I2I Landscaping report:

Mini Chamber test

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Acronym List

AI	Active Ingredient
HPLC	High-Performance Liquid Chromatography
IHI	Ifakara Health Institute
ITN	Insecticide-treated net
LSTM	Liverpool School of Tropical Medicine
QC	Quality control
SOP	Standard operating procedure
WHO	World Health Organisation

Summary

Aim and key questions addressed	- Optimisation of a novel bioassay (alternative to cone and tunnel tests) to evaluate the mortality effect of ITNs with a delayed mortality effect- chlorfenapyr ITNs
Context	- Laboratory
Test item	- Mosquito
Mosquito population	- Laboratory reared
Number of mosquitoes per replicate	- 10
Endpoints measured	- Knockdown, mortality
Exposure time	- 16 hours
Holding time	- Up to 72 hours
Indicative of personal protection	- N/A
Suitable chemistries	- Insecticide-treated net (ITN)
Appropriate controls	- Negative control: untreated netting (made of polyethylene or polyester ideally equivalent fabric to test item) - Positive control: new, unused samples of relevant net product
Relevant stage of production pipeline	- Product development - Bio efficacy assessment - Durability monitoring
Characterisation of output	- Endpoints well defined. Adaptation required for active ingredients with a different mode of action.
Accessibility	- Materials and set up are easily accessible. Need to be sourced and training is required
Cost	- Cost of equipment low e.g. deli pots required to set up Mini Chamber. Staff training is required to carry out the assay
Level of validation and characterisation of outputs	- Completed preliminary development. Multi-site testing conducted based at LSTM and Ifakara Health Institute (IHI). Next stages of internal and external validation required

Outstanding questions, gaps and priorities	- Future research to include trialling blood feeding measurements using artificial feeding sources - The aim is to validate the method to produce data to support PQ submissions as an alternative to cone and tunnel tests for evaluation of chlorfenapyr ITNs
Key references, related SOPs, guidelines and publications	- I2I-SOP-044-Mini Chamber Test - Lewis, A., Jones-Brown, R., Harvey, G., Praulins, G., Reid, E., Lugenge, A. G., Mechan, F., Gleave, K., & Lees, R. S. (2026). The mini chamber test: a novel bioassay for bioefficacy testing of chlorfenapyr treated nets used in malaria control. *Gates Open Res*, *10*, 14. https://doi.org/10.12688/gatesopenres.16381.1 - Matope, A., Lees, R., Spiers, A. and Foster, G. 2023. 'A bioassay method validation framework for laboratory and semi-field tests used to evaluate vector control tools', Malaria Journal. BioMed Central.

Overview

Chlorfenapyr has been integrated into insecticide-treated nets (ITNs) as a secondary compound to pyrethroids in dual active ingredient (AI) ITNs. Efficacy and durability testing of ITNs using bioassays measures short term mortality responses and is therefore not suitable for measuring insecticides such as the pyrrole insecticide chlorfenapyr. For example, the WHO cone test measures mortality following a short exposure time of forced contact with the net in a limited space. WHO tunnel tests are also available for measuring chlorfenapyr bioavailability on ITNs however small animals are required for this which can cause issues with accessibility to the bioassay due to animal handling laws. Due to this gap in evaluating chlorfenapyr-based ITNs a novel bioassay named the 'Mini Chamber' has been developed and is being optimised.

This assay is carried out in a laboratory by releasing 3–5-day old, non-blood fed female mosquitoes into the mini-chamber compartments. The ITN sample, with two 1cm holes, is held in place between two compartments and secured using tape and parafilm. A sugar source is available for the mosquitoes on the top of the chamber. Ten mosquitoes are introduced into compartment one of the chambers. They are free to fly in the chambers but must contact the net sample and navigate the holes to get to the sugar supply. A chamber with untreated netting is always run in parallel as a negative control. This set up is shown in Figure 1. Knockdown is measured 1 hour post exposure and mortality at 24, 48 and 72 hours.

During the tests, the chambers are held at 27°C ± 2°C and 75% ± 10% relative humidity in full darkness. After a six-hour exposure period, the mosquitoes are removed from each compartment using a HEPA-filtered mouth aspirator.



Figure 1: Mini chamber set up. Net piece is held in the middle of the two compartments with parafilm. Compartment two is the top compartment with removable mesh where the sugar source is placed.

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

An SOP has been developed by I2I:

- Mini Chamber test- I2I-SOP-044. Available on Github at https://github.com/i2i-Data-Repository/Mini-Chamber-Test-paper-data-R-code-and-SOP/blob/main/Mini%20chamber%20test%20SOP.docx
- Cambrex: Standard Operation Procedure for Cambrex Developed Mini Tunnel Apparatus Version 2.2

Are these sufficiently detailed?

The SOP contains a sufficient level of detail and includes thorough step-by-step guidelines on performing the assay.

Do these methods require specialised/non-standardised equipment and/or training?

Training is required for the performance of the Mini Chamber test and mosquito handling, including aspiration.

Specialised equipment is required to construct the Mini Chamber via two deli pots, with pre-prepared netting in-between (containing 2x 1 cm^2 holes). This set up is shown in Figure 1.

Are there issues with the methods or their interpretation?

The SOP was based on parameters and endpoints that have been optimised during experimental testing including the number of mosquitoes per test, length of time of exposure and vertical positioning of the assay.

The assay is conducted in the dark which is suitable for this assay as this could potentially increase host seeking behaviour as *Anopheles* host-seek at night which will impact flight behaviour and effectiveness of the pro-insecticide chlorfenapyr.

What AIs or combinations of AIs have the tests been used for?

Anopheles gambiae sensu lato (insecticide susceptible: Kisumu and Ifakara and resistant: Tiassalé 13 and Kingani) and the resistant *Anopheles coluzzii* Tiefora strains have been tested on untreated, PermaNet 2.0 (deltamethrin 1.4g/kg), PermaNet 3.0 (deltamethrin 4g/kg and piperonyl-butoxide 25g/kg) and Permanent Dual (Deltamethrin-2.1 g/kg and chlorfenapyr-5g/kg).

Is the assay validated, for which AIs/entomological effects, and to what extent?

Experimental work conducted using the Mini Chamber assay so far followed the Methods Validation Framework developed by Matope et al. (2023) to evaluate the precision and accuracy of the Mini Chamber Test. In brief, this is achieved by measuring the variability of proposed methods under different conditions and identifying operating procedures that produce the least variability.

The Methods Validation Framework developed by Matope et al. (2023) consists of four stages:

1. Preliminary development- this involves defining the method, endpoints, environmental conditions, controls and vector conditions.
2. Feasibility experiments- these perform the bioassay multiple times to generate data for sample size calculations, and to measure variability.
3. Internal validation- during this formal sample size calculations are done, and experiments ran to determine reproducibility within a laboratory. Also, during this stage, a data

package is compiled including the draft SOP, the method claims, examples of study designs, defined controls, and criteria for strain selection.
4. External validation-this involves at least two other laboratories performing the assay using the proposed experimental design to assess the outcomes and analytical parameters associated with the method claim.

Experimental work to date has completed the preliminary development stage after assessing the effect of varying the number of mosquitoes per chamber, the ITN exposure length, and orientation of the chambers.

During preliminary development, the number of mosquitoes was defined using an overnight exposure time (10 mosquitoes). The exposure time was then determined (6 hours) and the orientation of the assay (horizontal). Parts of the feasibility study have also been conducted: a standard operating procedure has been generated, and two comparator studies completed at LSTM and IHI.

What inputs need to be characterised? e.g., samples, mosquitoes, equipment

The equipment required is detailed in the SOP. The SOP details that the bioassay requires ten non-blood-fed adult female mosquitoes aged 3-5 days and includes the use of a control (untreated) net. A 6-hour exposure is stated in the SOP (a range of 2, 4, 6, 8 and 16 hours has been evaluated experimentally to select this test length).

The quantity of replicates to conduct per test has not been standardised. The overall sample size is dependent on the study, and the mosquito-insecticide combinations are determined by the study protocol.

The mosquitoes used for testing should be characterised and quality control (QC) measured according to I2I-SOP-016: Strain characterisation of resistant mosquitoes for monitoring bioefficacy in ITNs treated with two active ingredients (Dual-AI ITNs).

The net pieces used for testing should be characterised according to I2I-SOP-047: Net Characterisation for Mosquito Control Experiments.

Are endpoints clearly defined and appropriate? Who were they defined by?

The endpoints are clearly defined in the SOP, with knock down being measured 1 hour after exposure and the mortality again at 24 hours later or an extended period dependent upon the active ingredient. This is appropriate when testing with chlorfenapyr as mortality measurement beyond 24 hours is required.

Are there supporting SOPs? e.g., cleaning SOPs, mosquito rearing SOPs.

- LITSOP123- Test preparation, detailing the set-up of equipment required including holding cups.
- Detailed in I2I-SOP-044-Mini Chamber test includes instructions on equipment cleaning, as follows:
    - After the assay is complete remove parafilm, net pieces and cotton wool.
    - Soak the chambers in 5% Decon solution for two hours.
    - Rinse the chambers and mesh pieces twice using tap water and a final third time using distilled. Leave the chambers to air dry.
    - Repeat this process at the end of every test week or when testing with new net pieces.
- I2I-SOP-047: Net Characterisation for Mosquito Control Experiments.
- I2I-SOP-016: Strain characterisation of resistant mosquitoes for monitoring bioefficacy in ITNs treated with two active ingredients (Dual-AI ITNs).

Define Current Use Practices

Does everybody use the same SOP?

Yes The SOP has been developed following experimental work conducted multi-site at IHI and LSTM.

Are there differences of interpretation of the method?

Interpretation of methods from the SOP is clear. The experimental set up is detailed, the age of testing mosquitoes, the acclimatisation period prior to testing and the number of mosquitoes per assay are defined.

Are the results obtained largely consistent between studies?

The comparator study conducted at IHI, used a *An. arabiensis* pyrethroid resistant strain and an *An. gambiae* pyrethroid susceptible strain. There was a difference in mortality observed between the four-hour exposure tests performed at LSTM compared to IHI, this was attributed to the time of day the bioassays were performed with testing at LSTM occurring during the mosquitoes' night phase and testing at IHI during the day.

Is further development, refinement or validation of the method required? Based on priority, significance, and relevance of method.

The experimental work conducted so far used the Methods Validation Framework developed by Matope et al. (2023) as discussed in the section "Are they validated, for which AIs/entomological effects, and to what extent?"

Preliminary development and some parts of the feasibility experiments have been conducted, as detailed in Figure 2.

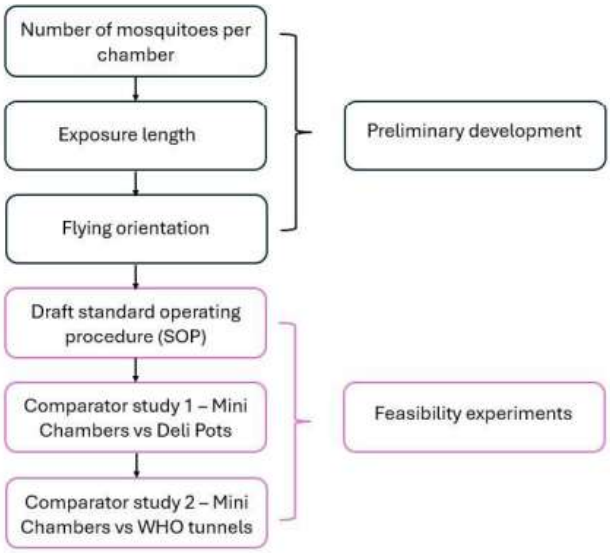


Figure 2: The flow of development for the mini chamber

The additional two stages of the Methods Validation framework need to be completed as next stages:

- Internal validation (making use of the draft SOP developed)
  - formal sample size calculations
  - experiments to determine reproducibility within a laboratory
  - a data package compiled (draft SOP already completed)
  - the method claims
  - examples of study design
  - defined controls
  - criteria for strain selection
- External validation- at least two other laboratories performing the assay using the proposed experimental design to assess the outcomes and analytical parameters associated with the method claim.

Identify Potential Sources of Variation

What are the sources of variability in the method and are there means to minimise or characterise these?

Variations in surface insecticide content between net pieces cut even from the same net is a potential source of variability in testing, even when obtained from the manufacturer. HPLC could be performed to determine the concentration of insecticide on the net surface and account for any variation.

Do current method/s need to be adapted for new active ingredients/MoA/types of tool?

The SOP states to measure knock down 1 hour after exposure and mortality at 24 hours or longer dependent upon the active ingredient tested with different modes of action. 72 hours is suitable for the use of chlorfenapyr, a pro-insecticide, when measuring mortality beyond 24 hours is required.

Gaps in biological or other understanding that hinder method development or validation

The data collected show that mini chambers have similar efficacy to WHO tunnel tests when measuring the mortality effect of chlorfenapyr ITNs. However, unlike the WHO tunnel test the Mini Chamber test is not able to measure blood feeding inhibition post exposure.

References

Lewis, A., Jones-Brown, R., Harvey, G., Praulins, G., Reid, E., Lugenge, A. G., Mechan, F., Gleave, K., & Lees, R. S. (2026). The mini chamber test: a novel bioassay for bioefficacy testing of chlorfenapyr treated nets used in malaria control. *Gates Open Res*, *10*, 14. https://doi.org/10.12688/gatesopenres.16381.1

Matope, A., Lees, R., Spiers, A. and Foster, G. 2023. 'A bioassay method validation framework for laboratory and semi-field tests used to evaluate vector control tools', *Malaria Journal*. BioMed Central.



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