**Title: Bioassays to determine residual efficacy of alphacypermethrin and chlorfenapyr on Interceptor G2 nets**

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* 1. Glossary

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| ***Alive*** | ***Knockdown (recorded 60 minutes after exposure)*** | ***Dead (recorded 24 hours after exposure)*** |
| -Can both stand and fly in a coordinated manner | -Any mosquito that cannot stand (e.g. has 1 or 2 legs) -Any mosquito that cannot fly in a coordinated manner -A mosquito that lies on its back, moving legs and wings but unable to take off -A mosquito that can stand and take of briefly but falls down immediately  | -No sign of life: immobile; cannot stand -A mosquito that lies on its back, moving legs and wings but unable to take off  |

**Table 1. Classification of adult mosquitoes as alive, knocked down or dead in Phase I WHO Cone bioassays**

1. **Purpose**

This SOP outlines the procedures for conducting bioassays to evaluate the residual efficacy of alphacypermethrin and chlorfenapyr on Interceptor G2 nets. The Interceptor G2 long-lasting insecticidal net (BASF, Ludwigshafen) is a multifilament polyester net produced with a proprietary polymer system. The net is coated with 200mg/m2 of chlorfenapyr and 100mg/m2 alphacypermethrin. Little is known about the bioefficacy of these two insecticides on Interceptor G2 nets that have been used in field conditions. To assess the residual bio-efficacy of both alphacypermethrin and chlorfenapyr, it is necessary to have a protocol that separates the actions of these two compounds.

Standard cone bioassays with standard susceptible strains will not allow a separation of the effects of alphacypermethrin and chlorfenapyr. In general, alphacypermethrin will have a rapid action whereas chlorfenapyr typically takes a longer time to affect the mosquitoes. In 3-minute cone bioassays on nets treated with chlorfenapyr at 200mg/m2 (the same dose present on the Interceptor G2), knockdown of *An. gambiae* s.s. Kisumu strain was 0% when measured one hour after the bioassay, and mortality was only 2% at 24 hours (WHO 2017). N’Guessan et al. (2007) found less than 20% mortality at 24 hours, which rose to nearly 80% at 72 hours. Other studies have found a more significant impact at 24 hours; Mosha et al. (2008) found increases of only 15-25% between mortalities recorded at 24 and 72 hours for the doses of 100, 250, and 500mg/m2. For alphacypermethrin, Tungu et al. (2016) found that Interceptor ITNs (with 200mg/m2 of alphacypermethrin) resulted in 100% knockdown of *An. gambiae* Kisumu at baseline. A similar result was found for *An. gambiae* Kisumu exposed to netting treated with alphacypermethrin at 100mg/m2 which resulted in 98% knockdown (WHO 2017c). We propose that the residual efficacy of alphacypermethrin on Interceptor G2 nets be assessed using the knockdown of pyrethroid-susceptible mosquitoes at 60 minutes after a 3-minute exposure to netting.

To measure the efficacy of the chlorfenapyr on netting will require more effort and the use of alphacypermethrin-resistant strains of mosquitoes. Oxborough et al. (2015) found that standard 3-minute cone bioassays conducted during the day were not reflective of the impact of chlorfenapyr in experimental hut studies. However, when nets were attached to filter papers and tested in WHO cylinders and the testing time was extended to 30 minutes at a temperature of 27°C (testing and holding), the results at 72 hours post-test were more representative of results in experimental huts (complete mortality).

Another option, described here, is the use of tunnel tests. As with the 30-minute cylinder bioassays, when tunnel tests were used, the results were much more reflective of the hut results, perhaps because the insecticide was available at night when mosquitoes were more metabolically active. Insecticide resistant *Culex quinquefasciatus* were used in tunnel tests to determine the regeneration time of Interceptor G2 nets (WHO 2017c), and the same principles can be used for determining the bioefficacy of these nets.

**Equipment and Materials**

* 1. WHO cones
	2. Aspirators (separate aspirators for introduction and removal of mosquitoes from the cone)
	3. Plastic cups
	4. Rubber bands
	5. Untreated netting
	6. Cotton
	7. Timers
	8. Plastic plates, 30cm x 30cm, solid
	9. Plastic plates, 30cm x 30cm, with 4 holes 10 cm in diameter
	10. Binder clips
	11. Sugar or honey solution (5-10%)
	12. Stapler
	13. Paper for labels
	14. Permanent marker
	15. Laboratory coat
	16. Glass tunnel
	17. Rabbit / guinea pig
	18. Latex-free gloves
	19. Data recording form
	20. Tiny Tag Data logger

**MOSQUITOES NEEDED**

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| **Strain** | **Characteristics** |
| Susceptible | Higher than 98% mortality when tested in standard WHO tube tests with alphacypermethrin-treated papers (0.05%) |
| Resistant | Either an alphacypermethrin-resistant insectary strain or wild mosquitoes collected from the field that have less than 70% mortality when tested in a tunnel test with a new Interceptor net (200mg/m2 alphacypermethrin). Ideally a malaria vector species should be used, but a well characterized *Aedes* or *Culex* species might also be used. |

1. **Safety**

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| **HAZARDS** |
| List items that are risks, e.g., manual handling, sharps, chemical, biological, radiation1. *Hazard –* Insecticide and hazardous reagents (Insecticide treated netting)
2. *Hazard –* Handling of animals may result in bites to humans or injury to animals.
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| **RISK CONTROL** |
| List what controls are put in place to minimise or lower the risk level, e.g., PPE, restrict use of item/chemical to trained persons, specific training and induction processes, designated waste disposal guidelines etc.1. *Risk control –* Wear lab coat and gloves at all times when handing insecticide and other reagents.
2. *Risk control –* Protocols in place to safely handle animals before, during, and after tunnel tests
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1. **Procedures**

**Testing the residual efficacy of alphacypermethrin:**

**Preparing for cone bioassays**

* 1. Prepare the plastic cups by covering them with pieces of untreated netting and secure the netting with a rubber band. Cut a small slit in the netting in the center of the cup to allow the end of the aspirator to pass through the netting and plug this hole with a small wad of cotton.
	2. Prepare sugar/honey solution by adding 20g of sugar or 20ml of honey to 180ml of water. Mix well.
	3. Cut the four pieces of netting from the nets of interest. Be sure to wear gloves while handling the nets and cut 30x30cm pieces of the net. This should be done according to WHO protocols (2013) as shown in Figure 1 (excluding position 1 as it may be exposed to excessive abrasion from being tucked under the bed). Label the netting immediately using paper labels stapled onto the corner of a net. Store in aluminium foil when not in use in refrigerator (4°C±3°).

**Figure 1: Recommended positions from which netting pieces should be taken (WHO 2013).**



* 1. Use the plastic plates to fix the cones onto the netting to be tested in place. Place the solid plastic plate on the bench top and then place the netting on top of the plate. Place the 4 cones on top of the netting and secure them by placing the plastic plate with 4 holes over the cones. Use binder clips to secure the plates to each other. The plates should then be placed at a 60° angle that allow access of the mosquitoes to the netting and clamp the plates into place.
	2. For each cone, 5 susceptible mosquitoes should be introduced into the cone and the cone blocked with a piece of cotton. The timer can be started as soon as all mosquitoes are in the cone. Ideally a separate timer should be used for each cone.
	3. Once the timer reaches 3 minutes, the mosquitoes should be aspirated gently from the cone and into the plastic cup through the slit cut in the netting. Cover the slit with cotton wool after the mosquitoes are in the cup. Provide mosquitoes with honey/sugar solution by moistening a piece of cotton wool, squeezing it to remove excess solution, and placing it on top of the cup.
	4. Knockdown of mosquitoes should be read by observing the number of mosquitoes unable to stand or fly at 60 minutes after the end of the cone bioassay.
	5. For each of the four net pieces tested, 10 mosquitoes (2 cones) should be completed, resulting in 40 mosquitoes used for testing all four net pieces (plus 50 mosquitoes exposed to control (untreated) netting in 3-minute cone tests to ensure mortality is due to the insecticide). See Figure 2. One set of controls can be used for all tests conducted in a day.

Figure 2: A matrix for recording bioassay results, each empty cell should be filled with the results (number responding/number tested).

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| **KNOCKDOWN** | Cone 1 | Cone 2 | Cone 3 | Cone 4 | Cone 5 |
| Piece 2 (side) |   |   |   |   |   |
| Piece 3 (side) |   |   |   |   |   |
| Piece 4 (side) |   |   |   |   |   |
| Piece 5 (roof) |   |   |   |   |   |
| Control 1 |   |   |   |   |   |
| Control 2 |  |  |  |  |  |

**Testing the residual efficacy of chlorfenapyr:**

**Determination of a resistant strain**

* 1. The testing of chlorfenapyr bioefficacy is challenging and requires either a wild resistant strain or laboratory-reared resistant strain. The use of a resistant strain introduces considerable variability into the bioassay, as even laboratory resistant strains can vary in their degree of resistance. For this reason, before each series of tunnel tests, the resistant strain should be tested in a tunnel test.
	2. Tunnel tests consist of 60x25x25cm glass or plexiglass containers. At each end of the tunnel, a 30x30cm mosquito cage is fitted. The LN netting sample, held in a disposable cardboard frame, is placed at one third the length of the glass tunnel. The surface of netting available to the mosquitoes is 400 cm2 (20 cm x 20 cm), with nine holes 1 cm in diameter; one hole is located at the center of the square, and the other eight are equidistant and located 5 cm from the border. In the shorter section of the tunnel, a suitable bait (e.g. guinea-pig or rabbit) is placed, which is unable to move and is available for mosquito biting (WHO 2013).
	3. Prior to commencing any tunnel tests, ensure that the temperature and humidity can be monitored throughout the testing and holding periods. This can be done using the TinyTag data loggers or similar loggers.
	4. To determine the resistant strain, two tunnels will be used. The first will have a piece of netting from a new Interceptor (alphacypermethrin 200mg/m2)(aired for a few days before use) and the second will have a piece of untreated netting.
	5. One hundred nulliparous female mosquitoes, aged 5-8 days and sugar-starved for 6 hours, will be introduced into the end of each tunnel opposite the bait at 18:00. The lights of the room will be turned off, and only turned on when the tunnel test is finished the following morning at 7:00. According to WHO guidelines (2013) the overall exposure period should be 12-15h. The environmental conditions in the room during the night should be 27 ± 2 °C and 75% ± 10% relative humidity
	6. At 7:00, a narrow insert will be slid down between the two compartments of the tunnel, to prevent mosquitoes from moving between the compartments. All mosquitoes will be carefully collected from the tunnel, noting the compartment in which the mosquitoes were collected (initial compartment/animal compartment), the bloodfeeding status (fed/unfed), and mortality (living/dead). The results can be recorded as shown in Figure 3.
	7. The following formula will used to be assess mortality in the tunnel tests:

Let X = the percent living in the control tunnels

Let Y = the percent living in the alphacypermethrin tunnels

The corrected mortality due to alphacypermethrin will be (X-Y)/X × 100

If control mortality is greater than 20%, the test should be repeated.

* 1. If the resistant strain shows a mortality of less than 70% in a tunnel with a new Interceptor net, then it can be used for testing of chlorfenapyr in Interceptor G2 testing. If this threshold is not met, either another field strain should be used after testing, or the net pieces should be tested at a laboratory that maintains a suitable strain of resistant mosquitoes. Note that it is not necessary that the vector strain be used, as the tests are meant to assess the quantity of insecticide on the netting, not the susceptibility of the mosquitoes. Many field sites may have easier access to *Culex quinquefasciatus* or *Aedes aegypti* that meet these criteria and use of these strains are encouraged. Once a suitable resistant strain has been found, it can be used in tunnel tests.

**Figure 3: Table for recording data from tunnel tests**

**Tunnel tests**

* 1. The resistant strain should be used for the four tunnels described here.
	2. Prior to commencing any tunnel tests, ensure that the temperature and humidity can be monitored throughout the testing and holding periods. This can be done using the TinyTag data loggers or similar logger.
	3. Four tunnels will be used for each net piece to be evaluated. The first one will be a tunnel with an untreated control net piece. The second will have the G2 piece to be evaluated. The piece of the G2 to be used (from amongst the four pieces cut above) will be determined using a random number generator. The third will have a piece of a new Interceptor net. The fourth will be from a new G2 net. Note that if there is the possibility to run more than 4 tunnels per night, only one control, one new G2, and one new Interceptor tunnels need to be run, and the results can be shared amongst all of the field G2s being tested.
	4. One hundred resistant nulliparous female mosquitoes, aged 5-8 days and sugar-starved for 6 hours, will be introduced into the end opposite the bait at 18:00. The lights of the room will be turned off, and only turned on when the tunnel test is finished the following morning at 7:00. According to WHO guidelines (2013) the overall exposure period should be 12-15h. The environmental conditions in the room during the night should be 27 ± 2 °C and 75% ± 10% relative humidity
	5. At 7:00, a narrow insert will be slid down between the two compartments of the tunnel, to prevent mosquitoes from moving between the compartments. All mosquitoes will be carefully collected from the tunnel, noting the compartment in which the mosquitoes were collected (initial compartment/animal compartment), the bloodfeeding status (fed/unfed), and mortality (living/dead). The results can be recorded as shown in Figure 4.
	6. The living mosquitoes from the tunnels will be put into cups covered with untreated netting, and cotton wool soaked in sugar solution will be placed on top of the cups, allowing mosquitoes to feed *ad libitum*. The mortality will be recorded at 18:00 (24 hours after the tunnel test started), and then again at 48 and 72 hours.
	7. The following formula will used to be assess mortality in the tunnel tests:

Let X = the percent living in the alphacypermethrin (Interceptor) tunnels

Let Y = the percent living in the G2 (field collected) tunnels

The corrected mortality due to chlorfenapyr will be (X-Y)/X × 100

Note that the equation above does not adjust for any mortality in the control tests. If control mortality is greater than 20%, the test should be repeated. The results of the field G2 net will be considered looking at the results of the four tests together.

* 1. And for analysis of bloodfeeding inhibition in the tunnel tests, the following formula will used:

Let X = the percent feeding in the alphacypermethrin tunnels

Let Y = the percent feeding in the G2 tunnels

The corrected bloodfeeding inhibition due to chlorfenapyr will be

(X-Y)/X × 100

Note that the formula above does not adjust for any lack of bloodfeeding in the control tests. If control bloodfeeding is less than 50%, the tests should be repeated

* 1. The cutoff for G2 nets will be defined at a later point after consideration of preliminary data.

Figure 4: Table for recording data from tunnel tests.



1. **QUALITY CONTROL**
	1.
	2. As described above, the susceptible strains should be characterized within 2 month of the bioassays. The resistant strain characterization should occur just prior to testing to ensure that the current resistance status of the mosquito strain is being captured.
	3. The net pieces from the new Interceptor (Step 3.12) and new Interceptor G2 (Step 3.19) should be cut from new unused nets. Since the insecticide concentration should be the same throughout the net, any 30x30cm square piece can be used for the controls. Each piece can be used up to 10 times before a new piece should be used. The number of times the net piece has been used can be recorded on the cardboard frame of the net or a notebook.
2. **SOP Copy Control Log**

**Purpose:** The log records the number of certified copies of this SOP printed and where they were distributed.

**When:** Whenever the SOP is reviewed: annually or more often when necessary.

**By whom:** By QA staff / designee

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| **SOP Distribution (location and number of certified copies)** |
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1. **Appendices**

**Appendix I: SOP Training Log for Personnel Training Files**

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| Date: | SOP Number and Title | Employee Signature | Supervisor Initials |
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