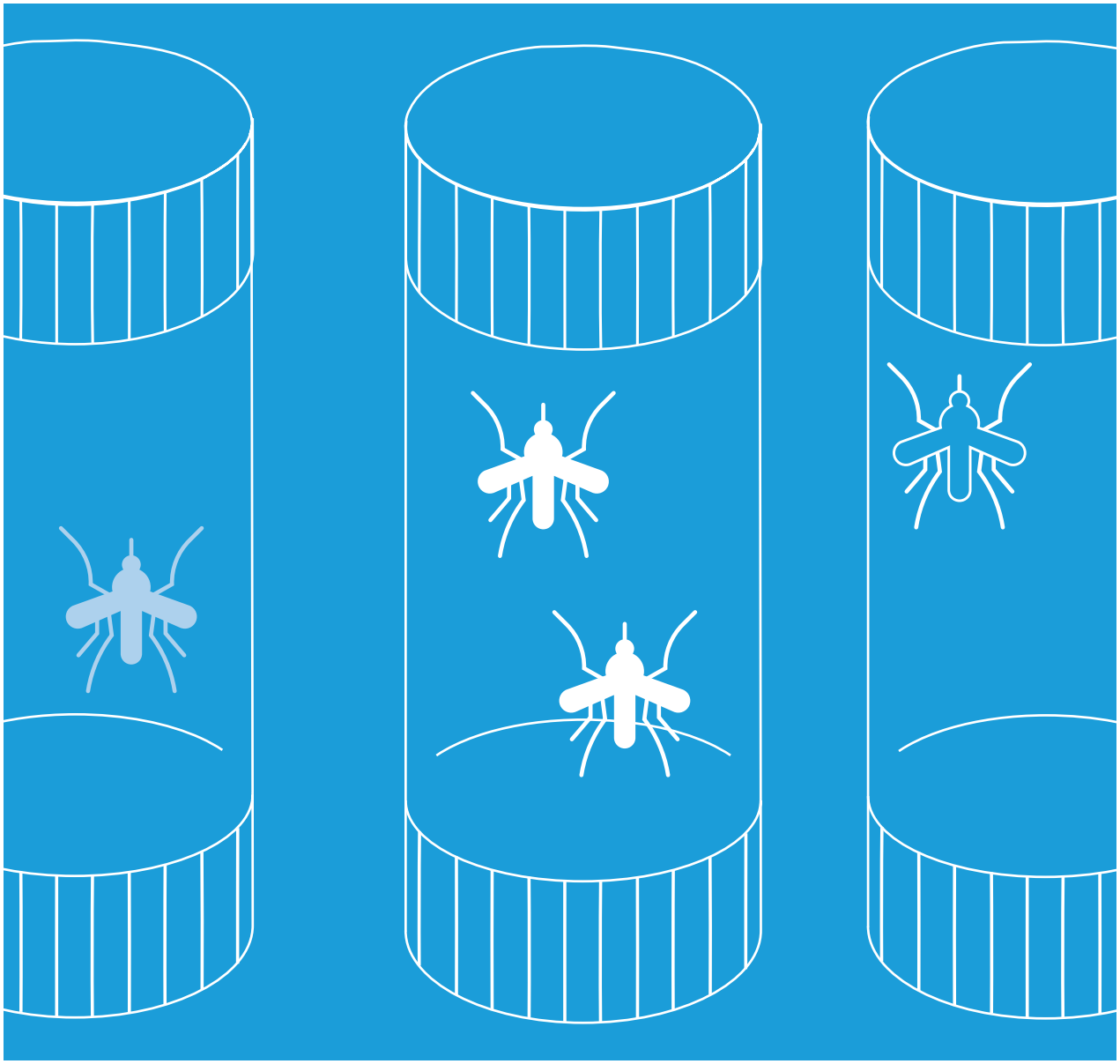
World Health Organization

Standard operating procedure for impregnation of filter papers for testing insecticide susceptibility of adult mosquitoes in WHO tube tests

SOP version: Paper-Impreg/01/14 January 2022



1. Introduction, scope and purpose

This standard operating procedure (SOP) describes the process for impregnating filter papers with insecticides and synergists to be used in World Health Organization (WHO) tube tests for testing insecticide susceptibility of adult mosquitoes. It is primarily aimed at researchers involved in the validation of discriminating concentrations, and is not intended for use by national programs for routine insecticide resistance monitoring.

The quality of the impregnated papers will affect the results of WHO tube tests and, consequently, the comparability of results over time and across sites. To minimize the risk of such variability in results, programs should purchase filter papers impregnated with the validated discriminating concentrations of various insecticides, as well as test kits for conducting WHO tube tests, from the Universiti Sains Malaysia (USM) – Vector Control Research Unit (http://www.inreskit.usm.my).

2. Equipment

☐	balance (precision 0.01 mg), spatulas and weighing boats for weighing insecticide (aluminium micro weighing dishes)
☐	refrigerator for storing impregnated filter papers
☐	fume hood for all insecticide work
☐	glass bottles, caps and labels
☐	a set of calibrated micropipettes (e.g. 100 µL, 200 µL and 1000 µL) for preparing stock solutions
☐	2 mL glass pipettes for impregnation of papers
☐	drying line and pegs for drying filter papers in the fume hood (optional)
☐	paper holding rack (optional: cannot be procured and has to be made locally, example in Fig. 1)

3. Reagents and consumables

☐	paper cutter for cutting filter paper to the appropriate size
☐	pencil for marking filter papers

☐	waste disposal bins, as necessary
☐	permanent marker pens
☐	Whatman No. 1 filter papers (catalogue number 1001 917)
☐	disposable plastic tips for micropipettes (e.g. 100 µL, 200 µL and 1000 µL)
☐	disposable glass pipettes of various volumes
☐	acetone, analytical grade or purer
☐	aluminium foil to keep filter paper dry and/or to wrap the filter paper for storage
☐	technical grade insecticide or insecticide active ingredient (AI)
☐	carrier oils (see Table 1)
☐	paper roll (optional)
☐	70% isopropanol wipes (for cleaning fumes)
☐	antibacterial cleaner such as 70% isopropyl alcohol or ethanol
☐	TFD4 or Decon 90 (for cleaning equipment in contact with chemical compounds)

4. Health, safety and environmental protection

✓	Before using any chemical compound, laboratory staff should read and understand the risk assessment, material safety data sheets and the control of substances hazardous to health assessment for each chemical used.
✓	Appropriate personal protective equipment must be worn at all times when handling insecticides, including laboratory coat, gloves, safety glasses and a face mask when weighing out chemicals.
✓	Ensure all working areas are clear of other materials before impregnating the papers.
✓	All staff working in the laboratory must have received laboratory induction training and the training must be documented in the individual's training file.
✓	All staff using this procedure must be trained in the safe operation of chemical fume hoods.
✓	Dispose of all waste materials appropriately following the national/ institutional safety guidelines.

5. Calculations for making the solution for paper impregnation

☐	5.1. Select the appropriate carrier oil for the preparation of the solvent solution for the insecticide to be treated on Whatman No. 1 filter paper (Table 1).
☐	5.2. Take note of the degree of purity of the AI to be used for the impregnation. This information is essential for calculating the exact quantity of AI.
☐	5.3. Choose the final concentration of AI in the solution of carrier oil and/ or solvent necessary for impregnating the filter papers (Table 1). Prepare the initial stock solutions with the highest concentration and then make serial dilutions to obtain the required serial concentrations. Calculations for the amount of AI, solvent and carrier oil to prepare the initial stock solution, and subsequent dilutions for the serial concentrations, are given in Annex 1 of this SOP.

Table 1. Insecticide and carrier oil required to impregnate papers

Insecticide class/synergist	Insecticide/synergist	Carrier oil/ solvent required
Organophosphates (OP)	Malathion	Olive oil and acetone
	Chlorpyrifos-ethyl	
	Pirimiphos-methyl	Acetone only[a]
Carbamates (C)	Bendiocarb	Olive oil and acetone
Pyrethroids (PY)	Permethrin 40:60	Silicone oil (Dow Corning 556 cosmetic grade fluid) and acetone
	Deltamethrin	
	Lambda-cyhalothrin	
	Alpha-cypermethrin	
Organophosphates (OP) control	N/A	Olive oil and acetone
Carbamates (C) control	N/A	Acetone only
Pyrethroids (PY) control	N/A	Silicone oil (Dow Corning 556 cosmetic grade fluid) and acetone
Synergist	Piperonyl butoxide (PBO)	Silicone oil (Dow Corning 556 cosmetic grade fluid) and acetone

[a] Note that, although in the past olive oil was recommended as a carrier oil, no carrier oil should be used as per the manufacturer's instructions (pirimiphos-methyl is supplied in a liquid state and is mixed with acetone to easily treat papers); N/A, not applicable

Notes:

i. A carrier oil enables a stable, thin, homogeneous layer of the AI to spread on the filter paper and prevents crystallization of the AI, which is usually solid at room temperature. Concentrations are generally expressed as the percentage of AI per unit volume of the carrier oil on the filter paper (acetone is volatile). An exception is pirimiphos-methyl, which is in liquid state; it is mixed only with acetone as a solvent and does not require any carrier oil for paper impregnation. Accordingly, its concentration is expressed as the quantity of insecticide in mg per m^2.

ii. According to the calculations shown in Annex 1:

   a. For compounds intended to be mixed with silicone oil (Dow Corning 556 cosmetic grade fluid), i.e. pyrethroids and PBO, Whatman No. 1 filter papers are impregnated with 3.6 mg (oil density 0.980) of the carrier oil/cm^2, i.e. 648 mg per 180 cm^2 filter paper, or 0.66 mL silicone oil per paper taking the silicone oil density of 0.98. (Note: Always check the density of the oil for the correct calculation.) Therefore, a 180 cm^2 filter paper impregnated at 1% concentration contains 6.61 mg of technical grade AI, or 367.22 mg AI/m^2.

   b. For insecticides intended to be mixed with olive oil, i.e. organophosphates and carbamates, papers are impregnated with 3.6 mg of the carrier oil/cm^2, i.e. 648 mg oil/180 cm^2 paper, or 0.71 mL olive oil/paper taking the olive oil density of 0.91. (Note: Always check the density of the oil for the correct calculation.) Therefore, a 15 cm x 12 cm (180 cm^2) filter paper impregnated at 1% concentration contains 7.12 mg of technical grade AI, or 395.6 mg AI/m^2.

iii. If serial dilution is needed to achieve the desired insecticide concentration, consider the example as follows: If the stock solution has a 95% concentration and the final required test concentration is 0.001%, the best practice is to prepare a 1% stock solution and dilute it with solvent solution (a mixture of carrier oil and acetone or acetone alone for pirimiphos-methyl – see Annex 1) to 0.05% and from this to the final 0.001% dilution. Remember not to dilute directly from 1% to 0.001%.

iv. Small quantities of AIs, for example less than 10 mg (0.01 g), are extremely difficult to weigh out accurately on a micro-balance. A preferred method is to weigh an approximate quantity of the AI (slightly above the required amount) and adjust the volume of the solvent solution (oil and acetone or acetone alone) according to the actual weight of the AI.

v. If acetone and carrier oils are stored in cold conditions (4–8 °C), bring them to room temperature by letting the closed bottles sit for up to 1 hour. Directly opening cold bottles will result in condensation of water vapours, which may affect the stability of the AI on the impregnated paper.

6. Preparation of equipment, consumables, reagents and working space

Note: Wear appropriate personal protective equipment in the laboratory.

☐	6.1. Check the cleaning log to ensure decontamination of the fume hood and the area to be used for treating papers.
☐	6.2. Prepare all the equipment and consumables required (see the list of "Equipment, reagents and consumables" given at the beginning of this SOP).
☐	6.3. Hang a string in the fume hood for drying papers after impregnation. Always use new clean string and pegs for each batch of paper preparation, or arrange aluminium foils on a horizontal surface to place papers for drying.
☐	6.4. Record all details of the equipment, reagents and consumables to be used for the preparation of impregnated papers on the recording sheet.
☐	6.5. Label the necessary bottles for stock and final solutions with name of the AI, concentration, date, type of solvent/carrier oil and the name of the laboratory technician who prepared them.

7. Weighing insecticide/synergist

The stock solution of solvent and carrier oil (except for pirimiphos-methyl) needed for all dilutions should be prepared in glass bottles inside a fume hood. (For example, for impregnating papers with pyrethroids and PBO, mix 66 mL of silicone oil with 134 mL acetone to prepare 200 mL of solvent solution; for impregnating papers with organophosphates or carbamates, mix 71 mL of olive oil with 129 mL of acetone to prepare 200 mL of solvent solution).

☐	7.1. Calibrate the balance with its door closed. Put an aluminium micro weighing dish or piece of aluminium foil (for a small quantity of AI) or the stock solution bottle (for a large quantity of AI) on the balance and adjust/tare the balance.
☐	7.2. Weigh the amount of AI required using a pipette if the test AI is a liquid (e.g. pirimiphos-methyl; PBO) or a spatula if the AI is a powder. Put the amount of AI/synergist in the weighing container (micro weighing dish, foil or bottle) and close the door to check the weight. If required, add more quantity of the AI to reach the approximate desired quantity. Press the button on the printer of the balance, and then press the print sign on the balance to obtain the date, time and amount weighed. Print out a copy and attach it to the recording sheet. If the balance is not connected to a printing machine, make a note in the lab book. Record the actual weight of the AI/synergist in the calculation table and adjust the volumes of the stock solution.
☐	7.3. Put the aluminium micro weighing dish with the AI or synergist into a glass bottle to prepare the initial stock solution.
☐	7.4. Using a pipette, add the required volume of the solvent solution (acetone + oil) or acetone (for pirimiphos-methyl) into the stock bottle containing the AI or synergist and vortex/shake well until fully homogenized. (Note: Let the micro dish remain in the stock solution until the AI is fully dissolved). Repeat steps 7.1–7.4 if more than 1 test concentration of the AI or synergist is required.
☐	7.5. Prepare the dilution solutions according to the calculation sheet (Annex 1). **Note**: Always ensure that separate gloves are used for different AIs or synergists, and never handle papers with contaminated gloves.

8. Cutting and preparing Whatman filter papers

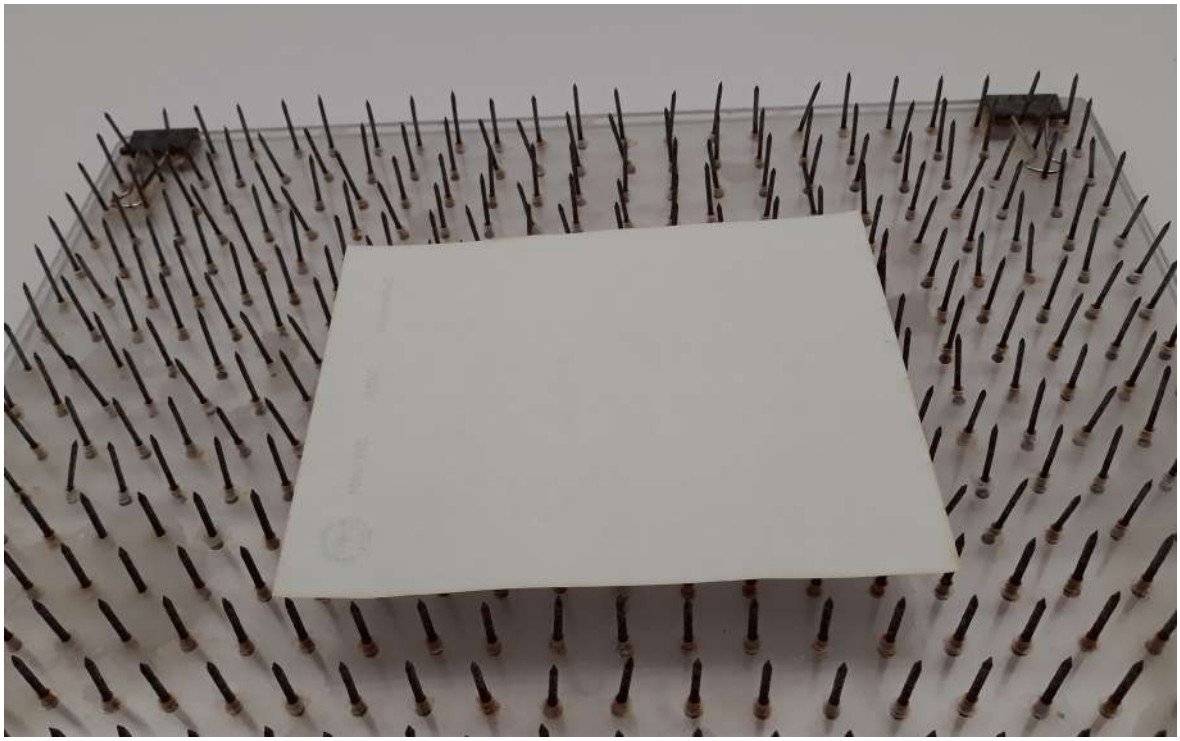
☐	8.1. Using a paper cutter, cut a sufficient number of 12 cm x 15 cm pieces of Whatman No. 1 filter paper. **Note:** Be careful with fingers and hands on the sharp edges of the cutter.
☐	8.2. With a pencil, on each paper to be impregnated, write the name of the insecticide or synergist and the percentage concentration on the bottom half of the paper along the 12 cm edge. For control papers, write the insecticide class group (namely, PY control, OP control, C control). The papers are now ready to be impregnated.

9. Procedure for impregnation of papers

☐ 9.1. Put on gloves and place a paper on a holding rack with its stamped or labelled side down (Fig. 1).

Note: Always impregnate the control papers first, using the same solvent that will be used for impregnating the insecticide/synergist papers.

Fig 1. A filter paper held on metal pins of a holding rack for impregnation



Source: photo courtesy of Institut de Recherche pour le Développement, Montpellier, France

☐ 9.2. Shake the solution well or vortex for 10 seconds before impregnating the paper; if the same solution is to be used for impregnating more than 1 paper, the solution should be vortexed before each impregnation.

☐ 9.3. To impregnate each paper, 2 mL of the final stock solution should be used. Using a glass pipette, slowly draw up 2 mL of the stock solution. Close the stock solution bottle to prevent evaporation of the acetone and open it again only when ready to draw solution for treating the next paper.

☐ 9.4. To impregnate the paper, slowly discharge the solution onto it by moving the pipette tip in rows until the surface of the paper is fully treated. To ensure even impregnation of the paper surface, do not re-treat the same column/row. Do not touch the paper with your fingers while treating it. Do not put treated papers on top of each other. If all of the 2 mL solution is exhausted before the full 12 x 15 cm surface of the filter paper has been impregnated, discard this paper safely and impregnate a new one.

Note: When papers are to be impregnated with serial concentrations of insecticides/synergist, always begin treating papers with the lowest concentration and treat other papers with higher serial concentrations using the same gloves and pipettes. Always change gloves and pipettes between the handling of papers with different insecticides.

☐ 9.5. Leave the treated paper to dry on aluminium foil or a metal drying rack (Fig. 1) at room temperature and in dark conditions for 24 hours. Ensure that the impregnated papers do not overlap/touch each other. Place control papers to dry in a separate place. Do not leave the filter papers to dry for a longer period, as several insecticide chemicals deteriorate rapidly with exposure to daylight.

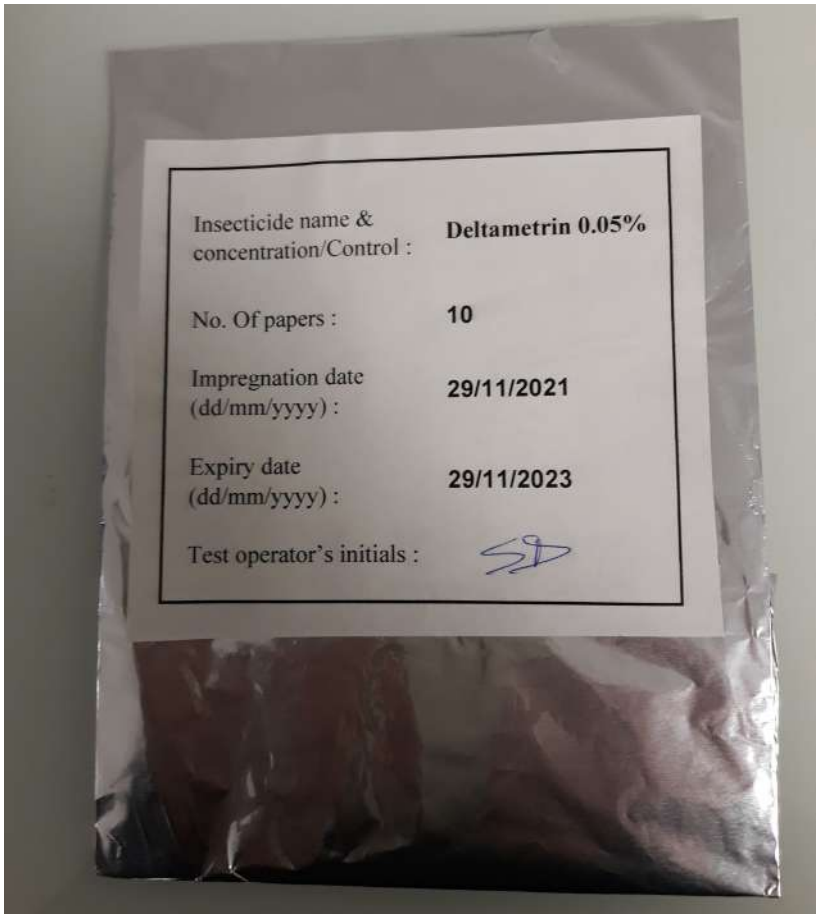
☐ 9.6. If impregnated papers or control papers are not used immediately after the drying period, wearing appropriate personal protective equipment, wrap them in pre-labelled aluminium foil (Figs. 2 and 3). For this, take 2 treated papers of the same concentration/carrier oil at a time, put them together in such a way that the impregnated sides of the papers face each other, while the labels are visible on the outside. Now, wrap them in aluminium foil and put them in a plastic bag or closed container to store in a refrigerator at 4–8 °C for future use. Do not keep them in storage beyond the recommended shelf-life (Table 2). Label each aluminium-wrapped packet with details such as the name of the insecticide/synergist, % concentration, date of impregnation, number of papers that it contains and name of the technician who impregnated the papers. Begin by packing the control papers followed by the insecticide papers, starting from the insecticide/synergist papers with the lowest concentrations. A batch of the number of papers required for 1 resistance test can be stored together in each hermetically sealed plastic bag or container.

Note: Ensure that gloves are changed between the packaging of different compounds, and always serially wrap papers from lower to higher concentrations of the same compound.

Fig. 2. Example of a label

Insecticide name & concentration/Control:
No. of papers:
Impregnation date (dd/mm/yyyy):
Expiry date (dd/mm/yyyy):
Test operator's initials:

Fig. 3. Impregnated papers wrapped in aluminium foil and labelled with the necessary details



Source: photo courtesy of Institut de Recherche pour le Développement, Montpellier, France

Table 2. Shelf-life of different impregnated papers

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

[a] Tentative (needs reconfirmation)

[b] WHO report, 2021 *(1)*

10. Cleaning procedure

Use the following method, as feasible:

- Clean the interior of the fume hood and wipe out the balance with 70% isopropanol wipes. Ensure that all paper-drying metal racks are left to soak for the appropriate amount of time for decontamination, as detailed below.
- Decontamination: Soak overnight in the decontaminant – a 20% alkaline solution (TFD4 or Decon 90) if the equipment is in direct contact with the insecticide, or 10% solution for equipment used for handling. On the following day, rinse 3 times with tap water and dry at room temperature.
- The benches and fixed equipment should be decontaminated with ethanol.

Note: The 20% and 10% decontaminant solutions should be changed at least once per month, or more often if necessary.

11. Acknowledgements

WHO acknowledges the contributions of Dr Vincent Corbel, Mr Stephane Duchon and Ms Laura Andreo, Institut de Recherche pour le Développement, Montpellier, France, and Dr Rosemary Lees, Liverpool School of Tropical Medicine, United Kingdom of Great Britain and Northern Ireland for drafting this SOP. The revision and harmonization of this SOP was done by Ms Lucia Fernandez Montoya, WHO Global Malaria Programme, Geneva, Switzerland. The development of this SOP was coordinated by Dr Rajpal S. Yadav, WHO Department of Control of Neglected Tropical Diseases, Geneva, Switzerland.

12. References

1. Determining discriminating concentrations of insecticides for monitoring of resistance in mosquitoes: report of a multi-centre laboratory study with recommendations of WHO consultations. Geneva: World Health Organization; 2021 (in press).

For further information, please contact: vectorsurveillance@who.int

Annex 1. Calculations for solutions to impregnate filter papers for WHO tube tests

A. Calculations for impregnating Whatman no. 1 filter papers with carrier oil

The Excel calculation spreadsheet is available online at: https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/calculation-tables-paper-impregnation-bottles-17jan2022-locked.xlsx

In the Excel spreadsheet, you will enter values in the green cells and the values in the white cells will be automatically calculated.

											Calculation for weight of AI in g adjusted for its percentage of purity					Calculation for weight of AI in mg (adjusted for AI purity)			Calculation for AI content in impregnated paper per m2		
Insecticide class group	Carrier oil	Insecticide or synergist	Targeted concentration of AI in % #	No. of papers to be treated	Weight of oil per paper (mg)	Total weight of oil needed for all papers to be treated (g)	Density of oil [a]	Volume of oil (mL)	Volume of acetone (mL)	Total volume of oil + acetone (mL)	Amount of AI required (g)	Purity of AI (%)	Amount of AI to weigh (g)*	Exact weight of AI (g)**	Total adjusted volume of acetone + oil (mL)***	Amount of AI to weigh (mg)*	Exact weight of AI (mg)**	Total adjusted volume of acetone + oil (mL)***	Area of 1 filter paper of 15 cm x 12 cm (m)	Quantity of AI per unit surface	
																				in g/m²	in mg/m²
			a	b	c	d = (b c)/1000	e	f = d / e	g = (b 2)-f	h = f + g	i = (a x f) /100	j	k = i x (100/j)	l	m = (l x h) / k	n = k x 1000	o	p = (o x h) / n	q	r = (i / b) / q	r x 1000
OP/C	Olive oil		1	1	648	0.648	0.910	0.712	1.29	2	0.0071	99.1	0.0072	0.0072	2.00	7.19	10	2.78	0.018	0.39560	395.60
		Control		1	648	0.648	0.910	0.712	1.29	2											
PY	Silicone oil		1	1	648	0.648	0.980	0.661	1.34	2	0.0066	99.4	0.0067		0.00	6.65	10	3.01	0.018	0.36735	367.35
		Control		1	648	0.648	0.980	0.661	1.34	2											
OC	Risella oil	DDT	4	1	648	0.648	0.806	0.804	1.20	2	0.0322	79.06	0.0407		0.00	40.68	10	0.49	0.018	1.78660	1786.60
		Control		1	648	0.648	0.806	0.804	1.20	2											
Synergist	Silicone oil	PBO	1	1	648	0.648	0.980	0.661	1.34	2	0.0066	99.3	0.0067		0.00	6.66	10	3.00	0.018	0.36735	367.35
		Control		1	648	0.648	0.980	0.661	1.34	2											

AI, insecticide or synergist active ingredient
OP/C, organophosphate or carbamate
PY, pyrethroids
OC, organochlorine
in mg AI per unit volume of oil (w/v)
[a] Always verify density of oil according to manufacturer's specifications
* This is the weight adjusted for the percentage purity of the AI
** Exact weight of AI shown on the electronic balance
*** Use 2 mL of total volume of the final solution to impregnate a paper of 15 x 12 cm size

B. Calculations for impregnating Whatman no. 1 filter papers without oil (example pirimiphos-methyl)

The Excel calculation spreadsheet is available online at: https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/calculation-tables-paper-impregnation-bottles-17jan2022-locked.xlsx

In the Excel spreadsheet, you will enter values in the green cells and the values in the white cells will be automatically calculated..

						Calculation for weight of AI adjusted for percentage of purity (in g)						Calculation for AI weight in mg		
Insecticide class group	Insecticide	Targeted concentration of AI on the paper		No. of papers to be treated	Area of 1 filter paper of 15 cm x 12cm (m)	Quantity of AI for the number of papers to be treated (g)	Purity of insecticide AI (%)	Total volume of solvent (acetone) (mL)	Amount of AI to weigh (g)**	Exact weight of AI (g)***	Total adjusted volume of solvent (acetone)* (mL)	Amount of AI to weigh (mg)**	Exact weight of AI (mg) ***	Exact volume of solvent (acetone)* (mL)
		in mg/m^2	in g/m^2											
		a	a/1000	b	c	d = (a/1000) x c x b	e	f = b x 2	g = d x (100 / e)	h	i = (h x f) / g	j= g x 1000	k	l = (k f) / j
OP	Pirimiphosmethyl	100.00	0.10000	1	0,018	0.0018	90.5	2	0.001989	0.02	20.11	1.99	20	20.11
	Control			1	0,018			2						

AI, active ingredient
OP, organophosphate
* Use 2 mL of total volume of the final solution to impregnate a paper of 15 x 12 cm size
** Considering purity of the AI
*** This is the exact weight of AI shown on the electronic balance

C. Dilutions of stock solution with acetone or acetone + carrier oil to prepare serial concentrations of insecticides for impregnating filter papers

The Excel calculation spreadsheet is available online at: https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/calculation-tables-paper-impregnation-bottles-17jan2022-locked.xlsx

In the Excel spreadsheet, you will enter values in the green cells and the values in the white cells will be automatically calculated.

a. Without carrier oil i.e. acetone alone (example pirimiphos-methyl)

	Final concentration of AI (mg/m^2)**	Final volume of acetone (mL)*	AI concentration of the initial stock solution (mg/m^2)**	Volume to take from the initial stock solution (mL)	Volume (mL) of acetone to add
	a	b	c	d = (a x b)/c	e = b - d
Stock solution*			**100**		
Serial dilution no. 1	**50**	10	**100**	5.00	5.00
Serial dilution no. 2	**30**	10	**100**	3.00	7.00
Serial dilution no. 3	**20**	10	**100**	2.00	8.00
Serial dilution no. 4	**10**	10	**100**	1.00	9.00
Serial dilution no. 5	**5**	10	**100**	0.50	9.50

b. With carrier oil and acetone

	Final concentration of AI (µg/ bottle)	Final volume ofacetone + oil (mL)*	AI concentration of the initial stock solution (µg/bottle)	Volume to take from the initial stock solution (mL)	Volume of acetone + oil to add (mL)
	a	b	c	d = (a x b)/c	e = b - d
Stock solution*			**0.5**		
Serial dilution no. 1	**50**	10	**0.5**	4.00	6.00
Serial dilution no. 2	**30**	10	**0.5**	2.00	8.00
Serial dilution no. 3	**20**	10	**0.5**	1.60	8.40
Serial dilution no. 4	**10**	10	**0.5**	1.00	9.00
Serial dilution no. 5	**5**	10	**0.5**	0.40	9.60

AI, active ingredient

* Initial stock solution is prepared by weighing the adequate AI amount and adjusting required volume of acetone alone or acetone + oil.

Note: Prepare the final volume according to the number of papers to impregnate (e.g. to impregnate 4 papers with a given concentration, prepare at least 10 mL solution to account for procedural loss of some solution)

** The AI concentrations are shown in mg/m2 (and not in percentages) as no oil is used

Standard operating procedure for impregnation of filter papers for testing insecticide susceptibility of adult mosquitoes in WHO tube tests

ISBN 978-92-4-004381-7 (electronic version)
ISBN 978-92-4-004382-4 (print version)