	Reduction of the effect that an insecticide is expected to have on a mosquito due to an alteration of the protein receptor targeted by the insecticide that prevents the insecticide from binding to the receptor.
vector control	Measures of any kind against disease-transmitting mosquitoes, intended to limit their ability to transmit the disease.
vector, principal	The species of mosquito mainly responsible for transmitting a specific disease in any particular circumstance. *Note:* Principal vectors may overlap seasonally or alternate in importance.
vector, secondary or subsidiary	Mosquito species thought to play a lesser role in the transmission of a disease than the principal vector; capable of maintaining disease transmission at a reduced level.

1. Introduction

Vector-borne diseases are major causes of sickness, disability and death worldwide. More than 80% of the world's population lives in areas with transmission of one or more of these diseases. The main vector-borne diseases are malaria, dengue, chikungunya, leishmaniases, Chagas disease, human African trypanosomiasis, lymphatic filariasis, onchocerciasis, Zika virus disease, yellow fever, Japanese encephalitis and schistosomiasis. These diseases are caused by parasites, bacteria or viruses that are transmitted to humans by various vectors: mosquitoes, sandflies, fleas, ticks, lice, triatome bugs, blackflies, tsetse flies and snails *(1)*.

Vector control plays a key role in reducing the burden of these diseases. Prevention of diseases for which vaccines or treatments (either prophylactic or curative) are not available relies heavily on vector control. For diseases for which treatments are available, such as malaria, vector control remains the most widely used prevention strategy and has historically led to the greatest reductions in disease burden.

Insecticides are the most widely used and effective vector control interventions. They are delivered through insecticide-treated nets (ITNs) and indoor residual spraying (IRS), as well as through supplementary interventions such as larviciding or space spraying. Currently, nine classes of chemical insecticides are used in vector control products prequalified by the World Health Organization (WHO) for public health use: pyrethroids, carbamates, organophosphates, organochlorines, neonicotinoids, pyrroles, butenolides, juvenile hormone mimics and spinosyns *(2)*.

Traditionally, ITNs have relied solely on pyrethroids; pyrethroids, carbamates, organophosphates and organochlorines have been widely used for IRS; organophosphates, juvenile hormone mimics and spinosyns have been used for larviciding; and pyrethroids and organophosphates have been used for space spraying.

Neonicotinoids and butenolides are used in vector control products prequalified by WHO since 2017. They are IRS products containing a neonicotinoid (clothianidin alone or in a mixture with a pyrethroid), and space spray products with a mixture of a pyrethroid and a neonicotinoid (imidacloprid) or a butenolide (flupyradifurone). ITNs containing a pyrrole (chlorfenapyr) or a juvenile hormone mimic (pyriproxyfen) in combination with a pyrethroid were prequalified in 2018 and 2019, respectively, although WHO recommendations for their deployment in vector control are pending until their epidemiological impact has been demonstrated.

As a result of the wide use of insecticides for disease vector control and in agriculture, mosquito resistance to insecticides in four classes traditionally widely used for vector control – organophosphates, carbamates, pyrethroids and the organochlorine dichlorodiphenyltrichloroethane (DDT) – has emerged in major disease vectors and spread across all regions of the world *(3–6)*. The status of vector resistance to newer insecticides contained in WHO prequalified vector control products – neonicotinoids, pyrroles, butenolides and spinosyns – is unknown because no standard test procedures and discriminating concentrations (DCs) have been available to monitor resistance to these compounds. However, resistance to these compounds is likely to develop as their use is scaled up, or may already be present as a result of the selection pressure exerted by their use in agriculture or as a result of cross-resistance with insecticides in other classes.

Resistance is caused by the evolutionary selection of specific genetic mutations in mosquitoes that allow them to survive exposure to insecticides. At present, the best understood mechanisms of resistance are:

- target site mechanisms, which are modifications of the molecular target site of the insecticide in the insect body (i.e. mutations in the voltage-sensitive sodium channel gene, known as "kdr" mutations); and
- metabolic mechanisms, which are an increase in the activity of insecticide-detoxifying enzymes within the insect body (i.e. cytochrome P450 monooxygenases, esterases, carboxylesterases and glutathione S-transferases).

Other, but less frequently studied, mechanisms that can lead to phenotypic resistance in some mosquito vectors are cuticular thickening that reduces absorption of the insecticide (cuticular resistance) *(7)* and changes in vector behaviour that allow vectors to avoid contact with the insecticide (behavioural resistance) *(8)*.

Knowledge of vector resistance to insecticides, the associated molecular mechanisms and changes in resistance over time is crucial for the design of effective vector control interventions. Specifically, insecticide resistance data are essential for selection of appropriate vector control interventions, to inform resistance prevention strategies or to prompt changes in vector control strategies when resistance emerges. Generating data for decision-making involves monitoring resistance in local vector species before and during implementation of vector control interventions, collating and analysing the data, and interpreting the findings.

To ensure adequate monitoring and management of insecticide resistance, including securing the necessary funds, control programmes need to develop national insecticide resistance monitoring and management plans. Guidance on how to develop such plans is provided in the WHO *Framework for a national plan for monitoring and management of insecticide resistance in malaria vectors (9)*. The plans should consider all vectors of disease and should be part of overall national vector control strategies. Where possible, they should be integrated with other sectors whose activities may affect the development of resistance, such as the agricultural sector.

Since the 1950s, WHO has coordinated efforts to provide standard test procedures and guidance to help countries monitor and manage insecticide resistance in several disease vectors *(10–19)*. These procedures and guidance have evolved over time. The current revision of WHO guidance is motivated by:

- the need to establish resistance monitoring procedures and DCs for insecticides recently recommended for disease vector control or for which large-scale evaluation is ongoing (e.g. neonicotinoids for IRS, pyrroles and juvenile hormone mimics for ITNs, and butenolides and neonicotinoids for space spraying);
- the need to integrate guidance for different disease vectors in one document, to ease use by integrated vector control programmes;
- the lack of DCs to monitor *Aedes* resistance to commonly used insecticides;
- the need to confirm some DCs for *Anopheles* mosquitoes that were still tentative;
- the need, expressed through questions and comments received from users of the previous WHO guidance, for better advice on how to plan and prioritize resistance monitoring in contexts with limited resources or where few mosquitoes are available for evaluating insecticide resistance, as well as for improved

direction on how to use resistance monitoring data to guide the design of malaria control strategies.

This document combines, updates and supersedes previous WHO guidance for monitoring resistance in *Anopheles*, *Culex* and *Aedes* mosquitoes provided in the *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Second edition (18)*, and *Monitoring and managing insecticide resistance in Aedes mosquito populations: interim guidance for entomologists (19)*. The updates with respect to former guidance are:

- description of the WHO bottle bioassay, a new bioassay that should be used for identifying the presence of resistance to insecticides that cannot be impregnated on filter papers; it was developed based on the United States Centers for Disease Control and Prevention (CDC) bottle bioassay, but aligning mosquitoe exposure and holding times with those of the WHO tube test;
- description of a new procedure to monitor *Anopheles* resistance to pyriproxyfen;
- new concentrations for monitoring *Anopheles* resistance to transfluthrin, chlothianidin, flupyradifurone, chlorfenapyr and pyriproxyfen;
- updated concentrations for monitoring *Anopheles* resistance to pirimiphos-methyl and alpha-cypermethrin
- new concentrations for monitoring *Aedes* resistance to transfluthrin, metofluthrin, prallethrin, bendiocarb, chlorpyrifos-ethyl, clothianidin and flupyradifurone;
- updated concentrations for monitoring *Aedes* resistance to alpha-cypermethrin, deltamethrin, lambda-cyhalothrin, permethrin, malathion and pirimiphos-methyl;
- improved guidance on how to plan resistance monitoring and prioritize bioassays in the context of limited resources or mosquitoes; and
- improved guidance on how to use insecticide resistance monitoring data to guide the selection of vector control interventions.

The new testing procedures and DCs presented here were established in 2022 based on a WHO-coordinated multicentre study conducted between 2017 and 2021 *(20)*. All other updates are based on recent WHO guidance documents on the control of vector-borne diseases (all of which are listed in this manual) and on current best practices in insecticide resistance monitoring; many of the updates aim to clarify guidance already provided in the two WHO publications mentioned above *(18,19)*. The draft manual was reviewed by external experts to ensure that its content accurately reflects current knowledge in the field. All names and affiliations are provided in the acknowledgments.

The scope of this document does not include testing resistance of mosquito larvae or of other non-mosquito vectors, but it will be updated when DCs and test procedures for mosquito larvae or other vector species are validated by WHO.

The document is aimed at field entomologists and biologists within ministries of health or partner institutions responsible for monitoring vector resistance to insecticides. It is also directed at programme managers and others in charge of designing and implementing vector control strategies who need to draw on resistance data to inform their decisions. Lastly, it can help researchers and the pesticide industry to assess, in a standardized way, vector resistance to compounds used in existing and new insecticide-based vector control products.

2. Evaluating insecticide resistance

Understanding the type and level of resistance of local vector populations to insecticides in current use, or planned for use, is crucial to ensuring that effective vector control interventions are selected and their effectiveness is preserved for as long as possible.

Vector resistance to insecticides and the prevalence of molecular resistance mechanisms can vary markedly across vector populations in different regions and at different times within a country. This reflects the different levels of selection pressure exerted by the use of insecticides in vector control and/or agriculture; changes in species composition; changes in vector behaviour that may increase or decrease contact with insecticides; and other evolutionary, ecological and climatic conditions. Local vector populations can become resistant to one or more insecticides at the same time; they will generally not revert to being susceptible, at least not at a rate that is of immediate relevance to programmatic decision-making *(21)*.

Test procedures are available to characterize the following aspects of vector resistance to insecticides:

- the presence of phenotypic insecticide resistance in a vector population;
- the intensity of phenotypic resistance;
- the effect of a synergist in restoring susceptibility to an insecticide in a resistant vector population; and
- the mechanisms responsible for phenotypic resistance.

This document provides detailed guidance on how to evaluate the presence and intensity of phenotypic resistance, and the effect of a synergist in restoring vector susceptibility to an insecticide. It provides an overview of the main mechanisms responsible for the phenotypic expression of resistance in disease vectors and on how to use mechanism data to guide decisions (section 7), but it does not provide details on the techniques available to identify each mechanism. Instead, it refers readers to external resources where these techniques are detailed.

The presence or absence of resistance, its intensity, and the ability of a synergist to restore vector susceptibility to insecticides can be evaluated using the following three standard procedures, the first two of which have been validated by WHO:

- WHO tube test
- WHO bottle bioassay
- CDC bottle bioassay.

The WHO tube test and the WHO bottle bioassay are described in detailed in section 6; standard operating procedures (SOPs) for these bioassays are separately provided on the WHO website *(22)*. Guidance to conduct the CDC bottle bioassay is provided on the CDC website *(23)*. The WHO bottle bioassay is a new method developed to determine mosquito resistance to certain insecticides that are not suitable for impregnation of filter papers. It is based on the CDC bottle bioassay but uses different test end-points aligned with those of the WHO tube test. Further details on the differences between the CDC bottle bioassay and the WHO bottle bioassay are provided in section 6.

These procedures only provide an indication of the presence of insecticide resistant in vector populations. They do not indicate the actual effectiveness of the tested insecticides when used as part of vector control interventions in the field. This is because the insecticide concentration and formulation used in vector control products are different from those used in resistance bioassays and because vector exposure to insecticides in the field is different from vector exposure to insecticides during a bioassay. If insecticide resistance is detected, additional investigations, including epidemiological assessment, will be required to study the impact of resistance on the effectiveness of vector control interventions in the field. In other words, the test procedures outlined here and the data they generate provide a first indication of a threat to the control of a vector-borne disease; they should be seen as the starting point for a more detailed and complex investigation if signs of insecticide resistance are detected.

Table 1. Insecticide discriminating concentrations (DCs) of pyrethroid insecticides and PBO for testing the ability of PBO to restore insecticide susceptibility in *Anopheles* mosquitoes

Aspect of resistance	Type of assay	Assay description	Assay outcome	Test procedures
Presence	Susceptibility bioassays	Measure mortality in a sample of mosquitoes that were exposed to a DC of an insecticide for a fixed period to estimate the percentage of resistant mosquitoes in a population	Confirmed or possible resistance, or susceptible	• WHO tube test with DCs • WHO bottle bioassay with DCs • CDC bottle bioassay with diagnostic doses • WHO susceptibility test for mosquito larvae with DCs
Intensity	Intensity bioassays	Compare mortality in mosquitoes that are exposed to different concentrations of an insecticide (normally the DC, as well as 5× and 10× the DC) to assess the intensity of resistance	High, moderate or low intensity	• WHO tube test with 5× and 10× the DC of insecticide • CDC bottle bioassay with increasing diagnostic dosages[a]
Effect of a synergist in restoring phenotypic susceptibility to an insecticide	Synergist–insecticide bioassays	Compare mortality in mosquitoes that are exposed to the DC of an insecticide to mortality in those pre-exposed to a synergist before exposure to the DC of the insecticide to assess the role of the synergist in restoring susceptibility to the insecticide	No restoration, partial restoration or total restoration of susceptibility	• WHO tube test with pre-exposure to PBO followed by exposure to a pyrethroid insecticide • CDC bottle bioassay with pre-exposure to a synergist followed by exposure to an insecticide[a]
Mechanism (molecular and biochemical)	Molecular and biochemical assays	Analyse enzyme expression or gene mutations in mosquitoes to detect anomalies that may cause phenotypic resistance	Presence or absence for each mechanism	• Molecular and biochemical assays for individual mechanisms

PBO: piperonyl butoxide.

[a] The WHO bottle bioassay has not yet been validated for measuring the resistance intensity or the effect of PBO.

3. Planning and setting priorities for resistance monitoring

Each of the test procedures referred to above requires trained personnel, a minimum number of mosquitoes, adequate equipment and consumables, and mosquito rearing facilities with optimal temperature and humidity conditions. Inadequate resources or insufficient mosquitoes often limit the extent of resistance monitoring that can be accomplished. Hence, it is important to properly plan insecticide resistance monitoring, prioritizing bioassays based on their importance for decision-making. By doing this, the data collected can inform the most important programmatic decisions, which tend to be about immediate- and medium-term deployment of interventions. Priorities for resistance monitoring are summarized in Box 1.

Box 1. Priorities for resistance monitoring

- Use available mosquitoes first to test whether insecticide resistance is present or absent in a given geographical area, because assessing resistance intensity or other aspects of resistance is only relevant in areas where vector resistance has already been confirmed.
- Test for vector resistance to insecticides currently in use and being considered for use. Testing for resistance to insecticides no longer in use is generally discouraged unless it can be well justified.
- Test for insecticide resistance in the primary disease vector(s); resistance of secondary vectors should only be tested if this is relevant to programmatic decisions.
- Use the same test procedure consistently to ensure comparability of results. This is essential to monitor and interpret observed changes in vector resistance.
- Monitor resistance when vector abundance is high to increase the likelihood of obtaining a large enough sample of mosquitoes to test their resistance to all relevant insecticides. This does not apply when spot checks of insecticide resistance need to be conducted as part of a larger investigation into the causes of an unexpected increase in incidence of vector-borne disease in a specific geographical area.

3.1 Priority end-points

Before any insecticide-based vector control intervention is deployed, programmes should ensure that local vectors are susceptible to insecticides in the relevant insecticide class. Once the intervention is in place, local vector susceptibility to insecticides in the insecticide class (or classes) in use should be monitored at least once a year to promptly detect any emergence of resistance and act to manage it.

The first priority should therefore be to determine the presence or absence of resistance in local vector populations to an insecticide in the insecticide class (or classes) used or planned for use for vector control in the area. If the local vector population proves to

be susceptible to an insecticide within a class, there is no need to evaluate resistance intensity or resistance mechanisms for insecticides in that class. When insecticide resistance is detected in a vector population, the importance of evaluating its intensity, its mechanisms or the role of a synergist in restoring susceptibility to the insecticide (see section 2) will depend on the available programmatic options to manage such resistance.

3.2 Selecting resistance monitoring sites

Countries should aim to evaluate vector resistance to insecticides in each operational area where insecticide-based vector control interventions are under consideration and to monitor it in each vector control operational area once interventions are deployed. However, monitoring resistance in each operational area (e.g. each district) is unlikely to be feasible in most countries due to resource constrains. A more feasible approach will be to group neighbouring areas with similar vector species composition, under similar selection pressures (e.g. the same vector control interventions and similar agricultural practices) and with similar history of insecticide resistance, and monitor resistance in one site within each group.

"Less is more" should be the founding principle for the establishment of sentinel sites – that is, the focus should be on generating high-quality data (i.e. strictly following SOPs) at a limited number of sites, rather than a greater quantity of data from more sites at the risk of reduced quality. For example, a country could start with one site per province in which insecticide-based vector control interventions are or will be deployed, prioritizing provinces where resistance selection pressure is likely to be highest. Further sites at lower administrative levels (e.g. district) could then be established as capacity for testing is built, especially if resistance is detected and more granular information is required.

Insecticide resistance may be also evaluated as part of a disease outbreak investigation, or when investigating the reasons for an unexpected increase in disease incidence. In these cases, resistance testing can be conducted ad hoc, without necessarily establishing a routine monitoring site.

3.3 Mosquito vector species

The capacity of different mosquito species to transmit pathogens to humans varies. Priority should be given to monitoring resistance of the species that play the greatest role in pathogen transmission. These species should be identified through entomological surveillance before starting insecticide resistance monitoring. As part of resistance monitoring, the species being tested need to be clearly identified and recorded. Details on mosquito sampling and species identification are provided in section 5.

3.4 Selecting insecticides

To determine whether there is resistance to a specific insecticide class, countries only need to test mosquitoes against one insecticide of the class. For example, to determine whether vectors are resistant to pyrethroids, countries should test mosquitoes against one pyrethroid insecticide only (e.g. deltamethrin, alpha-cypermethrin). If the test results confirm resistance to this pyrethroid, vectors can be considered resistant to pyrethroids

in general. Programmes should prioritize testing mosquitoes' susceptibility to insecticides in different insecticide classes, rather than testing susceptibility to different insecticides within the same insecticide class.

3.5 Consistency in procedures

To ensure reliable comparisons of assay results in different areas and at different times, it is recommended that a specific testing method is used consistently over time. The conditions under which a specific method is used, such as temperature and humidity, should also be as consistent as possible between tests; these conditions should be recorded with the test results to help with interpreting any discrepancies across test results. Further information on the difference between test procedures and recommended test conditions is provided in section 6.

Resistance can be monitored in both adult mosquitoes and larvae. Ideally, programmes should use the vector stage targeted by the insecticides being deployed or planned for deployment – that is, adult mosquitoes for interventions based on adulticides (e.g. ITNs, IRS) and larvae for interventions based on larvicides. Procedures for testing insecticide resistance of adult mosquitoes have been significantly improved since their development and extensively validated in a recent WHO multicentre study *(20)*. In contrast, test procedures for mosquito larvae were established by WHO in 1958 *(24)*, with further guidance provided in 1981 *(25)*, but these procedures have not been further validated since then.

3.6 Timing of resistance testing

Vector abundance and composition in a given geographical area may fluctuate during the year with changes in climatic conditions or other factors. Insecticide resistance monitoring should be timed to coincide with the peak vector density to increase the likelihood of obtaining a large enough sample of mosquitoes for testing resistance against all relevant insecticides.

The time of peak mosquito abundance will vary in different sites within a country and between countries. This time can be identified through regular and systematic entomological surveillance. For malaria vectors that breed in rural areas, the peak of vector abundance often occurs following the start of the rainy season, but this may not be the case in artificially irrigated areas, or in the case of urban vectors. *Aedes* spp. tend to breed in water storages, including artificial reservoirs near human habitations, which are often permanent or semi-permanent. Therefore, a peak in their density is in general less pronounced than for *Anopheles* spp. and does not necessarily follow rainfall patterns. *Culex quinquefasciatus* breeds throughout the year, but rainfall generally increases its population density as a result of expansion of breeding habitats.

4. Using insecticide resistance monitoring data to inform programmatic decisions

To maximize the impact of limited resources, disease control programmes need to choose cost-effective vector control interventions and prevent the emergence of vector resistance to the insecticides being used. Selection of vector control interventions will be driven by several factors in addition to vector resistance to insecticides – for example, feasibility of product procurement and registration in the country of use, availability of infrastructure and human resources, and budget constraints. However, insecticide resistance monitoring data can be useful to guide the selection of effective vector control interventions, develop suitable implementation strategies to delay the emergence of resistance and select replacement interventions when resistance is detected.

WHO recommended vector control interventions for different vector-borne diseases can be found in the WHO guidelines for malaria *(26)*, for dengue *(27)* and for lymphatic filariasis *(28)*.

Uses of insecticide resistance monitoring data are summarized in Box 2.

Box 2. Uses of insecticide resistance monitoring data

- Inform the selection of appropriate vector control interventions.
- Trigger a change in vector control intervention or insecticide when resistance is detected.
- Guide the introduction of novel or supplementary vector control interventions.
- Inform the development of resistance management strategies in areas where insecticide-based vector control is ongoing.
- Contribute to investigations of unexpected changes in vector-borne disease transmission and disease burden.

4.1 Selecting vector control interventions

This section provides brief guidance on selection of interventions for control of vectors of malaria, *Aedes*-borne diseases and *Culex*-borne diseases. The current document does not cover other disease vectors, such as sandflies and triatomine bugs; guidance on these vectors will be added as insecticide resistance monitoring procedures are developed for them.

4.1.1 Malaria control and elimination

Where local malaria vectors continue to be susceptible to pyrethroids

- ITNs can be considered, since they provide personal protection, and community-wide protection if high enough population coverage is achieved.
- IRS can be conducted with pyrethroids, or insecticides of other classes provided that vectors are susceptible to them. However, since pyrethroids are currently used in all ITNs, disease control programmes should minimize the use of pyrethroids in IRS to reduce selection pressure on the vector population and preserve the efficacy of ITNs over time.
- If ITNs and IRS are co-deployed, use of pyrethroids alone or in combination with other insecticides in IRS should be avoided.

Where local malaria vectors have developed resistance to pyrethroids

- Pyrethroid-only long-lasting insecticidal nets will still provide some protection, but their effectiveness may be reduced compared with deployment in areas of pyrethroid susceptibility.
- WHO suggests deployment of pyrethroid–piperonyl butoxide (PBO) nets instead of pyrethroid-only nets.
- IRS should be conducted with an insecticide that has a different mode of action from pyrethroids and to which the vector population is susceptible.
- WHO suggests not co-deploying ITNs and IRS, and that priority be given to delivering either ITNs or IRS at optimal coverage and to a high standard, rather than introducing the second intervention as a means to compensate for deficiencies in the implementation of the first intervention. However, a combination of these interventions may be considered for resistance prevention, mitigation or management if sufficient resources are available.
- WHO suggests the regular application of insecticides to water bodies (larviciding) for the prevention and control of malaria as a supplementary intervention to ITNs or IRS in areas with ongoing malaria transmission and where aquatic habitats are few, fixed and findable.

Where local malaria vectors are resistant to insecticide classes other than pyrethroids

- Disease programmes should avoid deploying interventions that use insecticides from such classes.

Changing malaria vector control interventions when insecticide resistance is detected

- **When using IRS for vector control.** If resistance in the local vector population to the insecticide used for IRS is detected, programmes should plan to change the insecticide used for one with a different mode of action, to which there is no locally known cross-resistance and to which vectors are susceptible. The modes of action and resistance mechanisms of commonly used insecticides are presented in Table 13.
- **When using pyrethroid-containing ITNs for vector control.** The ITNs should not be withdrawn if resistance to pyrethroids in the local vector population is confirmed, because they still protect against mosquito bites. In areas where local vectors are resistant to pyrethroids, WHO suggests deploying pyrethroid–PBO nets instead of pyrethroid-only ITNs. Alternatively, IRS using an insecticide with a different mode of action from pyrethroids could be deployed.

If pyrethroid resistance is detected, monitoring the intensity of resistance could help identify priority areas for a change of interventions or for deployment of supplementary interventions. However, such a decision should be taken as part of the formulation of an appropriate intervention package. The process for this will need to include several other considerations, such as the epidemiological situation; past and present intervention coverage; local parasites and vectors; and equality of access, efficacy, acceptability, cost and feasibility of implementation of various interventions – including those different from vector control.

4.1.2 Control of *Aedes*-borne diseases

The scientific evidence for the public health value of different vector control interventions against dengue, chikungunya and Zika virus disease is, in general, scarce. Approaches should target both immature and adult vector populations and not rely on a single intervention only. Control efforts should also target places of work and study, in addition to residential areas, because of the daytime biting habit of the vectors.

Environmental management is recommended to control dengue and other arboviral diseases, although more evidence for epidemiological impact is needed. Environmental management methods do not rely on the use of insecticides, and are therefore not affected by the presence or absence of insecticide resistance in local *Aedes* populations. Where feasible, environmental management to prevent and control mosquito breeding, and prevent contact between people and vectors could be more sustainable than use of insecticides in the long term, especially with community and intersectoral participation. Reducing sources of mosquito larvae, with active community support, should be the mainstay for controlling mosquito populations.

Where local *Aedes* vector species are susceptible to pyrethroids

- Application of chemical larvicides – to which vectors are susceptible – to water storage containers and other larval habitats can effectively reduce the population of immature mosquito stages.
- In emergencies, to suppress an ongoing epidemic or to prevent an incipient outbreak of disease, WHO recommends using indoor space spraying (fogging). Indoor space spraying using hand-held space spray applicators is more effective than outdoor space spraying. Outdoor space spraying with vehicle-mounted sprayers may have a low, if any, impact on disease transmission. Space spraying should not be applied for routine vector control operations.
- Targeted IRS with pyrethroids at high coverage and to a high standard can be used as a preventive or reactive intervention, especially where the endophagic mosquito *Ae. aegypti* is the primary vector. Such interventions eliminated *Aedes* species from several countries of South America as part of a campaign in the 1960s to 1970s against yellow fever.
- Personal protection with pyrethroid-containing ITNs should be promoted for young children, older people and unwell people who may sleep during the daytime, and to protect inpatients in hospitals and special wards who are undergoing treatment for *Aedes*-borne diseases. WHO also recommends the use of certain WHO prequalified skin-applied (topical) repellents for personal protection.

Where local adult *Aedes* vector species are resistant to pyrethroids

- If the mosquitoes are resistant to pyrethroids due to P450 mechanisms only, an organophosphate or a pyrethroid–PBO combination product should be used for space spraying for outbreak containment.
- If the local *Aedes* spp. are highly resistant to pyrethroids due to both knockdown resistance (kdr) and P450 mechanisms, space spraying may be conducted with a WHO prequalified organophosphate product such as malathion, a combination product containing non-pyrethroids (e.g. flupyradifurone and transfluthrin) or a pyrethroid and a non-pyrethroid (e.g. prallethrin and imidacloprid).

Where local *Aedes* vectors are susceptible to non-pyrethroid insecticides

- In this situation, non-pyrethroid chemical larvicides should be applied where *Aedes* breeding habitats are well known. Several non-pyrethroid larvicides (e.g. bacterial larvicides, benzoylureas, juvenile hormone mimics, organophosphates, spinosyns) and a monomolecular film–producing product have been prequalified by WHO, offering a choice of products from unrelated alternative chemical and microbial classes or a physical barrier, and with different durations of residual action in aquatic environments *(2)*. These include products for application in storage containers for non-potable water and drinking water.

Where *Aedes* vector species are resistant to any non-pyrethroid insecticides

- In this situation, disease programmes should avoid using insecticides from such classes for any control intervention. Rather, a product should be used from a class with an unrelated mode of action and to which there is no locally known cross-resistance.

4.1.3 Control of *Culex*-borne diseases

Some species of *Culex* are important vectors of disease – namely, *Culex quinquefasciatus, Cx. pipiens, Cx. tarsalis, Cx. tritaeniorhynchus* and *Cx. annulirostris*. The main *Culex*-borne diseases are Bancroftian filariasis, West Nile virus, Rift Valley fever, Japanese encephalitis, St Louis encephalitis, Murray Valley encephalitis and Ross River disease. Vector control can play an important role in elimination of lymphatic filariasis, which should complement or replace mass drug administration in some situations. *Culex* control options and their relation to insecticide resistance are as follows.

- Environmental management methods do not rely on the use of insecticides. Therefore, this option should be considered in all situations, irrespective of the presence or absence of insecticide resistance in local *Culex* species.
- Larviciding is the main recommended method for controlling most *Culex* species, especially in urban and semi-urban areas where breeding habitats are well known and findable. Larvicides should be applied in the main *Culex* breeding habitats, using products to which local *Culex* spp. mosquitoes are proven to be susceptible (e.g. bacterial larvicides, benzoylureas, juvenile hormone mimics, organophosphates, spinosyns) or amenable to control (e.g. monomolecular film–producing products).
- Space spraying is not recommended for routine control of *Cx. quinquefasciatus* or other *Culex* species. Where feasible, it can be considered to control outbreaks of arboviral diseases such as West Nile virus, using an insecticide to which *Culex* spp. mosquitoes are susceptible.

- In exceptional situations, such as for containment of an outbreak of Japanese encephalitis, residual spraying of major shelters of *Cx. tritaeniorhynchus* may be considered, using an insecticide to which *Culex* spp. mosquitoes are susceptible. The options include a number of WHO prequalified pyrethroids. Where *Culex* spp. are resistance to pyrethroids, a WHO prequalified insecticide from an insecticide class with an unrelated mode of action should be used.
- Personal protection with skin-applied (topical) repellents can be considered to prevent West Nile virus infection.

There is a need to generate more insecticide resistance data over time and space, especially for *Aedes* and *Culex spp*. vectors, and to improve our understanding of the impact of resistance on the effectiveness of vector control interventions with the aim of improving the use of resistance data in designing vector control and insecticide resistance management strategies.

4.2 Understanding unexpected changes in disease transmission patterns and outbreaks

Vector resistance to insecticides can compromise the effectiveness of insecticide-based vector control interventions. When unexpected incidence patterns for a vector-borne disease are observed, insecticide resistance data can be useful to flag whether insecticide resistance is the cause or a contributing factor. For example, an IRS campaign may be conducted in an operational area where resistance testing has shown that local vectors are susceptible to the insecticide used. However, vectors could be resistant to the insecticide applied in some sub-areas within the IRS operational area. This may affect IRS effectiveness and lead to unexpectedly high levels of disease incidence post-intervention. Nonetheless, the effectiveness of vector control interventions also depends on operational factors such as intervention coverage, product and deployment quality, human behaviours (e.g. replastering of walls after IRS) and vector behaviours. Therefore, other types of data will be needed to determine the extent to which insecticide resistance may be the cause of the unexpected increase in disease incidence.

In these settings, insecticide resistance monitoring data can also guide the selection of vector control interventions to reduce disease transmission or to stop disease outbreaks.

Table 2 summarizes considerations for different programmatic scenarios.

Table 2. Priority aspects of resistance to monitor in common programmatic scenarios

Programmatic scenario	Programmatic needs	Priority	Other useful characteristics of resistance
Introducing an insecticide-based vector control intervention for the first time, or changing vector control interventions or insecticide after resistance is detected	Select an intervention that is effective against the local vector population	To evaluate presence of resistance in the local vector population to the insecticides in the interventions under consideration, before any intervention is deployed	Depending on the intervention under consideration, evaluation of other aspects can help, such as: • PBO involvement in restoration of susceptibility to pyrethroids – can help to identify suitable areas for deploying products containing PBO; • analysis of resistance mechanisms – can help with selecting an insecticide with a different mode of action; and • resistance intensity – could help prioritize areas for a change in vector control interventions when resources are not sufficient to implement a change in all areas where resistance has been detected.
Insecticide-based vector control is already in progress	Detect resistance as soon as it emerges to trigger a change of interventions	To evaluate presence of resistance in the local vector population to the insecticides in use	Monitoring molecular mechanisms that are known to be related to phenotypic resistance to the insecticide in use can help with early detection of resistance emergence.
Unexpected increase in disease transmission	Identify the causes of the increase	To evaluate presence of resistance to the insecticide used. This can indicate whether reduced vector control efficacy may be a cause for such unexpected transmission patterns.	Where resistance of some level is already present, data on resistance intensity can indicate whether an exacerbation of resistance could be contributing to the increase in disease incidence.

5. Testing a relevant and representative sample of mosquitoes

Insecticide resistance monitoring results will be relevant if they are conducted using mosquito vectors of epidemiological importance; reliable if they use a representative sample of local mosquitoes (i.e. avoiding the use of too many sibling mosquitoes in the same bioassay); and comparable across time if they are always conducted with healthy mosquitoes of the same sex, developmental stage and age, collected from the same site and following the same testing procedures (see Box 3).

Box 3. Recommendations on mosquitoes to be used for resistance testing

- Prioritize testing the main vector of disease first, then consider testing secondary vectors.
- Where possible, test each vector species separately. Alternatively, identify mosquitoes after the test to estimate test outcomes for each species.
- Conduct the test with unfed female mosquitoes that are 3–5 days of age and have been starved 6 h before the test (except for pyriproxyfen, when they should be 5–7 days of age and blood-fed 1 h before exposure).
- Use the vector developmental stage that is targeted by the vector control intervention implemented or under consideration (e.g. adults for IRS and ITNs, larvae for larviciding).
- Test individuals that are in good physical condition (e.g. well nurtured, kept in uncrowded trays and cages).
- Use a sample with enough genetic diversity to represent the local vector population (i.e. collect mosquitoes from several houses, larval habitats and areas to avoid having many siblings in the tested sample).

5.1 Vector developmental stage and origin

Procedures exist to evaluate insecticide resistance in both adult and larval stages of mosquitoes.

- If the vector control intervention under consideration targets adult mosquitoes (e.g. ITNs, IRS, space spraying for *Aedes* control), insecticide resistance testing should be performed using adults. Standardized test procedures for adults – that is, the WHO tube test and WHO bottle bioassay – are available and well validated.
- If the vector control intervention under consideration targets larval stages (e.g. chemical larviciding), insecticide resistance testing should be ideally conducted using larvae. However, susceptibility test procedures for larvae have not been further validated or updated since they were established in 1981. Therefore, at present, they are less standardized than susceptibility test procedures with adults.

When resistance is evaluated using adults, it is recommended to collect them using one of three methods. Table 3 outlines these methods, rationale for their use, and advantages and disadvantages of each one.

Table 3. Developmental stage and origin of mosquitoes for resistance testing

Stage and origin	Rationale for use	Advantages	Disadvantages
Adults reared from wild-caught blood-fed female mosquitoes, preferably collected indoors (F1)	The preferred method, and especially suitable when the main vector species of an area is not known, or if this information is outdated.	Increases the likelihood that a test is conducted with a representative sample of the main vector species. Species can be identified before testing to avoid wasting resources testing vectors that are not epidemiologically important.	• May not be feasible in settings with low mosquito densities. • Can be time-consuming to collect sufficient female mosquitoes. • Requires insectary or mosquito rearing facility.
Adults reared from field-collected mosquito larvae (F1)	To be used when collecting wild adult mosquitoes is not feasible, such as when numbers are insufficient, but the larval habitats of the main vector species can be found.	Easier to obtain recommended mosquito numbers and can be less time-consuming.	• If the breeding habitats of main vector species are not known and cannot be found, sampling in random larval habitats may lead to testing species that are not the main vectors of transmission (e.g. zoophilic species). • Requires insectary or mosquito rearing infrastructure. • Larvae may have had prior non-lethal exposure to insecticides, which may affect bioassay outcomes.
Wild-caught adult females, preferably collected indoors (F0)	Practical when infrastructure to rear mosquitoes in adequate conditions is lacking or inadequate. To be used in emergency settings when collecting wild larvae may not be feasible, and rearing facilities are not available (e.g. refugee camps).	The sample may better represent the wild vector population transmitting a given disease at a given time and location. Does not need rearing facilities.	• Age, physiological conditions and infection status of vectors cannot be controlled, reducing the comparability of results between sites and over time. • Mosquitoes may have had prior non-lethal exposure to insecticides, which may affect bioassay outcomes. • Wild-caught females may be infectious for a pathogen and will need to be handled with care.

5.2 Mosquito sampling recommendations

The recommendations in Box 4 should be followed to ensure resistance testing with a sample of mosquitoes that is representative of the local vector population – that is, a sample that contains mosquitoes with a similar genetic diversity to that present in the local vector population. Box 4 provides recommendations for sampling mosquitoes to obtain a representative sample.

Box 4. Recommendations for mosquito sampling

Collection of wild adult female mosquitoes

- Collect adult female mosquitoes indoors from rooms where people slept the night before, or from the known preferred indoor and outdoor resting places of the main vector species.
- If adults are collected with the aim of using their F1 offspring for susceptibility testing, at least 30 blood-fed adult female mosquitoes should be collected from a number of different houses within a sampling area, and their offspring should be pooled before testing. This is to avoid the inclusion of a large number of siblings or individuals from the same strain in the same test sample.
- If F0 adults are collected for immediate use in bioassays, they should be collected from several geographically separate locations within the village, avoiding collecting mosquitoes from adjacent houses. This is to avoid having too many siblings in the same sample.
- Characteristics of the locality where adult mosquitoes are collected (e.g. implemented vector control interventions, agricultural practices) should be recorded because pre-exposure of mosquitoes to insecticide may affect test results.

Collection of wild mosquito larvae

- Collect larvae from the preferred larval habitats of the main vectors of transmission.
- Larvae should ideally be collected from as many different and geographically separate larval habitats as possible.
- Larvae should be pooled and reared together to avoid having a high proportion of siblings in the same test sample.
- Characteristics of the locality where larvae are collected (e.g. implemented vector control interventions, agricultural practices) should be recorded because pre-exposure of larvae to insecticides may affect test results.

Recommendation to ensure comparability of results over time

- Mosquito adults and/or larvae should be sampled from the same houses/larval habitats over time, and any changes to the collection site that may have altered vector exposure to insecticides (e.g. implemented vector control interventions, new agricultural practices) should be well noted.

5.3 Mosquito rearing conditions

Mosquito rearing conditions, such as temperature, water quality, food, larval crowding and mosquito manipulation during rearing processes or during the performance of resistance tests, can affect test results *(29, 30)*. To ensure comparability of test results over time, it is recommended to consistently adhere to the same mosquito rearing and handling protocols.

Mosquitoes should be handled carefully at all times, but particularly during testing and during rearing when they are in the earliest instars. Immature larval stages should be kept in rearing facilities in uncrowded trays with a water temperature of 25 °C ± 5 °C. Adult mosquitoes should be maintained in uncrowded cages at 27 °C ± 2 °C and relative humidity of 75% ± 10%.

To obtain the recommended number of mosquitoes for a test, wild-caught adult female mosquitoes or F1 offspring of wild-caught mosquitoes can be accumulated in cages as they emerge from pupae, or as they are collected from the field. While in the cages, mosquitoes should be provided with access to 10% sugar solution (and a bloodmeal 1 h before testing with pyriproxyfen). The sugar–water meal should be removed about 6 h before conducting a test. As described in Box 3, tests with pyriproxyfen require using blood-fed females.

5.4 Physiological status, sex and age of adult mosquitoes

Female mosquitoes should be used for resistance monitoring. Use of males for resistance monitoring is not recommended because they are not epidemiologically relevant (as they do not transmit pathogens), they are more susceptible to insecticides than females *(31)*, and vector control interventions always target female mosquitoes. For testing pyriproxyfen, males cannot be used because the end-point of the test is the inhibition of oviposition by female mosquitoes.

Studies using adult female mosquitoes have repeatedly shown that both age and physiological status (i.e. whether they are non–blood fed, semi-gravid or gravid) have a marked effect on susceptibility to insecticides. Older mosquitoes have shown increased susceptibility to insecticides than younger ones *(32–37)*, particularly when resistance is conferred by the presence of a detoxifying enzyme, the activity of which may decline with age *(44)*. Blood-fed mosquitoes have exhibited higher resistance than their unfed counterparts *(36, 38)*. Hence, standardizing mosquito age and feeding status is important to ensure comparability of test results over time. For all insecticides, except pyriproxyfen, female test mosquitoes should be 3–5 days old and non–blood fed. For pyriproxyfen, female test mosquitoes should be 5–7 days old to allow them enough time to mate before the test, and blood-fed 1 h before exposure to ensure that they can lay eggs during the test.

5.5 Vector species

Vector control interventions aim to control the main vector of disease to reduce disease transmission. In many endemic regions for vector-borne diseases, several vector species may be present concurrently, but their contribution to disease transmission may be different. Hence, it is important to ensure that resistance is monitored first in the main vectors of disease and then in secondary vectors.

Resistance bioassays should ideally be conducted with mosquitoes of a single species. However, knowing the species of mosquitoes used in a test is generally only possible where offspring of wild-collected females or adults emerging from wild-collected larvae are used. In the first case, the species of F1 offspring can be identified by determining the species of the mother, as described in Table 4. In the second case, species can be determined from visual inspection of the larvae, although this requires extensive experience in larva identification and is prone to errors. Both of these scenarios require adequate rearing, laboratory and human capacity. Where species identification before the bioassay is not feasible, mosquitoes should be collected from the larval habitats and resting places of the main disease vectors to ensure that the tested sample contains mostly main vectors. For example, for malaria and dengue, indoor-resting mosquitoes will likely provide a sample of the epidemiologically relevant vectors.

When species are not identified ahead of a bioassay, and if resources allow, they should be identified after the bioassay. To estimate mosquito mortality for each individual species, all mosquitoes included in a test should be identified. When resources are insufficient, analysing either all survivors or a subset of survivors can help detect possible resistance in some species. However, full bioassays with a sufficient number of mosquitoes of the species will be required to confirm resistance.

Some species can be discriminated from one another by observing their morphological traits under a microscope using standard taxonomic keys. Some anopheline species are morphologically identical or very similar, and form groups of cryptic species. Examples of such groups are *Anopheles gambiae* s.l., *An. funestus* s.l., *An. culicifacies* s.l., *An. fluviatilis* s.l., *An. dirus* s.l., *An. minimus* s.l., *An. nuneztovari* s.l. and *An. albitarsis*. Species within these groups can only be distinguished using molecular techniques, which can detect genetic differences among them. Molecular methods for species identification are provided in the publication *Methods in Anopheles research manual (39)*. Such methods require special ways of preserving mosquito samples.

Table 4. Process for conducting bioassays with only one vector species (if F1 adult progeny are used) or identifying the species post-test (if F0 adults are used)

Mosquitoes used in tests	Process
F1 progeny of wild-caught adult females	Species can be identified before starting a test as follows. • Hold females in individual cages or cups, allowing them to lay eggs separately from other females. • After eggs have been laid, kill the females and identify their species. • Pool the progeny of females of the same species to conduct a susceptibility test. This will guarantee that all pooled mosquitoes used in the test are of the same species.
F0 adults reared from wild-caught larvae, or wild-caught females used directly	Species should be identified after the test as follows. • At the end of the test, separate mosquitoes into four groups: – mosquitoes that died after exposure to the insecticide; – mosquitoes that survived after exposure to the insecticide; – control mosquitoes that died; and – control mosquitoes that remained alive. • Store each specimen in an individual 0.5 mL microcentrifuge tube with silica gel or in 70% ethanol. Each mosquito should be clearly labelled as dead or alive, treated or control. • Identify individual mosquitoes by polymerase chain reaction using standard methods. • Calculate mortality (or oviposition inhibition in the case of pyriproxyfen) for individual species, considering their respective control mortality (i.e. correcting mortality with Abbott's formula, when needed).

6. Standard bioassays

Standard bioassays have been developed to generate insecticide resistance data that can be compared over time and across locations. These include two complementary bioassays developed by WHO – the WHO tube test and the WHO bottle bioassay – as well as a bottle bioassay developed by the CDC.

Standard bioassays are direct response-to-exposure tests, which measure the effect of exposure to an insecticide or a synergist on a sample of mosquitoes. They can be used to evaluate the following aspects of resistance in a mosquito sample:

- presence of resistance (susceptibility bioassays);
- intensity of resistance (intensity bioassays); and
- ability of a synergist to restore mosquito susceptibility to an insecticide to which the mosquitoes are otherwise resistant (synergist–insecticide bioassays).

SOPs for the WHO tube test and the WHO bottle bioassay to evaluate the presence of resistance, and for the WHO tube test to evaluate the ability of PBO to restore mosquito susceptibility to pyrethroids were developed as part of a WHO multicentre study in 2021 *(20)* and are provided in the WHO website *(22)*. Procedures for the CDC bottle bioassay to evaluate presence and intensity of resistance and a synergist's ability to restore vector susceptibility to insecticides are provided on the CDC website *(23)*.

6.1 WHO susceptibility bioassays

The WHO susceptibly bioassays (tube test and bottle bioassay) are used to detect the presence of resistance to an insecticide in a vector population. They measure the phenotypic response of a vector sample after exposure to an insecticide.

In these bioassays, mosquitoes are exposed for 1 h to filter papers or bottles treated with an insecticide at the DC. The desired insecticide phenotypic effects – such as mosquito mortality (for adulticides) or oviposition inhibition (for pyriproxyfen) – are measured after a fixed holding period post-exposure. The DCs are calibrated to differentiate between susceptible and resistant mosquitoes, based on the results at the end of the holding period. Mosquitoes that are still alive or, that show low or no changes in oviposition (for pyriproxyfen) at the end of the holding period are considered to be resistant to the insecticide. The tests allow estimation of the percentage of resistant mosquitoes in a population. The classification method presented in Fig. 4 (in section 6.3) can be used to determine whether the mosquito population is resistant or susceptible to the insecticide, or whether resistance is possible and should be confirmed by additional bioassays.

Until recently, the only available WHO standard susceptibility test procedure was the WHO tube test. This test involves exposing adult mosquitoes to papers impregnated with an insecticide DC in specially designed plastic tubes. However, the chemical properties of some insecticides prevent their impregnation on filter papers. To allow monitoring of vector resistance to these insecticides, a new susceptibility test, the WHO bottle bioassay, was developed between 2017 and 2021 through a WHO-coordinated multicentre study *(20)*.

The WHO bottle bioassay is a modified version of the CDC bottle bioassay *(23)*. In both bioassays, mosquitoes are exposed to an insecticide by holding them in glass bottles (250 mL volume) coated with a mixture of an insecticide and acetone (as a solvent). For some insecticides, a surfactant is also used for coating bottles to prevent crystallization of the insecticide. The differences between the two bottle bioassays are the length of exposure of mosquitoes in bottles, the insecticide concentration used for coating the bottles and the test end-point. For harmonization with the WHO tube test, which has long been used by entomological personnel in disease control programmes, the WHO bottle bioassay involves exposing mosquitoes to an insecticide DC for a fixed period of 1 h, and measuring mosquito mortality 24 h after the exposure period (or 72 h in the case of chlorfenapyr). In contrast, in the CDC bottle bioassay, mosquitoes are exposed to insecticide-coated bottles for 30 min (or 45 min for DDT), and the test end-point is the mosquito mortality at the end of the exposure time. The main differences between the two procedures are shown in Table 5.

Table 5. Differences between WHO bottle bioassay and CDC bottle bioassay to evaluate vector susceptibility to insecticides other than pyriproxyfen

Bioassay	Number of treated bottles	Number of control bottles	Duration of exposure	Time after exposure at which mosquito mortality is evaluated
WHO bottle bioassay	4	2	1 h	24 h (or 72 h for chlorfenapyr)
CDC bottle bioassay	4	1	30 min (or 45 min for DDT)	At end of 30 min exposure period (or 45 min for DDT)

Based on the evidence generated in the WHO multicentre study and taking into account some historical data, WHO now recommends the insecticide DCs presented in Tables 6, 7 and 8 for adult *Anopheles*, *Aedes* and *Culex* species respectively. These standard DCs cover several insecticides of public health importance and several of the main disease vector species. Consistent use of the same DC is crucial to detect and monitor the emergence and spread of insecticide resistance over time.

To monitor resistance in a vector species that is not listed in these tables or for which no WHO standard DC has been established, countries may use the highest insecticide DC recommended for a species in the same genus, until species-specific DCs are established.

Fig. 1 provides a schematic representation of a WHO susceptibility test conducted using the WHO tube test procedure.

Fig. 1. Steps in the WHO tube test with adult mosquitoes for insecticides with killing effect

Pre-exposure holding period (1 h): Hold mosquitoes in holding tubes lined with clean white paper.

Exposure period (1 h): Expose mosquitoes to insecticide-impregnated papers (red tubes) or control papers treated with oil or acetone alone (yellow tubes).

Post-exposure holding period (24 h): Transfer mosquitoes into the holding tubes. Provide them access to a water-sugar solution. Read mortality at the end of the 24 h.

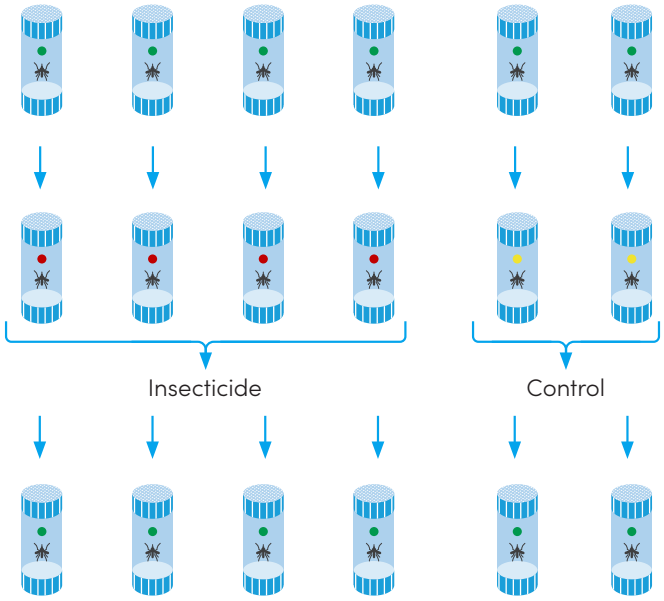


Fig. 2 provides a schematic representation of the phases of the generic WHO bottle bioassay and highlights the particularities for chlorfenapyr. A surfactant (81% rapeseed oil methyl ester – MERO) is needed to coat bottles with clothianidin and flupyradifurone to prevent crystallization of the active ingredients.

Due to the variability of results obtained while testing susceptible mosquitoes against chlorfenapyr during the multicentre study coordinated by WHO *(20)*, special requirements have been imposed on tests with chlorfenapyr for now:

1) a sample from a laboratory susceptible strain should be tested in parallel to the wild mosquito sample to validate the test. The reason for testing a susceptible mosquito strain in parallel is to validate the results of the test with the wild mosquito sample, because mortality in susceptible mosquitoes exposed to chlorfenapyr has sometimes been observed to be below the WHO susceptibility threshold. Both the test with the wild mosquitoes and that with the susceptible strain should include two control bottles each;
2) the temperature during bioassays with chlorfenapyr should be strictly held in the range 27 ± 2 °C, with humidity 75 ± 10%. This is because results across laboratories were inconsistent when the temperature during the test was below 25 °C; and
3) resistance to chlorfenapyr can only be confirmed when three WHO bottle bioassays, conducted with samples of the same vector population and at different time points, show a mosquito mortality <90% 72 h post exposure with mortality in the susceptible sample at the same time point being ≥98% in each test.

These special requirements may be withdrawn after more field data on susceptibility test with chlorfenapyr has been received and evaluated by WHO.

Fig. 2. Steps in the WHO bottle bioassay, highlighting the particularities of this test with chlorfenapyr

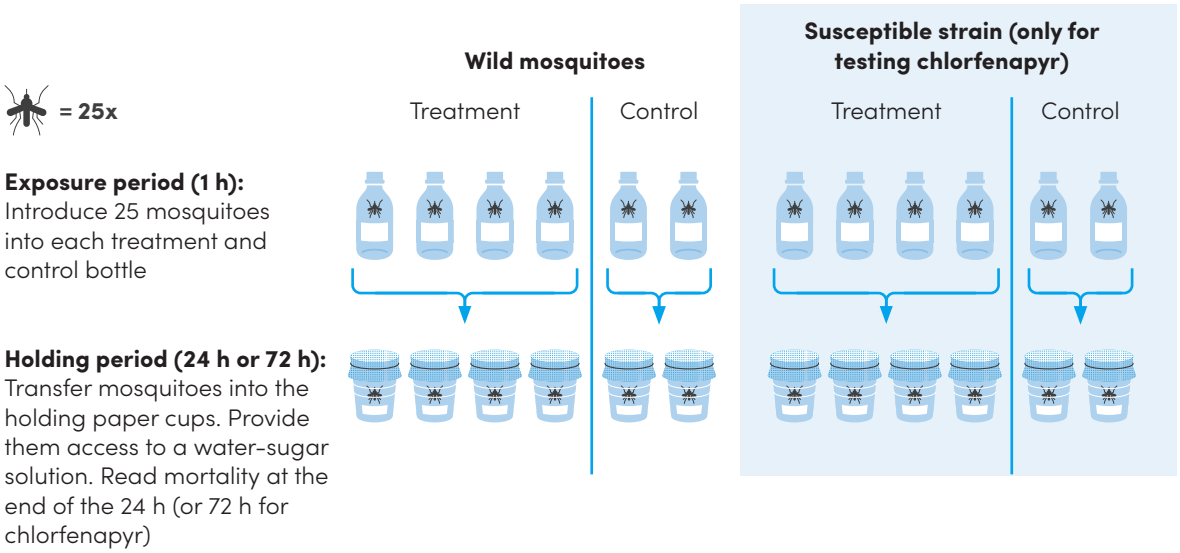


Fig. 3 provides a schematic representation of the WHO bottle bioassay to test vector resistance to pyriproxyfen, a juvenile hormone mimic compound. This procedure was developed between 2017 and 2021 through the WHO-coordinated multicentre study to establish procedures and DCs for new insecticides and mosquito species *(20)*. The procedure is more laborious and longer than the regular WHO bottle bioassay. This is because, in contrast to other insecticides whose aim is to kill mosquitoes, juvenile hormone mimics, such as pyriproxyfen, inhibit or reduce the fertility and fecundity of adult female mosquitoes. Although they can reduce a) the number of mosquitoes that lay eggs (oviposition rate), b) the number of eggs laid by each mosquito, c) the hatching of the laid eggs, d) the development of larvae to subsequent instars/pupae, or e) shorten mosquitoes' lifespan, the easiest effect to measure is the effect of pyriproxyfen on the number of mosquitoes that lay eggs *(39)*. This is measured by visual comparison of the proportion of treated and control mosquitoes that laid some eggs, as described in section 6.7. Hence, this bioassay involves the following steps:

- coating glass bottles with pyriproxyfen;
- drying bottles for 2 h;
- using female mosquitoes that are allowed to mate in cages during the previous 4 days before exposure;
- blood feeding the female mosquitoes 1 h before exposure;
- exposing the blood-fed females for 1 h in coated bottles, along with controls (Note: this test requires 16 bottles in total, i.e. four treatment bottles and four control bottles with wild-caught females and the same number with susceptible colonized strain; 400 mosquitoes in total);
- transferring the females into holding paper cups for 72 h after exposure to pyriproxyfen;
- chambering individual females in paper cups and monitor oviposition for 4 days; and
- counting the number of treated and control mosquitoes that laid eggs to calculate oviposition rate in treatments and control, and estimate the oviposition inhibition.

The test has been developed using laboratory strains, and concerns exist about the ability of wild-caught (or offspring of wild-caught) female mosquitoes to lay eggs during these laboratory procedures. If oviposition rates are low, dissection of ovaries can be considered as an alternative method to quantify oviposition rates. The procedures will be revised after data from tests conducted with wild mosquito populations have been generated and reported to WHO.

Fig. 3. Steps in the WHO bottle bioassay conducted with adult mosquitoes for juvenile hormone mimics

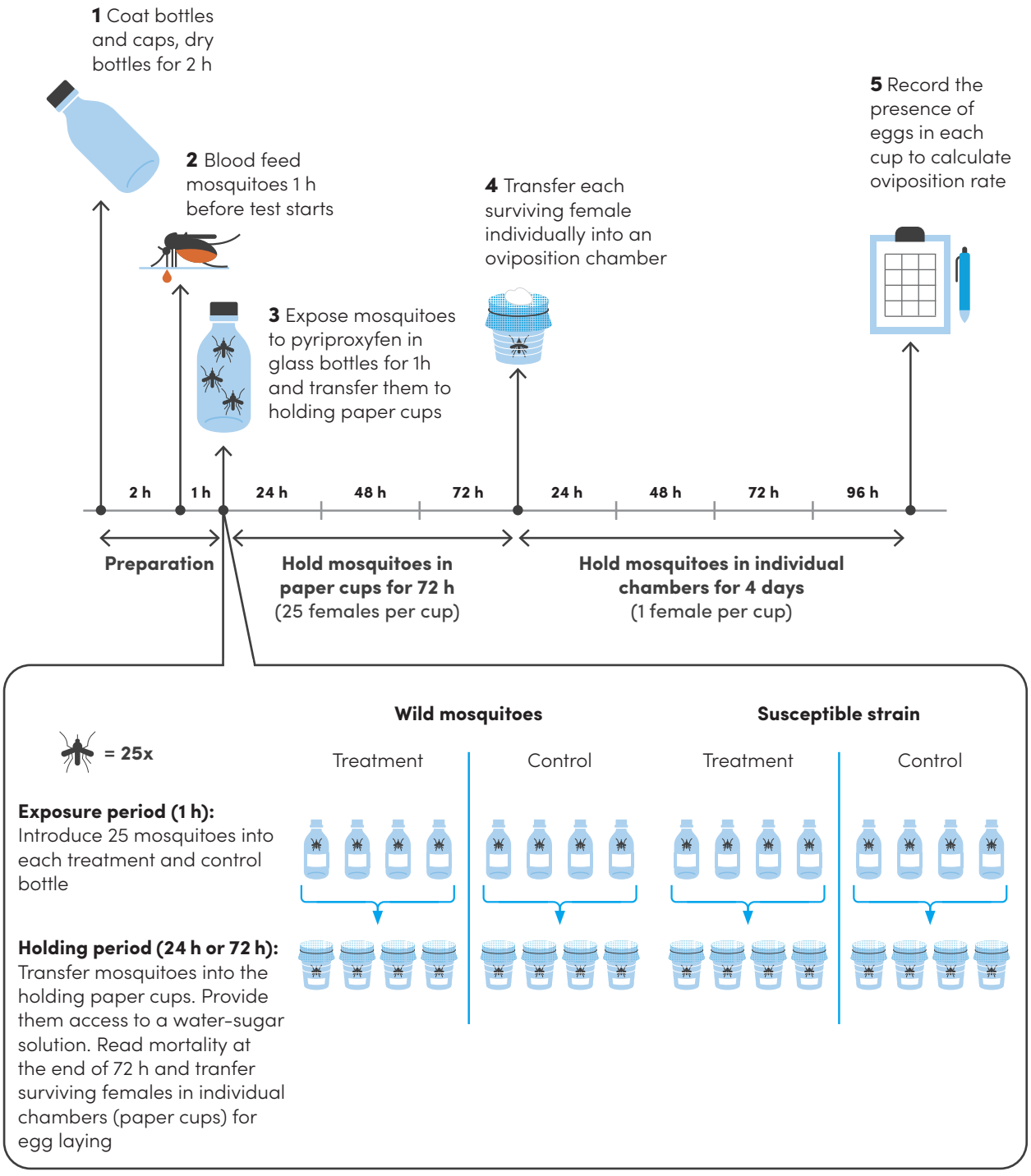


Table 6. Insecticide discriminating concentrations for WHO susceptibility bioassays with *Anopheles* mosquitoes

Insecticide class/ synergist	Test method	Insecticide	Species for which discriminating concentrations are validated	Discriminating concentration[a]	Available intensity concentrations on filter papers[b]	Exposure period	Holding period	Carrier oil/ solvent/ surfactant
Pyrethroids	WHO tube test	Alpha-cypermethrin	*An. funestus s.s., An. gambiae s.s., An. minimus*	0.05%	5× and 10×	1 h	24 h	Silicone oil
			An. albimanus, An. stephensi	0.30%[c]		1 h	24 h	Silicone oil
		Cyfluthrin	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus, An. stephensi*	0.15%	5× and 10×	1 h	24 h	Silicone oil
		Deltamethrin	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus, An. stephensi*	0.05%	5× and 10×	1 h	24 h	Silicone oil
		Etofenprox	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. stephensi*	0.50%	5× and 10×	1 h	24 h	Silicone oil
		Lambda-cyhalothrin	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus, An. stephensi*	0.05%	5× and 10×	1 h	24 h	Silicone oil
			An. sacharovi	0.10%		1 h	24 h	Silicone oil
		Permethrin (40:60 cis:trans isomer ratio)	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus, An. stephensi*	0.75%	5× and 10×	1 h	24 h	Silicone oil
	WHO bottle bioassay	Transfluthrin	*An. albimanus, An. stephensi, An. funestus, An. minimus, An. gambiae s.s.*	2 µg/bottle	–	1 h	24 h	Acetone only

Insecticide class/ synergist	Test method	Insecticide	Species for which discriminating concentrations are validated	Discriminating concentration[a]	Available intensity concentrations on filter papers[b]	Exposure period	Holding period	Carrier oil/ solvent/ surfactant
Carbamates	WHO tube test	Bendiocarb	NA	0.10%	5× and 10×	1 h	24 h	Olive oil
		Carbosulfan	NA	0.40%[d,e]	–	1 h	24 h	Olive oil
		Propoxur	NA	0.10%[e]	–	1 h	24 h	Olive oil
Organochlorines		DDT	NA	4.00%	–	1 h	24 h	Risella oil
		Dieldrin	NA	4.00% / 0.4%[e,f]	–	1 h	24 h	Risella oil
Organophosphates		Fenitrothion	NA	1.00%[e]	–	2 h	24 h	Olive oil
		Malathion	NA	5.00%	–	1 h	24 h	Olive oil
		Pirimiphos-methyl	*An. albimanus, An. stephensi, An. minimus, An. funestus s.s.*	100 mg/m^2 [c]	–	1 h	24 h	Acetone only
			An. gambiae s.s	170 mg/m^2 [c]	–	1 h	24 h	Acetone only
Synergist		Piperonyl butoxide	NA	4.00%	–	1 h	24 h	Silicone oil
Neonicotinoids	WHO bottle bioassay	Clothianidin	*An. albinamus, An. stephensi*	10 µg/bottle	–	1 h	24 h	Acetone + MERO 800 ppm
			An. funestus s.s., An. gambiae s.s.	4 µg/bottle	–	1 h	24 h	Acetone + MERO 800 ppm
			An. minimus	6 µg/bottle	–	1 h	24 h	Acetone + MERO 800 ppm
Butenolides		Flupyradifurone	*An. albimanus*	500 µg/bottle	–	1 h	24 h	Acetone + MERO 200 ppm
			An. stephensi, An. gambiae s.s.	60 µg/bottle	–	1 h	24 h	Acetone + MERO 200 ppm
			An. funestus s.s., An. minimus	100 µg/bottle	–	1 h	24 h	Acetone + MERO 200 ppm

Insecticide class/ synergist	Test method	Insecticide	Species for which discriminating concentrations are validated	Discriminating concentration[a]	Available intensity concentrations on filter papers[b]	Exposure period	Holding period	Carrier oil/ solvent/ surfactant
Pyrroles	WHO bottle bioassay	Chlorfenapyr	*An. gambiae s.s., An. stephensi, An. funestus s.s., An. albimanus*	100 µg/bottle	–	1 h	72 h	Acetone only
JH hormone mimics		Pyriproxyfen	*An. gambiae s.s., An. stephensi, An. funestus s.s.*	100 µg/bottle	–	1 h	72 h for mortality, 7 d for oviposition inhibition	Acetone only

–: not available; MERO: 81% rapeseed oil methyl ester (manufactured by Bayer CropScience); NA: species-specific data not available.

[a] Tube test: DC as percentage; for pirimiphos-methyl, in mg/m^2. Bottle bioassay: DC in µg/bottle (250 mL).

[b] These intensity concentrations have not been validated by WHO, but papers impregnated with pyrethroid intensity concentrations are available for procurement from Universiti Sains Malaysia for research purposes.

[c] These DCs supersede the tentative concentration presented in *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes* (second edition) *(18)*. Results obtained using the former tentative concentration should be validated against these new DCs.

[d] Tentative, needs confirmation; based on data published by N'Guessan et al. (2003) *(40)* and Ahoua Alou et al. (2010) *(41)*.

[e] Impregnated papers no longer supplied by Universiti Sains Malaysia.

[f] Exposure to dieldrin at 0.4% kills susceptible (SS) mosquitoes but not resistant heterozygotes (RS); exposure to dieldrin at 4% kills heterozygote (RS) but not homozygous (RR) resistant individuals *(16)*.

Table 7. Insecticide discriminating concentrations for WHO susceptibility bioassays with *Aedes* mosquitoes

Insecticide class	Test method	Insecticide	Species for which discriminating concentrations are validated	Discriminating cncentrations[a]	Exposure period	Holding period	Carrier oil/ solvent/ surfactant
Pyrethroids	WHO tube test	Alpha-cypermethrin	*Ae. aegypti*	0.05%[b]	1 h	24 h	Silicone oil
			Ae. albopictus	0.08%[b]	1 h	24 h	Silicone oil
		Deltamethrin	*Ae. aegypti, Ae. albopictus*	0.03%[b]	1 h	24 h	Silicone oil
		Lambda-cyhalothrin	*Ae. aegypti*	0.05%[b]	1 h	24 h	Silicone oil
			Ae. albopictus	0.08%[b]	1 h	24 h	Silicone oil
		Permethrin (40:60 cis:trans isomer ratio)	*Ae. aegypti, Ae. albopictus*	0.40%[b]	1 h	24 h	Silicone oil
	WHO bottle bioassay	Transfluthrin	*Ae. aegypti, Ae. albopictus*	3 µg/bottle	1 h	24 h	Acetone only
		Metofluthrin	*Ae. aegypti, Ae. albopictus*	1 µg/bottle	1 h	24 h	Acetone only
		Prallethrin	*Ae. aegypti, Ae. albopictus*	30 µg/bottle	1 h	24 h	Acetone only
Carbamates	WHO tube test	Bendiocarb	*Ae. aegypti, Ae. albopictus*	0.20%	1 h	24 h	Olive oil
		Propoxur	*Ae. aegypti*	0.10%[c]	1 h	24 h	Olive oil
Organophosphates		Chlorpyrifos-ethyl	*Ae. aegypti, Ae. albopictus*	1.00%	1 h	24 h	Olive oil
		Pirimiphos-methyl	*Ae. aegypti, Ae. albopictus*	60 mg/m^2 [b]	1 h	24 h	Acetone only
		Malathion	*Ae. aegypti*	1.50%[b]	1 h	24 h	Olive Oil
			Ae. albopictus	5.00%[b]	1 h	24 h	Olive Oil

Insecticide class	Test method	Insecticide	Species for which discriminating concentrations are validated	Discriminating cncentrations[a]	Exposure period	Holding period	Carrier oil/ solvent/ surfactant
Neonicotinoids	WHO bottle bioassay	Clothianidin	*Ae. aegypti*	20 µg/bottle	1 h	24 h	Acetone + MERO 1500 ppm
			Ae. albopictus	10 µg/bottle	1 h	24 h	Acetone + MERO 1500 ppm
Butenolides		Flupyradifurone	*Ae. aegypti, Ae. albopictus*	80 µg/bottle	1 h	24 h	Acetone + MERO 1500 ppm

MERO: 81% rapeseed oil methyl ester (manufactured by Bayer CropScience).

[a] Tube test: DC as percentage. Bottle bioassay: DC in µg/bottle (250 mL).

[b] These DCs supersede the tentative concentration presented in *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes* (second edition) *(13)*. Results obtained using the former tentative concentration should be validated against these new DCs.

[c] Impregnated papers no longer supplied by Universiti Sains Malaysia.

Table 8. Insecticide discriminating concentrations for WHO susceptibility bioassays with *Culex quinquefasciatus*[a]

Insecticide class	Test method	Insecticide	Species for which discriminating concentrations are validated	Discriminating concentration	Exposure period	Holding period	Carrier oil
Organochlorines	WHO tube test	DDT	*Cx. quinquefasciatus*	0.04%	4 h	24 h	Risella oil
Pyrethroids		Deltamethrin[a]		0.025%	1 h	24 h	Silicone oil
		Lambda-cyhalothrin		0.025%	1 h	24 h	Silicone oil
		Permethrin (40:60 cis:trans isomer ratio)		0.25%	3 h	24 h	Silicone oil
Carbamates		Propoxur[b]		0.10%	1 h	24 h	Olive oil
Organochlorines		Fenitrothion[b]		1%	2 h	24 h	Olive oil
		Malathion[a]		5%	1 h	24 h	Olive oil

[a] These concentrations are currently being re-evaluated by WHO.

[b] Impregnated papers no longer supplied by Universiti Sains Malaysia.

6.2 WHO intensity bioassays

WHO intensity bioassays are used to measure the intensity of mosquito resistance to an insecticide once resistance has already been established within a vector population.

WHO intensity bioassays follow the procedure previously described for susceptibility bioassay (Fig. 1) but require incremental testing – that is, exposing samples of mosquitoes to an increasing insecticide concentration, from 1× the DC to 5× and then 10× the DC. Intensity bioassays compare mosquito mortality after exposure to these successive insecticide concentrations using the algorithm presented in Fig. 5 (in section 6.8) to determine whether resistance intensity is low, moderate or high. Intensity bioassays should be conducted using the WHO tube test procedure or the CDC bottle bioassay procedure because the WHO bottle bioassay procedure has not yet been validated for the evaluation of resistance intensity. To date, papers impregnated with multiples (5× and 10×) of the DC are available for procurement only for pyrethroid insecticides.

6.3 WHO synergist–insecticide bioassays

WHO synergist–insecticide bioassays are used to measure the ability of a synergist to restore mosquito susceptibility to an insecticide to which they are resistant. In this context, a synergist is a compound that inhibits the activity of certain enzymes responsible for detoxifying insecticides in the insect's body. It is not an insecticide in itself and, hence, it does not have direct toxic effects on mosquitoes, although it may aid cuticular penetration of insecticides. If the mechanism responsible for mosquito resistance to a certain insecticide is metabolic, pre-exposure to a synergist may restore susceptibility to the insecticide.

PBO is currently used as a synergist in some WHO prequalified vector control products – namely, pyrethroid–PBO nets and some space spray products. Hence, synergist–insecticide bioassays can guide the deployment of these products.

In the WHO synergist–insecticide bioassay, a sample of mosquitoes is exposed first to filter papers impregnated with a standard non-lethal concentration of a synergist for 1 h and then to papers impregnated with a DC of an insecticide for another 1 h. Another sample of mosquitoes is exposed only to papers impregnated with the DC of the insecticide. Mosquito mortality in the two treatment groups is measured at the end of a 24 h holding period. Differences in mortality between the two test samples are used to evaluate whether the synergist is able to restore vector susceptibility to the insecticide. The classification method presented in Fig. 5 (in section 6.8) can be used to determine whether the restoration of susceptibility is full, partial or none.

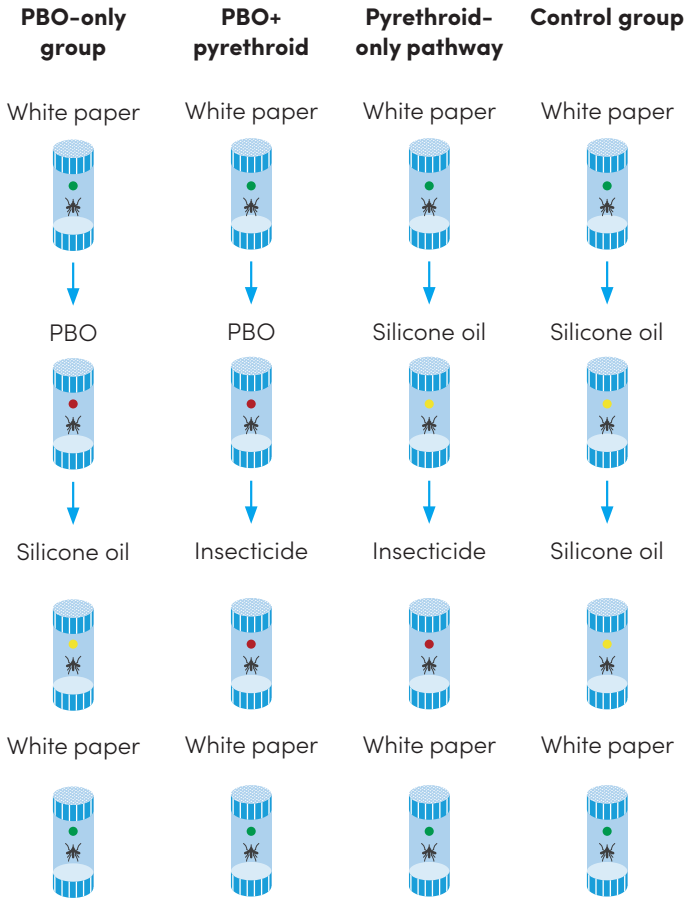
The exposure pathways for mosquito samples in the WHO synergist–insecticide bioassay are shown in Fig. 4. This procedure resembles the WHO susceptibility bioassays; the differences are that:

- four tubes (instead of six) are used in each test, one for exposure to synergist only, one for exposure to insecticide only, one for exposure to synergist followed by insecticide, and one for control; and
- the procedure (shown in Fig. 4) should be repeated four times until 100 mosquitoes have been exposed in each pathway.

A complete SOP for conducting the WHO synergist–insecticide bioassay to test the ability of PBO to restore susceptibility to pyrethroids using the WHO tube test can be found on the WHO website *(22)*.

Synergists are available for certain metabolic detoxification enzyme groups, including esterases, oxidases and glutathione S-transferases. However, a standard concentration for use in WHO synergist–insecticide bioassays has only been established for PBO (i.e. 4%), to measure its effect on resistant *Anopheles* vector species.

Fig. 4. Steps of a synergist–insecticide bioassay test



6.4 A note on the CDC bottle bioassay

The CDC bottle bioassay provides a complementary method to evaluate resistance in vector populations to insecticides other than juvenile hormone mimics, including detecting presence of resistance, measuring its intensity and evaluating the involvement of a synergist in restoring susceptibility to insecticides *(23)*. As with the WHO tube test or WHO bottle bioassay, this bioassay measures the phenotypic response of a vector population after exposure to an insecticide.

Because the tests have different end-points (see section 6.1), results of the CDC bottle bioassay cannot be compared with those of the WHO tube test or the WHO bottle bioassay. However, both tests can detect the presence or absence of insecticide

resistance in a vector population. In the same way that insecticide DCs have been established to detect insecticide resistance using WHO standard bioassays, standard diagnostic dosage and exposure times have been established to detect resistance using the CDC bottle bioassay. Differences in interpretation of results from the WHO tube test or WHO bottle bioassay and the CDC bottle bioassay are highlighted in Table 9. Because they use different materials and procedures, each procedure has advantages and disadvantages that are presented in table 10. Irrespective of what test is selected to assess vector susceptibility to insecticides, the same test should be consistently used over time to ensure comparability of results in a given area.

Table 9. Differences in interpretation of susceptibility results between WHO bioassays and the CDC bottle bioassay

Procedure	A vector population is considered to be resistant to an insecticide when:
WHO standard bioassays (tube test and bottle bioassay)	Mosquito mortality measured at the end of the holding time (usually 24 h) after exposure to a DC of insecticide is below 98% (possible resistance) or below 90% (confirmed resistance).
CDC bottle bioassay	Mosquito mortality measured immediately at the end of exposure to a diagnostic dose of insecticide for the diagnostic period of 30 min or 45 min, depending on the insecticide type, is below 98% (possible resistance) or below 90% (confirmed resistance).

Table 10. Advantages and disadvantages of CDC bottle bioassay compared with WHO tube test for insecticides for which both methods are suitable

Advantages of CDC bottle bioassay	Disadvantages of CDC bottle bioassay
• The procedure is quicker (e.g. no requirement for a 24 h or longer holding period). • Materials are more readily available: 250 mL Wheaton® bottles are widely available, and very small amounts of technical-grade insecticide are needed.	• Bottles are coated at each monitoring site by a technician, rather than being prepared by a central quality-assured production facility in a standard manner. This may affect quality of bottle coating and thus comparability of results. • Adequate human capacity to handle insecticides is required to avoid dangerous exposure to concentrated insecticide during coating of bottles. • Procuring or transporting glass bottles and insecticide aliquots to field sites or decentralized test laboratories is often more cumbersome than transporting plastic tubes and impregnated papers.

6.5 Considerations for conducting bioassays

To ensure that reliable and comparable resistance monitoring data are generated, it is imperative that standard testing conditions and procedures are followed consistently across laboratories. This section presents the recommended number of replicates and mosquitoes for each type of bioassay and the recommended ambient conditions for bioassays. It also provides guidance on the acquisition of testing materials, maximum uses and storage conditions of impregnated papers and coated bottles.

6.5.1 Number of test replicates

To obtain good-quality measurements of mosquito mortality in standard bioassays, the following guidance should be followed.

- **Include enough tubes or bottles in each bioassay** to control for biological random variations in test results. Use of multiple tubes or bottles will reduce the error in the measurement of mosquito mortality and improve confidence in test results.
- **Include control tubes or bottles in each bioassay** to control for the mosquito mortality that is not caused by the insecticides (i.e. caused by external factors such as mosquito handling, environmental test conditions, contamination of tubes or bottles, etc.) and that can lead to a false conclusion that a vector population is susceptible to an insecticide. Control tubes are tubes with papers treated with a mixture of a carrier oil and acetone or acetone alone. Control bottles are treated with acetone or a mixture of a surfactant and acetone. The mortality in control bottles or tubes is used to correct the mortality in treatment bottles or tubes using Abbott's formula.

Table 11 provides the recommended number of replicates (bottles or tubes) for conducting each type of bioassay.

6.5.2 Number of test mosquitoes

The flying behaviour of mosquitoes in tubes or bottles may be different depending on the number of specimens per tube or bottle. This will affect the insecticide dose that each insect receives. To standardize test results, it is recommended to always introduce 25 female mosquitoes into each tube or bottle. When the number of mosquitoes available for a bioassay is less than the recommended number, it is recommended to distribute them such that the number of mosquitoes in each tube or bottle is as close to 25 as possible. For example, if only 81 mosquitoes are available, distribute 75 of them in three tubes or bottles with 25 mosquitoes in each and discard the remaining 6 mosquitos. The optimal recommended numbers of mosquitoes for each type of bioassay are summarized in Table 11.

When the recommended mosquito numbers are not available

When it is not possible to test the recommended number of mosquitoes on a single day, tests can be spread over several days, provided that the mosquitoes tested come from the same vector population (i.e. are collected in the same village with the same collection method) and that, if the F1 generation is used, the mosquitoes are 3–5 days of age (or 5-7 days of age for pyriproxyfen).

- For WHO susceptibility bioassays, replicate tests can be conducted over a few days until the recommended sample size (Table 11) is reached, provided that at least two exposure and two control tubes or bottles are used in parallel in each test.
- For WHO intensity bioassays, the different insecticide concentrations can be tested on different days. For each concentration, sets of two exposure and two control tubes can be tested each day over a few days, as explained for WHO susceptibility bioassays.
- For WHO synergist–insecticide bioassays, repetitions of the process shown in Fig. 4 can be run on different days until the recommended sample size is reached. Each repetition should include at least four tubes – that is, one each for exposure to synergist only, synergist followed by insecticide, insecticide only and control.

If, even then, the number of mosquitoes available is less than the number required for completing a WHO bioassay, tests conducted with less than the recommended number of mosquitoes can still be helpful to identify areas of potential vector resistance. However, the ability of the test to identify the true presence of resistance decreases as the number of mosquitoes used decreases. Where resistance is detected using less than the recommended number of mosquitoes, further tests will be needed to confirm resistance. These can be conducted over the course of the year when mosquitoes become available. If insufficient mosquitoes are available to conduct the desired bioassay, the bioassays should be prioritized to inform the most relevant and immediate programmatic decisions following guidance given in section 3.

Table 11. Optimal numbers of adult mosquitoes recommended for monitoring resistance to insecticides using WHO standard bioassays

Bioassay	Total No. of mosquitoes per test	Control		Treatment	
		No. of mosquitoes per tube/bottle	No. of tubes/ bottles per test	No. of mosquitoes per tube/bottle	No. of tubes/ bottles per test
WHO susceptibility bioassay (for all insecticides except pyriproxyfen and chlorfenapyr)	150	25	2	25	4
WHO susceptibility bioassays with chlorfenapyr	900 (450 wild mosquitoes and 450 from a susceptible colony to complete the 3 tests required to confirm resistance)	25	12 (4 tubes/ bottles per test – 2 for wild mosquitoes, 2 for susceptible colony – for the 3 tests required to confirm resistance)	25	24 (8 tubes/ bottles per test – 4 for wild mosquitoes 4 for susceptible colony – for the 3 tests required to confirm resistance)
WHO susceptibility bioassays with pyriproxyfen	400 (200 wild mosquitoes and 200 from a susceptible colony)	25	8 (4 for wild mosquitoes 4 for susceptible colony)	25	8 (4 for wild mosquitoes and 4 for susceptible colony)
WHO intensity bioassays	250 (to test 5× and 10× DC simultaneously)	25	2	25	4 per concentration tested (8 for testing 5× and 10× DC)
	300 (to test 5× and 10× DC separately)	25	2 per concentration tested (4 for testing both 5× and 10× DC)		
WHO synergist–insecticide bioassays[a]	400 (100 × 4 test rounds)	25	4 (1 in each test round)	25	12 (1 tube for each of the 3 exposure pathways in each test round)

[a] If enough mosquitoes are available, each test round can be conducted with two or more tubes or bottles per exposure pathway, thereby reducing the number of control mosquitoes needed (control mosquitoes are needed each time a bioassay is conducted).

6.5.3 Ambient test conditions

Environmental temperature during mosquito rearing and resistance bioassays influences the toxicity of insecticides to insects *(42–44)*. Both temperature and relative humidity can affect the survival of mosquitoes during the test period. Therefore, these parameters should be controlled, and their values recorded during the exposure and holding periods in all bioassays. In the absence of an insectary or a climate-controlled room, during the bioassays, the tubes or bottles should be placed into in a container (e.g. cool box) covered with a wet towel placed in a sheltered, shaded location. A thermometer or temperature data logger and a hygrometer should be placed in the container to record the range of temperature and humidity during the bioassay.

Tests should be carried out at 27 °C ± 2 °C and relative humidity of 75% ± 10%.

Note: This is especially important for bioassays with chlorfenapyr, as bioassay results for this insecticide are highly sensitive to temperature.

6.5.4 Equipment and materials

Standard susceptibility test kits, insecticide-impregnated papers and other supplies to conduct WHO susceptibility tests with mosquito adults and larvae are currently produced and supplied by the Vector Control Research Unit, Universiti Sains Malaysia (USM), Penang, Malaysia, in coordination with WHO. Procedures for ordering test kits and supplies are specified on the USM website. Kits, impregnated papers and other supplies can be ordered using the catalogue and order form available on the WHO website (https://www.who.int/teams/control-of-neglected-tropical-diseases/vector-ecology-and-management/vector-control/insecticide-resistance or https://www.who.int/teams/global-malaria-programme/prevention/vector-control/insecticide-resistance) or on the USM website online (https://inreskit.usm.my/).

Insecticide-impregnated Whatman no. 1 filter papers or control papers come in boxes containing eight papers each for a particular insecticide or respective control. The range of insecticides for which impregnated test papers are available is expected to expand over time, as DCs for other insecticides and synergists are established. Users are encouraged to regularly consult updates to this document to see the latest list of standard DCs, and the WHO and USM websites to check the availability of papers impregnated with these concentrations and other relevant materials.

6.5.5 Number of uses of impregnated papers and coated bottles

The insecticide content and thereby efficacy of impregnated papers or coated bottles decline with the number of times they are used and the number of mosquitoes tested. Ensuring proper handling and use of impregnated and control papers, and coated and control bottles, during bioassays is vital to maintaining their quality and generating reliable data.

- For the WHO tube test, insecticide-impregnated or control papers should not be used more than 6 times , which is equivalent to exposing a maximum of 150 mosquitoes to each paper.
- For the WHO bottle bioassay, data still need to be generated on how many times a treated bottle can be used and for how long they remain usable after they are coated. Until the number of reuses is determined, before reusing a bottle, its validity should be checked by exposing some mosquitoes known to be susceptible

to the insecticide inside the bottle and verifying that they die. When a bottle is used, mosquito aspiration can generate moisture inside the bottle. Therefore, before reusing a bottle, it should be left open to dry with cap off for 2–4 h.

6.5.6 Storage of impregnated papers

Impregnated and control papers should be stored at a temperature of 4–8 °C in cool cabinets or refrigerators. Where possible, temperature data loggers should be kept in the storage cabinets to continuously track temperature variations during prolonged storage. If impregnated papers are stored at 4–8 °C (i.e. refrigerator temperature), their shelf life ranges from 2 to 5 years, depending on the insecticide, as determined by a recent study coordinated by WHO *(45)*. This study also evaluated the stability of the papers at warmer storage conditions for short periods (54 ± 2 °C for 2 weeks or 40 ± 2 °C for 8 weeks). Table 12 provides the shelf life and accelerated storage stability for insecticides to which mosquito resistance is commonly monitored.

Table 12. Shelf life for storage under optimal conditions, and storage stability of freshly treated papers at accelerated temperatures.

Class	Insecticide	Shelf life under optimal cold storage conditions (4–8 °C) (years)	Accelerated storage stability of freshly treated papers (54 ± 2 °C for 2 weeks or 40 ± 2 °C for 8 weeks)
Organochlorine	p,p'-DDT	5	Stable
Organophosphates	Malathion	3[a]	Stable[a]
	Pirimiphos-methyl	3[a]	–
Carbamate	Bendiocarb	3[a]	–
Pyrethroids	Alpha-cypermethrin	2	Stable
	Cyfluthrin	2	Stable
	Deltamethrin	2	Stable
	Etofenprox	2	Stable
	Lambda-cyhalothrin	2	Stable
Synergist	Piperonyl butoxide	3	Stable

–: Under WHO evaluation.

[a] Tentative (needs confirmation).

One hour before performing the test, the box with impregnated papers should be taken out of the cold cabinet or refrigerator and brought to room temperature unopened. This step is to avoid condensation of water on the surface of the papers, which can hydrolyse the insecticide if the boxes are opened immediately. Test papers should never be exposed to direct sunlight or to temperatures higher than 8 °C, except for short periods during usage or shipment by a courier agency (as shown in Table 12).

The date of expiry of each batch of papers is given on their box. Papers should not be used after their expiry date (see Table 12).

Storage of new papers

Unused impregnated papers, packaged in their original plastic boxes that are sealed with tape, should be stored in a refrigerator at 4–8 °C during and up to the end of the shelf-life period.

Storage of papers between testing rounds

When a paper is used, the date of use should be written with a pencil on the edge of the paper on the untreated side. Between insecticide resistance testing rounds, reusable papers (i.e. used less than 6 times) should be separated by aluminium foil from used ones and kept in their original plastic box, sealed with tape and stored in a cool container or refrigerator at 4–8 °C, or, if this is not possible, in a cool, dark cupboard. Storing them at higher temperature for long periods could compromise their quality.

Storage of papers between tests within the same testing round

To avoid excessive paper manipulations when bioassays are conducted over a few days, impregnated papers can be retained in the exposure tubes, provided the tubes are individually wrapped in aluminum foil after each use and kept at 4–8 °C or, if this is not possible, in a cool, dark place. Tubes with papers still wrapped in aluminum foil kept under cold conditions need to be brought to room temperature for 1 h before removing the aluminium wrapper.

6.5.7 Storage of coated bottles

Experience with CDC bottle bioassays shows that the shelf-life of bottles after coating depends on the insecticide used for coating *(23)*. For insecticides tested using CDC bottles, shelf-life is between 12 h and 5 days *(23)*. No data are available on the shelf-life of bottles coated with the new DCs presented in Tables 6 and 7. Hence, it is currently recommended to use bottles shortly after they are coated and dried. Before reusing a bottle, it should be left open to dry with cap off for 2–4 h. If a bottle is reused within a few days since first use, it is recommended to check that it is still usable by exposing some mosquitoes known to be susceptible to the insecticide inside the coated bottle and verifying that they die.

6.6 Mortality calculations and adjustments

In each bioassay, mortality should be calculated separately for the treated mosquitoes (i.e. those exposed to the insecticide and/or synergist) and the control mosquitoes. The number of mosquitoes killed at the end of the exposure period is then divided by the total number of mosquitoes initially exposed in the tubes or bottles and the result expressed as a percentage.

To interpret test results in intensity bioassays, mortality should be calculated separately for mosquitoes exposed to each multiple of the DC of insecticide (e.g. 5× and 10× the DC). In synergist–insecticide bioassays, mortality should be calculated separately for each exposure pathway (i.e. synergist only, synergist followed by insecticide, insecticide only and control).

Mortality for treated mosquitoes is calculated as:

$$\textit{Treatment mortality}\ (\%) = \frac{\textit{Number of treated mosquitoes that died}}{\textit{Total number of treated mosquitoes}} \times 100$$

Similarly, control mortality is calculated as:

$$\textit{Control mortality}\ (\%) = \frac{\textit{Number of control mosquitoes that died}}{\textit{Total number of control mosquitoes}} \times 100$$

Mortality-based criteria to validate tests

Criteria to validate susceptibility and intensity bioassays conducted with all insecticides except chlorfenapyr

- control mosquito mortality 24 h post-exposure is ≤20% (i.e. in solvent/oil control).

Criteria to validate susceptibility tests with chlorfenapyr

- control mosquito mortality 72 h post-exposure is ≤20% (i.e. in solvent/oil control); and/or
- mortality in the susceptible colony 72 h post-exposure is ≥98%; and/or
- tests are done strictly within the temperature range 27 °C ± 2 °C.

Criteria to validate synergist–insecticide bioassays

- control mortality is ≤20% (i.e. in solvent/oil control); and
- mortality in mosquitoes exposed to PBO only is ≤10%.

Mortality adjustments when control mortality is high

Treatment mortality adjustments should be carried out as follows.

- If the control mortality is <5%, no correction of mortality is necessary.
- When control mortality is ≥5% and ≤20%, the observed mortality in insecticide-exposed mosquitoes must be corrected using Abbott's formula:

$$\textit{Corrected treatment mortality}\ (\%) = \frac{(\%\ \textit{treatment mortality} - \%\ \textit{control mortality})}{(100 - \%\ \textit{control mortality})} \times 100$$

Standard data collection forms to record the results of bioassays, both mortality and knockdown rates, are provided in the WHO SOP on the WHO website *(22)*. District Health information System 2 (DHIS2)–based digital forms are also provided on the website of the Global Malaria Programme *(46)*.

Checking the quality of the papers when unexpected results are obtained

In the event that unexpectedly high numbers of survivors are found following exposure to an insecticide that is expected to kill all test specimens (based on knowledge of local vectors and vector control interventions):

- If the papers were procured from USM, Malaysia, contact them to reconfirm the quality of the papers using the quality control samples kept in their storage.
- If possible, test the papers against a susceptible mosquito laboratory strain in a research laboratory following standard procedures with the optimal number of mosquitoes.

6.7 Oviposition inhibition calculations

For tests with juvenile hormone mimics (e.g. pyriproxyfen), oviposition inhibition is measured by comparing the oviposition rate of a mosquito sample exposed to the juvenile hormone mimic compound with that of a control (untreated) mosquito sample.

During tests to monitor resistance of wild populations of mosquitoes to juvenile hormone mimics, a susceptible laboratory mosquito strain (e.g. Kisumu strain or other local strain) should be tested in parallel to validate the test (see explanation for WHO bottle bioassay with pyriproxyfen in section 6.1 and test validation criteria below).

Mosquito mortality 72 h post-exposure should be recorded both in the wild sample and in the sample from the susceptible laboratory strain as this is needed to validate test results. Mortality 72 h post-exposure is calculated as indicated in section 6.6.

Oviposition rates in treated and control samples are calculated as follows, both for the wild population and for the laboratory strain:

$$\textit{Oviposition rate}_{\textit{treatment}}\ (\%) = \frac{\textit{Number of treated females that laid eggs}}{\textit{Total number of treated females initially chambered}} \times 100$$

$$\textit{Oviposition rate}_{\textit{control}}\ (\%) = \frac{\textit{Number of control females that laid eggs}}{\textit{Total number of control females initially chambered}} \times 100$$

$$\textit{Oviposition inhibition}\ (\%) = \left(1 - \frac{\textit{Oviposition rate}_{\textit{treatment}}}{\textit{Oviposition rate}_{\textit{control}}}\right) \times 100$$

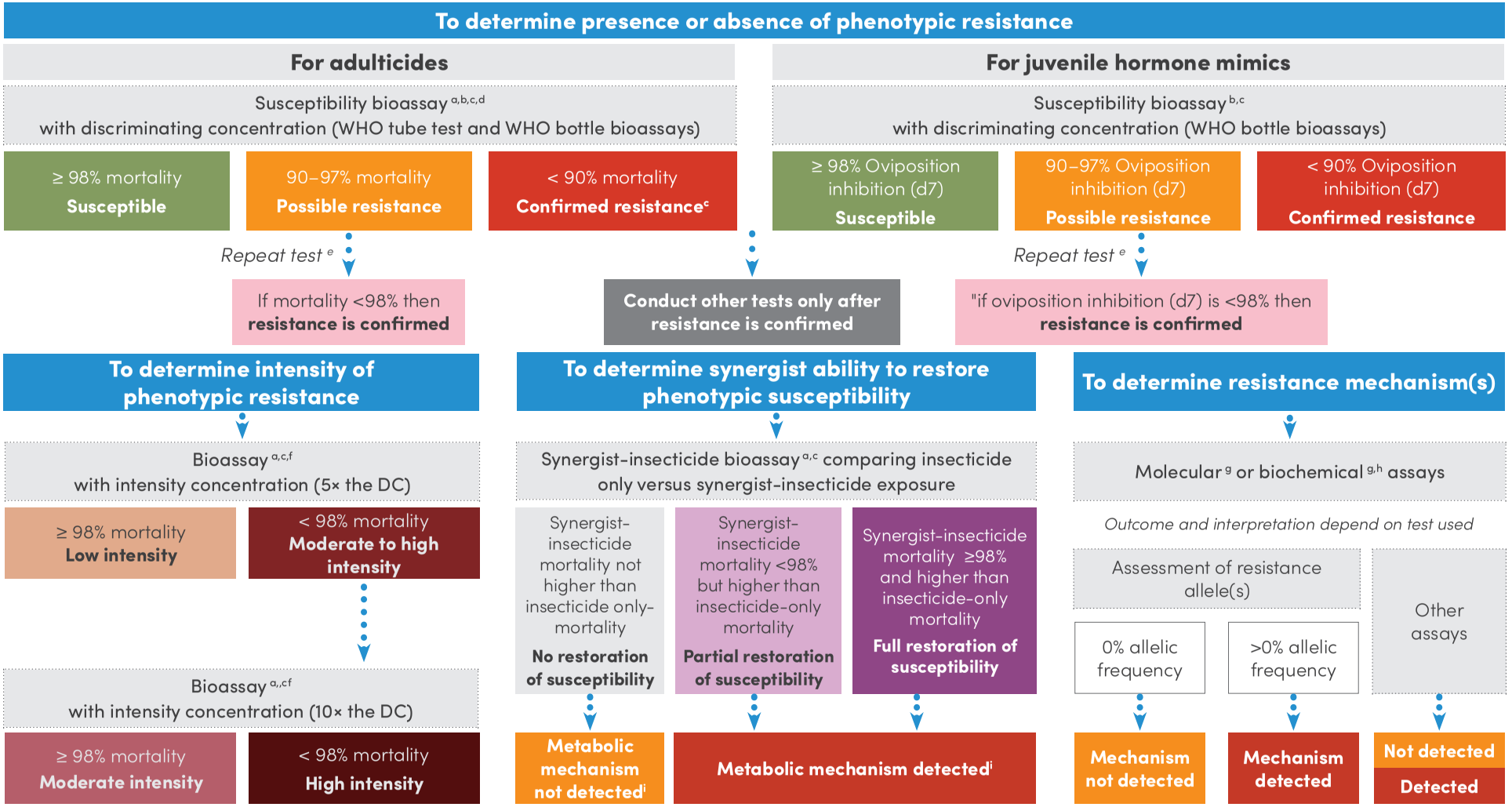
Criteria to validate test with pyriproxyfen

- mortality in control mosquitoes of the susceptible strain and of the wild population is ≤20% at 72 h post-exposure; and/or
- oviposition rate in control mosquitoes of the susceptible strain and the wild population is >30% at the end of the chambering period (i.e. day 7 after 1 h exposure to pyriproxyfen); and
- oviposition inhibition in the susceptible mosquito strain at the of the chambering period (i.e. end of day 7 after exposure to pyriproxyfen) is ≥98%.

6.8 Interpretation of bioassay results

Caution must be exercised when interpreting the results of bioassays. Conducting a test with less than the optimal number of mosquitoes (see Table 11 for optimal numbers) will increase the uncertainty in the test results and may lead to misclassification of the resistance status of a vector population. In addition, mosquito sampling, rearing techniques, handling, the quality of the impregnated papers or coating of bottles and test ambient conditions may influence the results, leading to underestimation or overestimation of mosquito mortality.

Fig. 5. Overview of bioassay types and outcomes for insecticide resistance monitoring in adult mosquitoes



Graph footnotes:

a Mortality should be adjusted using Abbott's formula if mortality in the control is 5–20%.

b Mortality is measured 24 h after 1 h exposure to the insecticide; with chlorfenapyr or pyriproxyfen, it is measured 72 h after exposure. In bioassays with chlorfenapyr or pyriproxyfen, a test with a susceptible mosquito strain should be conducted in parallel to the test with the wild mosquitoes.

c The test is discarded when control mortality is >20% either in the wild population or in the susceptible mosquito strain at the end of the holding period (note: susceptible strains are only tested for chlorfenapyr and pyriproxyfen). For pyriproxyfen, the test should be discarded if the oviposition rate 7 days after exposure to the insecticide is <30% in either of the two control groups (that of the wild population or the susceptible strain) or <98% in the exposed group of the susceptible strain.

d In bioassays with chlorfenapyr, resistance can only be confirmed if mortality 72 h after 1 h exposure is <90% in three tests conducted with the same vector population at different time points.

e Using a new sample from the same mosquito population.

f Papers impregnated with the 5× and 10× concentrations are available from USM, Malaysia, for pyrethroid insecticides only.

g Should be conducted using mosquitoes tested in resistance bioassays.

h Test for known resistance mechanisms only.

i Refers to mechanisms of the broad group(s) related to the specific synergist used in the bioassays (e.g. P450 monooxygenase for PBO). No restoration of susceptibility by the synergist implies that other resistance mechanisms are present.

6.8.1 Interpretation of susceptibility biossays

For adulticides (except chlorfenapyr)

Only tests conducted strictly following the relevant SOP *(22)* should be considered for interpretation. When mortalities need to be corrected with Abbott's formula, test results should be interpreted only after mortalities have been corrected.

- **Confirmed resistance:** If mortality (corrected, if necessary) is <90%, provided that at least 100 mosquitoes were tested, the vector population can be considered resistant to the insecticide.
- **Possible resistance:** If mortality (corrected, if necessary) is ≥90% but <98%, the presence of resistance is possible but not confirmed. Test results should be confirmed by repeating the test with a new sample from the same mosquito population. (Note: Avoid using F1 of the tested mosquitoes.) If two tests consistently show mortality <98%, resistance is confirmed.
- **Susceptibility:** If mortality (corrected, if necessary) is ≥98%, the vector population can be considered susceptible to the insecticide.

Exception for chlorfenapyr

The WHO bottle bioassay with chlorfenapyr has shown some interlaboratory variation in test results due to the strong influence of testing conditions (especially temperature during bioassays). Therefore, to confirm resistance to chlorfenapyr in a wild vector population, at least three WHO bottle bioassays need to be conducted with the same vector population. Furthermore these three tests should all meet the following criteria:

- the mortality of test mosquitoes 72 h post-exposure should be <90%;
- the mortality in the susceptible laboratory colony, tested in parallel to the wild mosquitoes, 72 h post-exposure should be ≥98%; and
- temperature during the bioassay was strictly held within the range 27 °C ± 2 °C.

For juvenile hormone mimics (e.g. pyriproxyfen)

Only tests in which a susceptible laboratory strain was tested in parallel to the wild mosquito sample should be considered for interpretation of results.

- **Confirmed resistance**: If oviposition inhibition in the wild mosquito sample is <90% at the end of the chambering period (i.e. day 7 after 1 h of exposure to the DC of the juvenile hormone mimic compound) and oviposition inhibition in the susceptible mosquito strain (tested in parallel) at the same time point is ≥98%, the mosquito population can be considered resistant to the insecticide.
- **Possible resistance**: If oviposition inhibition in the wild mosquito sample is ≥90% but <98% at the end of the chambering period (i.e. day 7 after 1 h of exposure to the DC of the juvenile hormone mimic compound) and oviposition inhibition in the susceptible mosquito strain (tested in parallel) at the same time point is ≥98%, the presence of resistance is possible but not confirmed. Test results should be confirmed by repeating the test with a new sample from the same mosquito population. (Note: Avoid using F1 of the tested mosquitoes.) If two tests consistently show that oviposition inhibition is <98% in the wild mosquitoes while oviposition inhibition is ≥98% in the susceptible mosquito strain (tested in parallel), resistance is confirmed.

- **Susceptibility**: If oviposition inhibition in the wild mosquito sample after at the end of the chambering period (i.e. day 7 after 1 h of exposure to the DC of the juvenile hormone mimic compound) is ≥98% and oviposition inhibition in the susceptible mosquito strain (tested in parallel) at the same time point is ≥98%, the mosquito population can be considered susceptible to the insecticide.

Note: procedures for testing vector resistance to pyriproxyfen are new and have been developed using laboratory-colonized mosquito strains. The procedures need to be further validated with field mosquito populations from multiple settings. Results obtained with field mosquitoes may lead to a change in the test procedure or the interpretation of its results.

6.8.2 Interpretation of intensity bioassays (5× and 10× discriminating concentrations)

Results of intensity bioassays can only be interpreted if resistance to the insecticide has been previously confirmed using susceptibility bioassays with the DC of the insecticide. A comparison of mortalities across mosquitoes exposed to papers or bottles treated with the 1×, 5× and 10× the DC is then used to evaluate the intensity of resistance to that insecticide. When mortalities need to be corrected with Abbott's formula, test results should be interpreted only after the mortalities have been corrected. The current recommendations for interpretation of results are as follows.

- **Low-intensity resistance:** If mosquito mortality (corrected, if necessary) is <90% after exposure to the DC (1×) and ≥98% after exposure to 5× the DC, results indicate low-intensity resistance. In this case, it is not necessary to conduct a bioassay with 10× the DC.
- **Moderate-intensity resistance:** If mosquito mortality (corrected, if necessary) is <90% after exposure to the DC (1×) and <98% after exposure to 5× the DC, results indicate a moderate-intensity resistance. An additional bioassay with 10× the DC should be conducted to determine whether intensity is indeed moderate or high instead. If mortality after exposure to 10× the DC is ≥98%, moderate-intensity resistance is confirmed.
- **High-intensity resistance:** If mosquito mortality (corrected, if necessary) is <90% after exposure to the DC (1×) and <98% after exposure both to 5× and to 10× the DC, results indicate high-intensity resistance.

6.8.3 Interpretation of synergist–insecticide bioassays

Synergist–insecticide bioassays should only be conducted in populations of mosquitoes that are resistant to the insecticide whose effect the synergist potentiates. When evaluating the ability of the synergist to restore susceptibility to the insecticide, the mortality in mosquitoes exposed to the synergist followed by the insecticide should be compared with the mortality in mosquitoes exposed only to the insecticide. If the mortality in mosquitoes exposed only to the insecticide is ≥90%, the effect of a synergist (e.g. PBO) cannot be reliably assessed. When mortalities need to be corrected with Abbott's formula, test results should be interpreted only once the mortalities have been corrected. If mortality in mosquitoes exposed only to the insecticide is <90%, the effect of the synergist can be interpreted according to the following criteria.

- **Full restoration of susceptibility:** If mortality (corrected, if necessary) in mosquitoes pre-exposed to a synergist and then to the insecticide is ≥98%, it can be considered that the synergist causes full restoration of susceptibility to the insecticide. This implies that the metabolic resistance mechanism targeted by the synergist is mainly responsible for the observed phenotypic resistance in the tested population.
- **Partial restoration of susceptibility:** If mortality (corrected, if necessary) in mosquitoes exposed first to a synergist and then to the insecticide is <98%, but greater than the mortality in mosquitoes exposed to the insecticide only by at least 10%, it can be considered that the synergist causes partial restoration of susceptibility to the insecticide. This implies that the metabolic resistance mechanism targeted by the synergist only partially accounts for the observed phenotypic resistance and that other resistance mechanisms are likely to be present in the tested population.
- **No restoration of susceptibility:** If mortality (corrected, if necessary) in mosquitoes pre-exposed to a synergist and then to the insecticide is equal to or lower than the mortality in the mosquitoes exposed only to the insecticide, it can be considered that the synergist does not restore susceptibility to the insecticide. This implies that the observed phenotypic resistance in the tested population is not caused by the metabolic resistance mechanism targeted by the synergist.

7. Resistance mechanisms and detection methods

Phenotypic resistance is caused by several different types of genetic alterations in the mosquito. These genetic alterations lead to functional changes in the mosquito that allow it to survive exposure to insecticides. Where insecticides are used for vector control, because mosquitoes with such alterations survive and the others die, these genetic alterations are passed on from generation to generation, thereby spreading through the mosquito population.

At present, the best understood resistance mechanisms are divided into two groups: metabolic and target site. Other mechanisms known to confer phenotypic resistance, but less studied, are cuticular thickening *(7)* and changes in vector behaviour *(8)*. These four types of mechanisms are described in Box 5.

Box 5. Insecticide resistance mechanisms

Metabolic resistance arises because of changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal. The detoxification prevents the insecticide from reaching its intended site of action within the mosquito, or protects the mosquito from toxic secondary metabolic products. In the case of malaria vectors, three enzyme systems are believed to be important metabolizers of insecticides: esterases, cytochrome P450 and glutathione S-transferases.

Target-site resistance occurs when the protein receptor that the insecticide is designed to attack is altered by a mutation. When this happens, the insecticide can no longer effectively bind to the intended target site of the receptor; thus, the insect is either unaffected or is less affected by the insecticide.

- For DDT and pyrethroids, the mutations occur in the sodium channel receptor, conferring "knockdown resistance" (mediated by *kdr* genes).
- For organophosphates and carbamates, the mutations occur in the protein acetylcholinesterase (a neurotransmitter), conferring *ace-1* resistance.
- For dieldrin and fipronil, the mutation occurs in the gamma aminobutyric acid receptor (*rdl* gene).

Cuticular resistance is a reduction in a mosquito's absorption of insecticide due to a thickening or alteration in composition of their cuticle. Cuticular modifications are attributed to overexpression of one or more of the diverse genes involved in the formation of the cuticle.

Behavioural resistance is a modification in mosquito behaviour that enhances avoidance of, or reduces contact with, insecticides. The genetic basis of behavioural resistance in mosquitoes is poorly understood.

Although WHO standard bioassays are sufficient in themselves to inform several common programmatic decisions, knowledge of the mechanisms responsible for resistance can be useful in certain situations. For example, since some insecticides have similar modes of action, some resistance mechanisms can confer resistance to more than one insecticide (see Table 13). This is called cross-resistance. When selecting a new intervention after resistance to an insecticide(s) in use has been detected, the identification of resistance mechanisms is important to select an intervention with an insecticide that does not share the mode of action impeded by the detected resistance mechanism. Other uses of resistance mechanisms data are listed in Box 6.

Since these tests require a certain laboratory capacity and can be costly, national disease control programmes should aim to identify resistance mechanisms when there is a clear use for such data. When there is no national capacity for investigating the mechanisms of resistance, but such information is considered valuable, help can be sought from partner institutions.

Box 6. Application of resistance mechanisms data

- Highlight areas where phenotypic resistance may be present, based on prior knowledge of the association between resistance markers and phenotypic resistance.
- Confirm presence of resistance where well-calibrated markers for resistance mechanisms are available.
- Track the temporal evolution of resistance via the detection of changes in the frequency of resistance markers in a time series of samples.
- Monitor spatial variation in resistance at scales where phenotypic resistance testing is impractical by monitoring changes in the frequency of resistance mechanisms in samples from spatially separated locations.
- Help to select alternative vector control interventions once resistance to an insecticide in use has been confirmed, by evaluating prospects for resistance to candidate replacement insecticides.

Some mechanisms have been found to be strongly predictive of, or significantly correlated with, mosquitoes' phenotypic resistance to insecticides. In *Anopheles gambiae*, well-known *kdr* mutations (L1014L/S) are widespread on the African continent *(47)* and are known to confer resistance to various pyrethroids. Mutations in *kdr* genes are also present in other *Anopheles* species worldwide *(48)*, some of which have a local role in malaria transmission (e.g. *An. albimanus, An. stephensi, An. sinensis, An. culicifacies*). *Ace-1* duplicated resistance alleles have been observed to be associated with phenotypic resistance to carbamates or organophosphate in *An. gambiae (49)*. Higher expression of CYP6P9 in *An. funestus (50)* and CYP6M2 in *An. gambiae (51)* is known to metabolize pyrethroids, causing high levels of resistance to these insecticides.

In *Aedes*, at least six different substitutions in voltage-sensitive sodium channel genes (V1016G/I, F1534C/S, V410L and S989P) are corelated with pyrethroid resistance. Transcriptomic studies showed that overexpression of cytochrome P450 genes, especially in CYP6 and CYP9 families, highly correlates with deltamethrin resistance in the dengue vector *Ae. aegypti (52,53)*. Overexpression of carboxyl/cholinesterase (CCE) genes (CCEae3a and CCEae6a) in Aedes is also strongly associated with resistance to the organophosphate insecticide temephos *(54, 55)*, and these genetic markers represent promising candidates to monitor temephos resistance in the field.

Finally, *Culex* mosquito species with a role in transmission of human diseases (e.g. *Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus*) also developed resistance to public health insecticides due to *kdr* mutations (1014F/S/C), and higher expression of CYP450, CCE genes and glutathione S-transferase *(56)*. Mutations in the gene coding for an insensitive acetylcholinesterase (G119S, F290V, F331W) cause various levels of resistance to carbamates and organophosphates *(49, 57, 58)*.

As a result of the correlation of some of these mechanism with phenotypic resistance, their detection and frequency could become a good proxy for the emergence and prevalence of resistance in a vector population. Emergence of, or dramatic changes in, other mechanisms, such as *kdr* or several metabolic markers, whose associations with phenotypic resistance are more variable, could serve as an early warning of the emergence, development of, or potential shifts in, resistance status.

Table 13. Main insecticides used for vector control and associated resistance mechanisms

Insecticide class	Biochemical target				Known resistance mechanisms						
					Target site mutations				Metabolic mechanisms		
	Sodium channel	AChE	GABA receptor	ACh receptor	*kdr*	*ace-1*	*rdl*	*Nla*	COE	GST	P450
Organochlorines	X				++					++	+
Cyclodiens			X				++				+
Organophosphates		X				++			++	+	+
Carbamates		X				++					+
Neonicotinoids				X				+	++		++
Pyrethroids	X				++				+	+	++
Phenylpyrazole			X				++				+
Avermectines			X		Unknown						+
Juvenile hormone mimics	Hormone receptors and chitin biosynthetic pathway				Target site mutations						+
Bti toxins Bs toxin	Intestinal wall receptors of mosquito larvae				Receptor mutations				Probable mechanisms: alteration of toxins, immunity		

ace-1: gene encoding acetylcholinesterase; ACh: acetylcholine; AChE: acetylcholinesterase; COE: carboxylesterase; GST: glutathione S-transferase; *kdr:* knockdown resistance gene; *rdl:* resistance to dieldrin gene; *Nlα:* nicotinic acetylcholine receptor subunit. X indicates the correspondence between insecticide family and biochemical target; +/++ indicates the strength of the association between the resistance mechanism and the phenotypic resistance to the insecticide class.

Source: Adapted from Table 3: Principaux insecticides utilisés contre les vecteurs et mécanismes de résistance associés from the ANSES report *Resistance des moustiques vecteurs aux insecticides* (October 2021) *(59)*, translated to English by the World Health Organization.

A range of biochemical and molecular assays are available to detect and quantify the presence and frequency of these mechanisms. A detailed description of these techniques is outside the scope of this document and can be found in *Methods in Anopheles research manual (39)* or other published reports *(60)*. These assays require adequate preservation of mosquito samples. Biochemical assays to detect enzyme activity are best performed on fresh samples or samples stored at –80 °C. For detection of DNA-based mechanisms, mosquito samples should be preserved in ≥70% ethanol or in Eppendorf tubes with silica gel. For advanced molecular detection methods (e.g. RNA-based gene expression analysis), samples should be preserved either in RNAlater at –20 °C or without RNAlater at –80 °C.

8. Data management and reporting

Vector control programmes should establish robust systems for the collection, management and analysis of insecticide resistance monitoring data. It is imperative that these data are available in a timely way to decisions-makers. The data should be interpreted jointly with vector control monitoring data, epidemiological data and other relevant types of data to understand the impact of insecticide resistance on the effectiveness of disease control and to inform programmatic decisions.

Where there is limited capacity in national programmes to monitor insecticide resistance, these functions may be totally or partially delegated to partner institutions. Solid memorandums of understanding should be in place to ensure that partners share resistance monitoring data with programmes in a timely manner. Priorities and procedures for data collection, and the frequency and format of data reporting should be agreed between vector control programmes and partner institutions up front.

To support countries in collecting insecticide resistance monitoring data, collating data from partners, making data available to decisions-makers and helping them to interpret it, WHO has developed standard paper forms, excel forms as well as digital tools based on the health information software system DHIS2. The paper forms are provided as annexes to the SOPs for insecticide resistance monitoring available on the WHO website *(22)*. The excel forms are available on the same website. The digital tools include digital data collection forms, automatically calculated indicators and dashboards for data visualization and interpretation. These tools are free of charge and can be easily installed in existing national DHIS2 implementations. They are available on the WHO website *(46)*. They support the collection of data both online and offline, and from multiple devices (e.g. phones, tables, computers).

Countries are encouraged to regularly (at least annually) share their insecticide resistance monitoring data with WHO for its inclusion in the WHO global database *(61)*. Standard Excel templates are available for national programmes to report their resistance monitoring data to WHO *(61)*. The global database includes information provided by countries and partners, as well as data regularly extracted from scientific publications and reports. These data with several visualizations and interactive maps, are made publicly available through the interactive data visualization platform Malaria Threats Map *(3)*, a platform dedicated to the dynamic monitoring of biological challenges to malaria control. For malaria, WHO analyses these data once a year and provides an overview of the global status of vector resistance to insecticides in the World malaria report and through the Malaria Toolkit App.

9. Knowledge gaps

Opportunities exist to improve insecticide resistance monitoring and the use of monitoring data for programmatic decision-making. The most important knowledge gaps and information needs, including the gaps identified in the WHO multicentre study *(20)*, are listed below.

Testing procedures

- DC and/or alternative standard procedures to test resistance to emerging compounds should be developed at the product testing stage, so that vector resistance to these compounds can be programmatically monitored as soon as they are deployed in countries.
- Procedures and DCs for monitoring resistance in other disease vectors (e.g. sandflies, triatomine bugs) are lacking and should be promptly developed.
- Laboratories participating in the WHO multicentre study to establish new procedures and DCs to monitor resistance raised concern about difficulties in getting wild female mosquitoes to lay eggs in laboratory settings for testing resistance against pyriproxyfen. To address this problem, the test protocol should be validated with field-caught populations in various settings.
- Further work should be conducted to determine the suitability of other surfactants, such as SPAN 80, for use in the WHO bottle bioassay, and on the role and capacity of surfactants, such as MERO, in facilitating cuticular penetration of insecticide, and any impact this might have on bioassay outcomes.
- The suitability of treating filter papers or coating bottles with 5× and 10× the DC of certain compounds is currently unknown. For bottle bioassays, further investigations are necessary to ensure that these test compounds do not crystallize at these higher concentrations. For filter paper tests, the stability of papers impregnated with the 5x and 10x DC of pirimiphos-methyl should be carefully investigated as no carrier oil is used for impregnating the filter papers.
- The shelf life of Whatman no. 1 filter papers treated with the newly recommended DCs of insecticides is unknown for most compounds and should be investigated. This is particularly important for papers treated with pirimiphos-methyl that do not contain a carrier oil. Similarly, the shelf-life of bottles coated with the newly established DCs and the number of times that coated bottles can be used is unknown. The length of storage of stock solutions should also be determined, especially for compounds tested in WHO bottle bioassays. This information should be collected to ensure adoption of adequate storage conditions, ease procurement processes and ensure the quality of test results.
- The dynamics of PBO oxidases–pyrethroids in mosquitoes are not well understood. Better understanding could improve the procedures and mortality thresholds for synergist-insecticide bioassays, and facilitate collection and interpretation of more useful data. Key factors to be considered include simultaneous versus sequential exposure to PBO, use of different concentrations and addition of surfactants or adjuvants.
- More evidence is needed to establish adequate bottle drying times and procedures, especially for volatile compounds that may evaporate more quickly than other insecticides.
- A detailed assessment of the main factors that drive the variability of test results is needed to improve test procedures and the interpretation and comparability of results.

- Research on methods to evaluate behavioural resistance in mosquito vectors should continue until validated methods are available that can be used to programmatically monitor this type of resistance.

Using insecticide resistance monitoring data to inform programmatic decisions

- The impact of insecticide resistance on the effectiveness of vector control interventions remains unclear *(62, 63)*. Understanding this impact is of upmost importance to guide vector control strategies.
- The use of resistance intensity data for decision-making is still unclear. The relationship between resistance intensity and the efficacy of vector control interventions should be further investigated to guide the use of these data in the formulation of vector control strategies.
- Methods to extrapolate resistance monitoring data to areas where such data are lacking, using a realistic number of predictive variables, are needed. This will help to improve the available information for decision-making and reduce the resources needed for insecticide resistance monitoring once a product is recommended for public health use.
- Associations between certain overexpressed genes in resistant mosquitoes and the resulting phenotypic response are not well understood. Obtaining validated markers of resistance could simplify resistance monitoring in the future.

Global resistance monitoring

- There is a global need to increase monitoring of insecticide resistance in *Aedes* spp. vectors and report data to WHO for evaluation of the status of insecticide resistance in these species.
- Countries still lack adequate financial resources, infrastructure and capacity to monitor and manage insecticide resistance in malaria vectors. Donors and partners are encouraged to support countries with this endeavour.

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