
**Protocol for Evaluation of the Efficacy of Long-Lasting
Insecticide Treated Nets under field conditions in Ethiopia**

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Abbreviations and acronyms

ACTs	Artemisinin compound treatment
ANOVA	Analysis of variance
DDT	Dichloro Diphenyl Trichloroethane
EPHI	Ethiopian Public Health Institute
GPS	Global Positioning System
HPLC	High Pressure Liquid Chromatography
IRS	Indoor Residual Spray
KD	Knock Down
Kdr	Knock down resistance
LLINs	Long Lasting Insecticide Treated Bed Net
NGO	Non-Governmental Organizations
RH	Relative Humidity
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

1.Introduction

The Global technical strategy for malaria 2016–2030, approved by the World Health Assembly in May 2015, set ambitious but achievable targets for 2030, including a reduction of at least 90% in global malaria incidence and mortality (WHO, 2015a). The World Health Organization (WHO) recommended malaria interventions namely quality assured vector control, chemoprevention, diagnosing and treatment of the interventions recommends implementing to attain the intended target (WHO, 2015c). In sub-Saharan Africa, the recommended malaria interventions namely Long Last Insecticide treated Nets (LLINs) delivered by manufacturers have increased in recent years, rising from 5.6 million in 2004 to 145 million in 2010. Nearly 300 million LLINs were delivered to African countries between 2008 and the end of 2010. Meanwhile, the number of people protected by Indoor Residual Spray (IRS) in the WHO African Region increased from 10 million to 78 million in 2010. Globally by 2005, 185 million people were protected by IRS and by 2010, representing 6% of the global population at risk were protected by the same intervention. In 2013, 124 million people were protected by IRS, representing 4% of the global population at risk (WHO 2015b).

On previous literature IRS and LLINs can build its acceptability in a community by its killing and knock down effect of insects but recent studies indicate that target vector populations are developing resistance to the chemicals used in IRS and LLINs. Consequently, malaria intervention tools are considered not as effective and trusted in these intervention methods as a malaria protective tool. These newly observed challenge may be an obstacle in attaining the strategic goal set by WHO (Baume C. *et. al.*, 2007).

Use of LLINs substantially reduces malaria mortality and morbidity. Currently manufacturers have developed treatment processes, in which the insecticide is more stably bound to fabrics that are more wash resistant, resulting in long-lasting. The introduction of these LLINs, ideally lasting the entire lifetime of a mosquito net, is considered a possible solution to this problem. With the support of the World Health Organization (WHO) the use of LLINs in the vector control strategy was adopted in Ethiopia during 1997/98 with the intention of gradual scaling up. Following this the National Malaria Control Programme has distributed.

LLINs and curtains have repeatedly been shown to reduce malaria morbidity and mortality in sub-Saharan Africa (Lengeler, 2004). Wide use of LLINs was shown to reduce incidence and provide significant protection in children against overall mortality, mortality attributed to malaria, clinical attacks of malaria and malaria infection (Alonso *et al.*, 1993; D'Alessandro *et al.*, 1995; Maxwell *et al.*, 1999).

Guidelines for testing long-lasting insecticidal nets (LNs) were first published by WHO in 2005. The original guidelines were designed for pyrethroid-treated nets and were based on the state of knowledge and LN technology at the time. Considerable experience in testing LNs has since been gained, and WHO published additional guidelines for monitoring the durability of LNs under operational conditions by 2013. No new guide line is released by WHO since 2013. WHO Pesticide Evaluation Scheme (WHOPES) gives full or interim recommendation LLINs that used for malaria control purposes. However, their performance needs to be monitored under various cultural and environmental settings to assess durability and long term effectiveness for malaria prevention and control. In view of the current wide scale distribution this protocol proposes to evaluate the wash resistance and efficacy of LLINs under laboratory and field conditions in Ethiopia.

The efficacy of the LLINs in killing mosquitoes and their wash resistance will be assessed using bioassay tests. The study will help to generate data on the efficacy of LLINs under local conditions which is important for the planning and implementation of the national malaria control programme. Therefore, in view of the current wide scale distribution, this study proposes to evaluate the wash resistance and efficacy of different brands of LLINs under field conditions in Ethiopia.

2.Purpose of the LLINs Protocol

The main purpose of this protocol is to assist national vector-borne disease control programme, and other relevant agencies, in monitoring the durability of LLINs under operational conditions. The information will be useful in planning the replacement of worn-out nets in LLINs programme, making decisions to procure the most suitable LLINs for the setting and understanding the factors associated with the durability of LLINs products. The principles and methods outlined in this document can, however, contribute to such evaluations.

This protocol is designed for monitoring various LLINs products. LLINs is a factory treated mosquito net that is expected to retain its biological activity for a minimum number of standard washes as defined by WHO and a minimum time under field conditions. Currently, a LLINs is expected to retain its biological activity for at least 20 standards WHO washes under laboratory conditions and 3 years of recommended use under field conditions, as defined in the WHO guidelines. In addition, the methods described here can be used in monitoring the performance of LLINs. The protocol provides guidance and to serve as a basis for conducting LLINs. The protocol is intended for use by those responsible for malaria control. These include national malaria control programme personnel; regional malaria control programme, health facilities, government sectors other than the health sector, Non-Governmental Organizations (NGO), and relevant international and bilateral agencies and other donors in support of malaria vector control.

MIS 2015 presents findings on ownership of LLINs in malarious areas >2000m and ≤2500m above sea level(asl) shows that 64 percent of all households owned at least one LLIN in malarious areas (areas <2000m asl). On average, households in malarious areas own 1.18 LLINs per household.

Ethiopian vector control programme is to maintain universal coverage with LLINs and/or IRS in targeted areas. Based on new Malaria NSP (2014-2020) stratification and targeting of interventions, LLIN and IRS are being implemented together in high transmission stratum to bring down the malaria burden. The vector control programme should build or strengthen their capacity to monitor and evaluate the durability of LLINs distributed to targeted populations as a routine programme management activity. This should include measurement of three essential outcomes, namely survivorship or attrition rate, fabric integrity and insecticidal activity of LLINs over time under field conditions in the country.

3.LLINs Protocol Objectives

3.1 General Objective

The main objective of this protocol is to provide current information and guide on assessing and evaluating LLINs registration by the Ministry of Animals and Natural Resources and procurement by the National Malaria Control Program/Federal Ministry of Health of Ethiopia that ultimately help in malaria prevention, control and elimination.

3.2 Specific objectives:

The specific objectives of this protocol is to:

- ✓ Determine the bio-efficacy of LLINs against *Anopheles arabiensis*, principal malaria vector insecticidal activity
- ✓ Evaluate wash resistance of LLINs through repeated community wash in the field setting
- ✓ Test the survivorship and fabric integrity of LLINs in various environments and cultural settings
- ✓ Evaluate the insecticide content at the beginning and over time as they are routinely used by people in various settings
- ✓ Determine the acceptability of LLINs by the community

4. Approach

Include a WHO position paper or protocol on which our protocol mainly relied on. If there is a need we could include other references here. Here where we should reflect our protocol mainly uses WHO guideline. Two main approaches can be used to study LLINs durability: (i) prospective longitudinal studies in which nets are followed from the time of distribution until a defined endpoint is reached; and (ii) retrospective, cross-sectional surveys to assess previously distributed nets in a representative sample of households. This protocol proposes to use prospective longitudinal monitoring.

4.1 Study site

The study can be conducted in all representative malarious areas of Ethiopia. The study site will be selected based on conditions of the location that may affect bed net usage, accessibility and local support and on-ground capacity. The sites should represent the environments and cultural settings in which LLINs can be distributed. The study site should be large enough to allow selection of sufficient numbers of nets during follow-up.

4.2 Sample size and net allocation

In order to estimate the number of LLINs for the study, the sample size necessary for a given degree of precision or to detect differences in durability between products should be calculated separately for each main outcome variable (e.g. Attrition rate, physical integrity). The sample size depends on the type of sampling: LLINs that are collected and taken away (after replacements are given) for bioassay and chemical residue analysis are lost to follow-up; therefore, the pool of nets at the start must be accordingly larger in order to have the same number of LLINs available for assessment of the core outcome variables; if nets are left in place after each assessment, the number needed will be smaller. The sample size should also be adjusted for the effect of a cluster design (between-cluster variation) of net assessment and for the expected attrition. If possible, the net products to be studied should be distributed in a fairly large population, in numbers that needed on the basis of the initial sample size calculation.

A subset of households will be selected from these for the follow-up study. Initial distribution to a large population and the selection of a sample at each follow-up should help to ensure that the results are representative and free of covert attempts to influence the attitudes or behaviour of the selected households. In studies in which more than one LLINs product is to be monitored, the different products can be allocated either to households or to individuals or sleeping places. When randomization is at household level, each household should be randomly assigned to receive one type of LLINs.

The number of LLINs that each household receives should be based on the national policy for universal coverage. Equal numbers of households should be assigned to receive each LLINs product to be monitored, although slightly different numbers of each product may distribute because of differences in the numbers of nets required for households in each treatment arm. Random allocation may be possible if a census is conducted well in advance and a master list of eligible households has been drawn up. Pre-printed lists with each household and LLINs product they are to receive should be provided at the time of distribution. If a list of households is not available at the time of distribution, LLINs products can be allocated to consecutive households in a systematic fashion, alternating the different products and providing sufficient nets to each household to cover all family members. Alternatively, different LLINs could be randomly assigned within the same household. This allows direct comparisons of net durability and preference and equal exposure of nets in different types of households (e.g. Rich and poor).

If, however, there is a strong preference for one type of LLINs in a household, which affects the use of different LLINs, this assignment could bias the estimate of LLINs durability. Allocation by this method should be random, if a roster of households with the number of nets required is generated during the census, or systematic as described above for household-level allocation. The LLINs to be distributed in the evaluation should be marked to distinguish them from those that will be (or have been) distributed during a campaign or bought or received from other sources. At a minimum, a label should be sewn into the net at the factory, showing (perhaps with a machine-readable bar-code) date of manufacture and batch number. Additional labels with a unique study code might be applied by the researcher when the nets are opened to identify them as study nets. The study code should be recorded on the master list. In some projects, it was found that labels tended to be lost over time; therefore, the use of permanent ink, car paint or coloured, tear-proof thread knotted into the netting is recommended for long term labelling. Owners should be asked to begin using their nets immediately and to store any existing nets for use when the LLINs provided are worn out. It might be beneficial to conduct a 'hang-up' campaign within one month of distribution to ensure that recipients are using their new nets.

4.3 Sampling

If sampling is done at a net level, a list of randomly selected nets with their unique code numbers and information on the household to which they distributed (e.g. Household identification, name of head of household, Global Positioning System (GPS)) should be given to the staff who are sampling the nets in the field, who should sample all the nets on the list. If sampling is one at the household level, the number of nets distributed to each household should be estimated, and then the number of households required to reach the estimated sample size should be determined. Sampling should be simple random sampling, so that there is an equal probability of selection of each household in the list. Determination of the sample size should take into account the precision and the difference necessary to detect each outcome measure for the different LLINs products.

The estimated sample size should also take into account variations between nets and between households. It should be based on the outcome that requires the largest sample, although subsamples can be taken for some measures (e.g. bio-efficacy). The sample net should be labelled by the study organizers or the manufacturer, allowing easy reading of stored information (product, batch, manufacturing data, distribution date), as long as it has not faded or the label has been lost. Sample size on the basis of attrition, for outcomes such as bio-efficacy, it may not be feasible to test large numbers of nets. If sampling is done at the household level and all nets in the household are sampled, the sample size should be adjusted for design effect and the questionnaire adjusted accordingly.

A sample of 250 LLINs per product will allow detection of a 10% point difference in LLINs attrition rate if the best-performing product has an attrition rate of 10%. This sample size will also allow detection of a 12% point difference in LLINs attrition rate if the best-performing product has an attrition rate of 20%. A list of additional households should be generated from the master list as alternatives for refusals and the absence of a person to interview. The number of nets available for monitoring fabric integrity will decrease over time; however, as observed in previous studies, the fabric integrity of surviving nets is not expected

to change much after a certain time if torn nets are thrown away (and hence lost to follow-up). With an attrition rate of 40% after 2 years, a sample of 250 LLINs per product will provide at least 150 nets for fabric integrity measurement, which is considered sufficient to detect major differences between products in a given setting. A subsample of nets that have been assessed for fabric integrity should be randomly selected and withdrawn (after replacement) for measuring insecticidal activity. In previous studies, 30 nets per LLINs product at each time were found sufficient for bio-efficacy testing, but a larger sample will provide more precision. As mentioned above, the overall number of nets needed for the study should be increased by the number withdrawn. Ideally, nets for bio-efficacy testing should be selected randomly from a roster of nets in the study. All registered products of LLINs by regulatory bodies will be included in the study.

4.4 Households and bed net distribution

LLINs to be evaluated will be distributed to all households included in the sample size in the areas. The total number of households required for the bio-efficacy and wash resistance study will be of equal number of samples of each type of nets. Each net type will be coded and randomly deployed to each study village and the number of nets required for the study will be calculated based on the outcome variables. Each household will be given a code relative to the code on the LLINs. This protocol can be used to monitor the durability of a LLINs product distributed in a country or for comparing several LLINs products. Users of the protocol encouraged to include one or more other LLINs for comparison, in order to provide information for future procurement. When a LLINs product is available in another denier and hence might be expected to have a different durability, it could be included for comparison.

4.5 Attrition

Attrition is the proportion of nets no longer in use as intended after a defined period after their distribution to the households. Attrition can be categorized by the main reasons why a net is no longer used, namely decay (e.g. Destroyed, so torn and worn out that it is considered useless for protection against mosquitoes), absence (e.g. Stolen, given away, moved) or used for other purposes.

4.6 Survivorship

Survivorship is the proportion of distributed nets still available for use as intended in the households to which they were given over a defined period, e.g. One year, two, three or more years.

4.7 Physical or fabric integrity

Reflects the number, location and size of holes in each net. When possible, the assessment can also be categorized by type of hole (burn, tear, seam failure, nibbled or chewed by animals). The physical or fabric integrity of the surviving nets can be assessed as a function of length of use, until deterioration leads to the net being discarded or used for another purpose.

4.8 Insecticidal activity (bio-efficacy)

Bio-efficacy is the degree of Knock Down (KD), mortality or inhibition of blood-feeding induced in susceptible mosquitoes, as determined by standard WHO test procedures and criteria (i.e. Cone bioassay, tunnel test). Insecticidal activity is associated with the type and content or availability of insecticide. The insecticide content is expressed as g/kg or mg/m² of the LLINs and is determined by the method outlined in WHO specifications for LLINs. This information is of value in interpreting data on bio-efficacy. Insecticidal activity can be assessed as a function of length of use. The interaction between insecticide type and content and the location, size and number of holes in ensuring personal protection has not been studied and remains a priority for research.

4.9 Evaluation of the efficacy of LLINs will be conducted in three cases:

The protocol more focuses on field studies to determine the fabric integrity, efficacy and operational acceptability of a LLINs, as summarized below. Although some observations on the safety of such nets will be carried out in the field, a preliminary safety assessment has to be undertaken, following the generic risk assessment model developed by WHO for this purpose, before any field study can be done. In addition, the physical properties of the fabric and factors relating to its structural integrity should conform to WHO specifications for netting materials.

4.9.1 Preliminary test

- Regeneration of insecticide and wash resistance
- Efficacy

4.9.2 Small-scale field trials

- Wash resistance
- Efficacy
- Fabric integrity
- Safety observations

4.9.3 Large-scale field trials

- Wash resistance
- Efficacy
- Fabric integrity
- Safety observations

5. LLINs study Procedure

5.1 Baseline Study

Feasibility study of the sites and collection of baseline information on the communities will be conducted in advance of the study. In line with this, a baseline bioassay and the chemical analysis using the procedure outlined below will be performed. In order to determine the time period required for regeneration of a LLINs after standard washing and holding at 30 °C, bioassays are carried out at 24-hour intervals on net samples washed and dried once and three times consecutively until initial biological activity is restored; nets washed three times are expected to deplete surface insecticide on the net, whereas nets washed once may not. Insecticide bioavailability (efficacy) curves will be established and compared to nets washed once and three times consecutively. The time required (in days) to reach the plateau is the period required for regeneration of the net. If the two curves are different, the longer period will be adopted as the washing interval in Phase I and Phase II studies to ensure that wash resistance is not overestimated.

Besides, the resistance of a LLINs to washing will be determined through standard bioassays carried out on nets washed at intervals required for regeneration (as determined above), using the standard WHO wash, and dried and held at 30 °C. Bioassays will be done after 0, 1, 5, 10, 15 and 20 washes or more as necessary. Each bioassay should be done just before the next wash. Regression curves should be drawn using respectively, percentage mortality and KD versus number of washes. The number of washes providing mortality and or KD above the cutoff point (more than 80% mortality after 24 hours and or above 95% KD after 60 minutes post-exposure) is reported. If a LLINs falls below the cutoff point, the study should continue until 20 washes are reached; a tunnel test (Annex 2) should then be conducted. If a WHO certificate of wash resistance or and regeneration time is provided, this procedure will be exempted.

Net samples (25 cm x 25 cm) will be individually introduced into one liter beakers containing 0.5 liters deionized water, with two grams per liter soap at PH 10–11) added just before and fully dissolved. Beakers will be immediately introduced into a water bath at 30 °C and shaken for 10 minutes at 55 movements per minute. The samples are then removed and rinsed twice for 10 minutes in clean, deionized water in the same shaking conditions as stated above. Nets dry at room temperature and stored at 30 °C in the dark between washes in a horizontal position.

5.1.1 CASE I

Nets will be distributed to each household of the study villages and efficacy will be determined at the start of the project and once every two months for a total of 12 months by a trained person with close supervision of the researchers. Households will be instructed not to wash their nets as this will be done for them during each bimonthly visit by the investigators and by the peasants. During each visit, a precise history of the bed net use, including type of bed, age and number of occupants, reported usage i.e. Daily, occasionally etc., Whether the net hangs or is tucked under the bed covering and whether during the day it hangs, is rolled up or is taken down will be noted with the use of the questionnaire outlined in Annex 1. In addition, during each household visit, each net will be subject to a physical inspection, washing and a bioassay tests as outlined below.

5.1.2 CASE II

Side by side in the selected villages the efficacy, longevity, fabric integrity as well as the community acceptance of the LLINs will be studied every six months in the household randomized trials lasting for three years.

5.2 Physical condition of the nets

Each net will be subjected to a physical inspection, which will include counting the number of holes graded in three size categories; Large, by measuring them by Meter, and noting the location of the holes according to horizontal quarter sections of the sides i.e. Lower quarter, lower middle, upper middle, upper quarter or top (as outlined in Annex 2). However, if nets become so deteriorated and offered no protection they will be replaced with new ones during the course of the study.

5.3 Net washing

Before the start of the field study, local washing conditions will be assessed and areas where washing is deemed excessively harsh will not be eligible for inclusion. The frequency and method of washing under field conditions will also be carefully monitored during the study. If washing conditions prove excessively harsh during the trial the result will be weighed accordingly.

Nets will be washed by trained persons using a standard procedure each time following the standard WHO procedure. The nets will be washed in clean well water with a small quantity of local soap (Soap commonly available in the community; no more than two grams per litre of water). The nets will not be left in the water to soak for longer than 10 minutes and agitated gently for no more than 5 minutes by hand, and the samples should be removed and rinsed twice for 10 minutes in clean water in the same shaking condition as stated above then dried horizontally in the shade. The PH of the water will be recorded. At the same time the Participant will wash their LLINs according to the method of washing following standard WHO procedure on the basis of washing schedule shown in Table 1.

Table 1. Washing schedule

Number of washes	Months of use
Baseline	0
1 wash	2
2washes	4
3washes	6
4washes	8
5washes	10
6washes	12

5.3.1 In case I

Each net will be tested for wash resistance and bio-efficacy once every two months for a total of 12 months (this is case I). The bimonthly washing and bio-efficacy will be conducted in two groups:

- a) Washing to be conducted by trained persons using the standard procedure with close supervision,
- b) Washing to be conducted by the users themselves by instructing them with the method of washing.

Each net will be washed using the protocol described above, according to the schedule outlined in Table 1. Standard WHO cone bioassay will be performed on 30 randomly selected nets of each type at the start of the project and every two months for bioassay, wash resistance and physical inspection. The bioassay will be performed according to the established regeneration time for each net type thereafter cones will be placed in sub sampling locations on each net as shown in Annex 2.

Five susceptible, non-blood fed, two to five day old female susceptible *Anopheles arebiensis* mosquitoes exposed to the net for three minutes, after which they are held for 24 hours with access to the sugar solution. At least 50 mosquitoes on each net (10 replicates) and samples from different type of nets will be tested.

The results will be reported for each net tested along with the pooled results (5 x 10 x 4 = 200 mosquitoes). The number of mosquitoes KD will be counted 60 minutes after exposure, and mortality (functional and effective) after 24 hours of recovery period will be recorded. An average mortality and KD value for each treatment group will then be calculated based on the number of nets for each group (WHO, 2013.1). Bioassay data for each of the sub-sampling locations on the net will also be analysed separately to see if there is a specific trend which may be indicative of more insecticide loss in lower parts of the net due to physical abrasion or lower and central parts of the net due to physical handling. Mosquitoes exposed to untreated nets will be used as controls. If mortality for a given day is between 5% and 20% on the control net it is corrected by an Abbot's formula, if mortality for a given day is > 20% all bioassays carried out on that day will be repeated. The same mosquito strain will be used throughout the study.

The bioassay tests will be carried out at a temperature of 25± 2°C and 70 ± 10% Relative Humidity (RH). If at least 80% of nets meet the cut-offs criteria for WHO cone bioassay test, then the product meets the definition for a LLINs. If a LLINs falls below the cut-off point of cone bioassays, particularly for repellent LLINs six nets with the lowest mortality or knockdown will be exposed in a tunnel test. If at least 80% fail to meet the WHO criteria for the tunnel test, then the net does not meet WHO criteria as a LLINs. The bio-cone test result using LLINs washed by trained persons using the WHO standard procedure will be used for evaluation purpose.

5.3.2 For case II

The same *Anophele* species and same procedure implemented in all cases regarding cone bioassay, following the regeneration of the insecticide or bioavailability given by the manufacturer. The bioassay will be carried on randomly selected 30 sample nets from each type every six months. At the end of three years, at least 80% of nets meet the cut off criteria for either the WHO cone bioassay test or the tunnel test, and then the product meets the definition for a LLINs.

5.4 Tunnel tests

Tunnel test done for insecticides that have a high excito-repellent effect, such as permethrin and etofenprox. Therefore, the efficacy or mortality and blood feeding inhibition of LLINs that no longer meet the criteria of standard cone bioassays will be studied in the laboratory, by releasing non-blood fed female *Anophele arebiansis* mosquitoes, aged 5 - 8 days, in a tunnel (square section 25cm x 25cm) made of glass, 60 cm length. At each end of the tunnel, a 25cm square cage is fitted with extension and covered with polyester netting.

At one third of the length, a disposable cardboard frame is placed in the treated netting sample. The surface of netting “available” to mosquitoes is 400 cm² (20cm x 20cm), with nine holes each one cm in diameter, one hole is located at the centre of the square; the other eight are equidistant and located at five cm from the border. In the short section of the tunnel, a bait unable to move is placed in the cage at the end of the long section of the tunnel, 100 females are introduced at 18:00. Females *An. arebiansis* will be released to fly in the tunnel, but have to make contact with the piece of netting and locate the holes in it before passing through to reach the bait.

The following morning, at 09:00, the mosquitoes are removed and counted separately from each section of the tunnel and the immediate mortality is recorded. Live females are placed in plastic cups with honey solution; delayed mortality is recorded after 24 hours. During tests, cages are maintained at 27°C \pm 2°C and 80% \pm 10% RH under subdued light. Several tunnels will be used simultaneously, one tunnel with untreated netting always being used as a negative control. Blood-feeding inhibition is assessed by comparing the proportion of blood-fed females alive or dead in treated and control tunnels. Overall mortality is measured by pooling the immediate and delayed (24hour) mortalities of mosquitoes from the two sections of the tunnel (Annex 2).

5.5 Chemical assay

5.5.1 For Case I

Chemical assays of the total insecticide content of the netting following the methodology recommended by the manufacturer and WHO before and after the wash resistance and longevity study will support better interpretation of the results. For this purpose, four samples (30cm x 30cm) will be cut from positions 1 up to 4 as outlined in Annex 2, using sharp scissors. The samples will be rolled up and placed in labelled, new, clean aluminium foil prior to analysis. The samples from each net will be combined to provide the average target concentration of the insecticide on the net. Where the target concentration of insecticide is not uniform for the whole net and/or if another chemical is used (i.e., For a combined net), the average target concentration will be calculated separately for the different sections of the net and for each chemical. The number of nets required for chemical analysis is given in table 2 for case I and table 3 for case II.

5.5.2 For Case II

Chemical assays (Mandatory) for case II have also done at the beginning of the trial to ensure that the target dose of the insecticide has been achieved, as well as at the completion of the project. For this purpose, four samples (30cm² x 30cm²) are cut along a diagonal across the roof and three samples along a diagonal across each side of 10 randomly selected nets, using sharp scissors. The samples are rolled up and placed in labelled, new, clean aluminium foil prior to assay. The samples from each net will be combined to provide the average target concentration of the insecticide on each net. The number of nets required for chemical analysis is given in table 2 and 3

Measuring the, levels of insecticide will be done using High Pressure Liquid Chromatography (HPLC) or other appropriate testing procedure recommended by the manufacturer of the specific net type at the London School of Hygiene and Tropical Medicine or elsewhere the service is available. An attempt will also be made to do the duplicate locally.

Table2. Number of nets required for chemical analysis for case I

	Number of nets required for code I	Number of nets required for code II
Baseline chemical analysis at the start of the study	5nets	5nets
LLINs after a year	10 nets	10 nets

Table3. Number of nets required for chemical analysis for case II

	Number of nets required for code I	Number of nets required for the code II
Base line chemical analysis at the start of the study	5 nets	5 nets
LLINs after three years	10 nets	10 nets

6. Adaptations for LLINs with novel insecticides, synergists and insecticide mixtures

LLINs with new active ingredients or mixed formulations may have different mechanisms of action and performance criteria. Some novel LLINs are well understood and familiar, and these can be assessed with established WHOPEs methods and criteria. LLINs that have new intended functions or purposes (e.g. formulations with a synergist), however, will require additional test procedures and criteria. The mechanism of action is entirely different and the conditions for effectiveness are not yet known (e.g. LLINs with slow-acting insecticides), epidemiological evidence of effect on malaria or other vector borne diseases (proof of principle) may be required. The present document provides guidance for evaluating anticipated LLINs products with new insecticides or combinations of insecticides that may become part of a resistance management strategy.

6.1 Characterization of insecticide resistance

New, non-pyrethroid insecticides brought to the public health pesticides market must be tested against a range of resistant mosquito strains in this studies. Therefore, new mosquito strains with novel resistance mechanisms should be established and characterized. Ideally, resistant strains should be characterized by target site modification (e.g. *kdr*) and the presence of different metabolic resistance mechanisms. Phenotypic resistance should be measured just before the trial or within the 6 months before the trial. In this studies, phenotypic resistance should be measured before distribution of nets, at the mid-point of the study and at the end of the study.

6.2 Efficacy testing of nets with insecticides other than pyrethroids

LLINs may contain compounds with entirely new modes of action on mosquitoes. If the new insecticide acts primarily through contact toxicity, like pyrethroids, causing rapid knock-down and mortality, the general framework for evaluating LLINs will be applicable, although modifications may be required in each step of testing. LLINs products that act by causing mortality alone, repellence alone or by an alternative mode of action on mosquitoes, may require, as proof of principle, epidemiological studies to demonstrate their efficacy in reducing malaria transmission or in controlling the disease. A number of modifications are recommended to these studies for LLINs containing novel insecticides. This type of testing is designed to assess the efficacy, wash resistance and regeneration time of the insecticide on the netting. The current guidelines recommend that LLINs be tested against susceptible strains of mosquitoes. When new insecticides are used in the manufacture of LLINs, cross-resistance to other insecticides should be assessed. It may be necessary to modify certain test procedures, depending on the mode of action of the new insecticide. For example, for LLINs with slow-acting insecticides, mortality may be recorded 24, 48 and 72 h after exposure. For LLINs that contain growth regulators, it may be necessary to measure the fertility and fecundity of females exposed to the netting. In these studies, the efficacy of LLINs should be determined against wild, free-flying mosquitoes susceptible both to pyrethroids (where possible, given the spread of pyrethroid resistance) and to the insecticide on the candidate LLINs. The recommendations for the studies of pyrethroid-treated LLINs should be followed, although some modifications may be required, depending on the mode of action of the insecticide on the novel LLINs. The reference LLINs should be a WHOPEs-recommended net with the same or similar specifications in terms of netting material, denier and mesh size. Currently, the reference LLINs will necessarily be a pyrethroid-treated LLINs. As LLINs containing novel insecticides with new modes of action become available, further modification of these guidelines and evaluation methods may be necessary.

A net will be considered to have met the requirements for WHO interim recommendation if the mortality and blood-feeding inhibition of the candidate LLINs washed 20 times is equal to or better than that of the positive control washed 20 times.²³ If the candidate LLINs meets these criteria when tested against a vector population that is susceptible to both pyrethroids and the novel compound, further tests should be conducted in areas where the vector population is resistant to pyrethroids but susceptible to the novel compound. Where pyrethroid-susceptible populations are not available for testing, a reference LLINs should still be included in the comparison as best

practice; however, the decision to recommend the novel product as an LLINs will be made on the basis of its own performance. This studies should include a positive control LLINs arm as recommended above, and WHO cone bioassays and tunnel tests should be done with pyrethroid-susceptible and pyrethroid-resistant strains. The susceptible strain serves for quality control, while the resistant strain is used to estimate the durability of the candidate LLINs under field conditions. Laboratory evaluations of novel LLINs may have to be modified. As noted above, candidate LLINs treated with insecticides with effects on mosquitoes that differ from those of pyrethroids may require proof of principle and new assays.

6.3 Efficacy testing of nets with a mixture of insecticides

The benefits of novel non-pyrethroid insecticidal nets that withstand fewer than 20 standard washes but meet the grave threat of pyrethroid resistance may have to be considered. It is anticipated that novel LLINs products will contain mixtures of at least two unrelated insecticides. ‘Mixtures’ are products in which at least two insecticides are co-formulated, such that an insect on contact is exposed to both insecticides at the same time. In all cases, the efficacy, wash-resistance and regeneration of the candidate LLINs should be determined for both the product as a mixture and for the individual components of the product. This is necessary in order to understand and demonstrate the benefit of combining them. The following modifications in the studies are recommended for LLINs products with mixtures of insecticides: At the beginning testing should be conducted against both a susceptible and one or more pyrethroid-resistant mosquito strains. The regeneration time and washing interval should be those of the final product. The following treatment arms are recommended for LLINs in which two compounds in the mixture are active against mosquitoes, in order to determine the efficacy of the individual insecticides and the added benefit of the mixture: Candidate LLINs with compounds A and B, candidate LLINs with compound A only or candidate LLINs with compound B only.

The trials should initially be conducted in an area with mosquitoes susceptible to both pyrethroids and the compounds in the mixture in the candidate LLINs. If the product is as effective as the reference LLINs, it should also be tested in an area with pyrethroid-resistant mosquito populations that show reduced mortality and blood-feeding inhibition when conventional LLINs with pyrethroid are used. The following treatment arms should be tested: An untreated net, preferably of the same material as the candidate LLINs; if not available, possibly a polyester net. Candidate mixture LLINs, unwashed, candidate mixture LN, washed 20 times, reference LN, unwashed and reference LN, washed 20 times.

The ultimate decision is based on a comparison of the candidate LLINs washed 20 times and the positive control washed 20 times. The efficacy of the candidate LLINs in terms of mortality and blood-feeding inhibition should be equal to or better than that of the positive control. Bioassays of nets before washing, after washing and after the field trial should be done with colony mosquitoes as well as with wild-caught pyrethroid-resistant mosquitoes. As noted above, mosquitoes collected in the field should be additional treatment arms may be required if more than two compounds are present in the candidate LLINs. If removal of one compound from the candidate LLINs significantly alters the migration or release of the other compound, conventionally treated nets might have to be included to test each compound individually (arms 2 and 3). The benefits of novel net products with mixtures of insecticides that withstand fewer than 20 standard washes but meet the grave threat of pyrethroid resistance will be considered. preserved for quality control or future studies of genetic markers of insecticide resistance and their relation to efficacy. The studies should include at least two LLINs products: the candidate LLINs and a reference LLINs. It is not necessary to test the component parts of the candidate LLINs separately. It is recommended that both pyrethroid-susceptible and pyrethroid resistant mosquitoes be tested in WHO bioassays and tunnel tests.

6.4 Efficacy testing of combination nets

Combination LLINs contain two or more different nettings, each of which has a different specification for fibres and/or active ingredient(s), with or without synergists. In case of trials each netting component must be assessed separately. The full product should be studied. When the netting contains a mixture of insecticides or insecticide plus a synergist, the principles for evaluating LLINs with mixtures as described above generally apply. The full product should be compared with a reference LLINs, but bioassays should be conducted separately on each netting component of the LLINs. Depending on the specifications of the net, the sampling scheme for bioassays and chemical assays may require modification of both type of testing procedure.

7. LLINs study statistical analysis

Analysis of variance Chi-square and t-test statistics will be used as appropriate to compare mosquito mortality between the different test series to evaluate the potential of LLINs with WHO cut off criteria. Other appropriate optional software can be used for the analysis.

8. LLINs project budget breakdown

For Evaluating LLINs project currently Birr 760,000.00 is needed detail budget breakdown is maintained in the Memorandum of understanding and it also possible to revise it based on the recent situation at the time of signature of agreement document.

9. LLINs project ethical considerations

The WHO and independent risk assessment of pesticide products have to show that the LLINs are safe to use; all study participants will be provided with a new net free of charge at the end of the study. The purpose of the study will be explained and householders will be asked to participate for the duration of the study. The head of each household will be asked to complete an informed consent form, which will be explained in the local language and read aloud if the person is illiterate (Annex 4). Scientific and Ethical Review Office of Ethiopian Health and Nutrition Research Institute will also approve the protocol and informed consent will be obtained from the study participants.

10. LLINs project reporting

Outcomes should be communicated to the community, to relevant national programme, other stakeholders and to the manufacturers. Investigators are also encouraged to publish the results of these studies in peer-reviewed journals.

11. Publication policy

We at EPHI, aspire to select research paper, through highest quality peer review. To achieve this, the entire peer review and publication process must be thorough, objective, and fair. Almost every aspect of this process involves important ethical principles and decisions, which are seldom explicitly stated and even less often shared with the readership. Journal's reputations depend on the trust of readers, authors, researchers, reviewers, editors, research subjects, funding agencies, and administrators of public health policy. This trust is enhanced by describing as explicitly as possible the journal's policies to ensure the ethical treatment of all participants in the publication process. We have been following Publications Guidelines.

We at EPHI aspire to select research paper, through highest quality peer review. Publications should strictly seek original work that has not been previously published or currently not under review at another journal or conference.

12. LLINs project implementation protocol by Phase

List of activities by phase		Duration	
Phase	Activities	Commencement	Completion
Phase 1	Project proposal development and Finalization	June 1	June 30
Phase 2	Memorandum of understanding to conduct the bio efficacy test	July 1	July 15
Phase 3	Procurement of nets and other essentials	July 1	July 30
Phase 4	Field evaluation of nets and Chemical analysis	August 1	May 30
Phase 5	Data entry and analysis for wash resistance	January 1	May 15
Phase 6	Case I or 12 months report	May 1	May 30
Phase 7	Case II final technical report	May 30/2019	
The total time required	3years		

The end date of LLINs project will be revised based on the starting date of data collection and it is possibly rescheduled based on the working physical year.

13. LLINs project Benefits

The protocol serves as a guide to evaluate LLINs under the local conditions of Ethiopia, which will eventually be used in the malaria control programme and regulatory procedure.

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Annex 1

Information on physical status of the net sampled and its use

1. ID No-----
2. Date (DD/MM/YY) -----
3. Region-----
4. Zone-----
5. District-----
6. Kebele -----
7. Name of the interviewee-----
8. Sex 1) Male 2) Female
9. Age-----
10. Marital status 1) Married 2) Single 3) Other
11. Educational level
 - 1) Illiterate 2) Can read and write 3) Grade 1-4
 - 4) Grade 5-8 5) Grade 9-12 6) above grade12
12. Occupational status?
 - 1)Housewife 2) Farmer 3) Merchant 4) Daily laborer 5) Government employee 6) Factory worker
 - 7) student 8) seeking a job 9) Other-----
13. Type of house? (Observation)
 - 1) House with separate bedroom and kitchen
 - 2) Tukul with kitchen /bedroom together
 - 3) Other-----
14. For how long have you usedthe bed net? -----
15. Do you have bed net? 1) Yes 2) No
16. If yes, type of the bed net 1) Circular 2) Rectangular 3) Combination
17. Number of nets-----
18. Can you tell me the age of each bed net? (Write from old to the recent one)
- 18.1. Bed net one-----
- 18.2. Bed net two-----
- 18.3. Bed net three-----

26.2. Bed net two----- Times

26.3. Bed net three-----Times

27. How often do you wash the net?

27.1. Bed net one

1) Once/year 2) Twice/ year 3) Three times/year 4) Four times/year

5) Never washed 6) other-----

27.2. Bed net two

1) Once/year 2) Twice/ year 3) Three times/year 4) Four times/year

5) Never washed 6) other-----

27.3. Bed net three

1) Once/year 2) Twice/ year 3) Three times/year 4) Four times/year

5) Never washed 6) other-----

28. Can you tell me the temperature of the water used to wash the bed net?

1) With cold water 2) With warm water 3) With hot water

29. Can you tell me the type of detergent or soap used to wash your bed net?

1. Commercial soap

2. Commercial powder

3. Mixture of soap and powder soaking

4. Other-----

30. For how long do you washed the bed net at a time?

1) For less than an hour 2) For more than an hour

31) Do you rinse the bed net before drying? 1) Yes 2) No

32. Do you rub the bed net against the rock? 1) Yes 2) No

33. Where do you dry the bed net after washing?

1) Inside home 2) Outside home 3) other

34. To dry, how do you hang the bed net after washing?

1) Flat 2) Hanging

35. How do you dry the bed net? 1) Sun light 2) Shade 3) other

36. Does the bed net have holes?

36.1. Bed net one 1) Yes 2) No

36.2. Bed net two 1) Yes 2) No

36.3. Bed net three 1) Yes 2) No

37. If Yes, Use the following code for size of holes:

37.1. Bed net one

1. Hole smaller (3mm up to 1cm)
2. Large hole, (1.1cm up to 5cm)
3. Large hole (>5cm)

37.2. Bed net two

1. Hole smaller (3mm up to 1cm)
2. Large hole, (1.1cm up to 5cm)
3. Large hole (>5cm)

37.3. Bed net three

1. Hole smaller (1cm up to 2cm)
2. Medium hole, (2.1cm up to 5cm)
3. Large hole (>5cm)

38. Number of holes on the bed net? (Observation, make right sign if the net is completely worn out (-----))

38.1. Bed net one

1) Lower ¼ 2) Lower middle 1/4 3) Upper middle 1/4 4) Upper 1/4

38.2. Bed net two

1) Lower ¼ 2) Lower middle 1/4 3) Upper middle 1/4 4) Upper 1/4

38.3. Bed net three

1) Lower ¼ 2) Lower middle 1/4 3) Upper middle 1/4 4) Upper 1/4

39. Total number of repaired holes?

39.1. Bed net one

- 1) With stitches-----
- 2) With Knots-----
- 3) With Patches-----

39.2. Bed net two

1) With stitches-----

2) With Knots-----

3) With Patches-----

39.3. Bed net three

1) With stitches-----

2) With Knots-----

3) With Patches-----

40. Neatness of the net? (Observation)

40.1. Bed net one

1) Clean 2) A bit dirty 3) Dirty 4) Very dirty

40.2. Bed net two

1) Clean 2) A bit dirty 3) Dirty 4) Very dirty

40.3. Bed net three

1) Clean 2) A bit dirty 3) Dirty 4) Very dirty

41. What type of net do you prefer?

1) Circular 2) Rectangular 3) both 4) No specific preference

42. Colour preference? 1) White 2) Blue 3) green 4) Other

43. How did you get the net? 1) Paying in cash 2) Loan 3) Free 4) Combination

44. How much birr do you suggest for a single net?

1) 10 Birr and above 2) 11-20 Birr 3) 21- 40 Birr 4) 41 Birr and above

45. Which body do you suggest for net distribution?

1) Health extension workers 2) Kebele administration 3) NGO's

4) Others-----

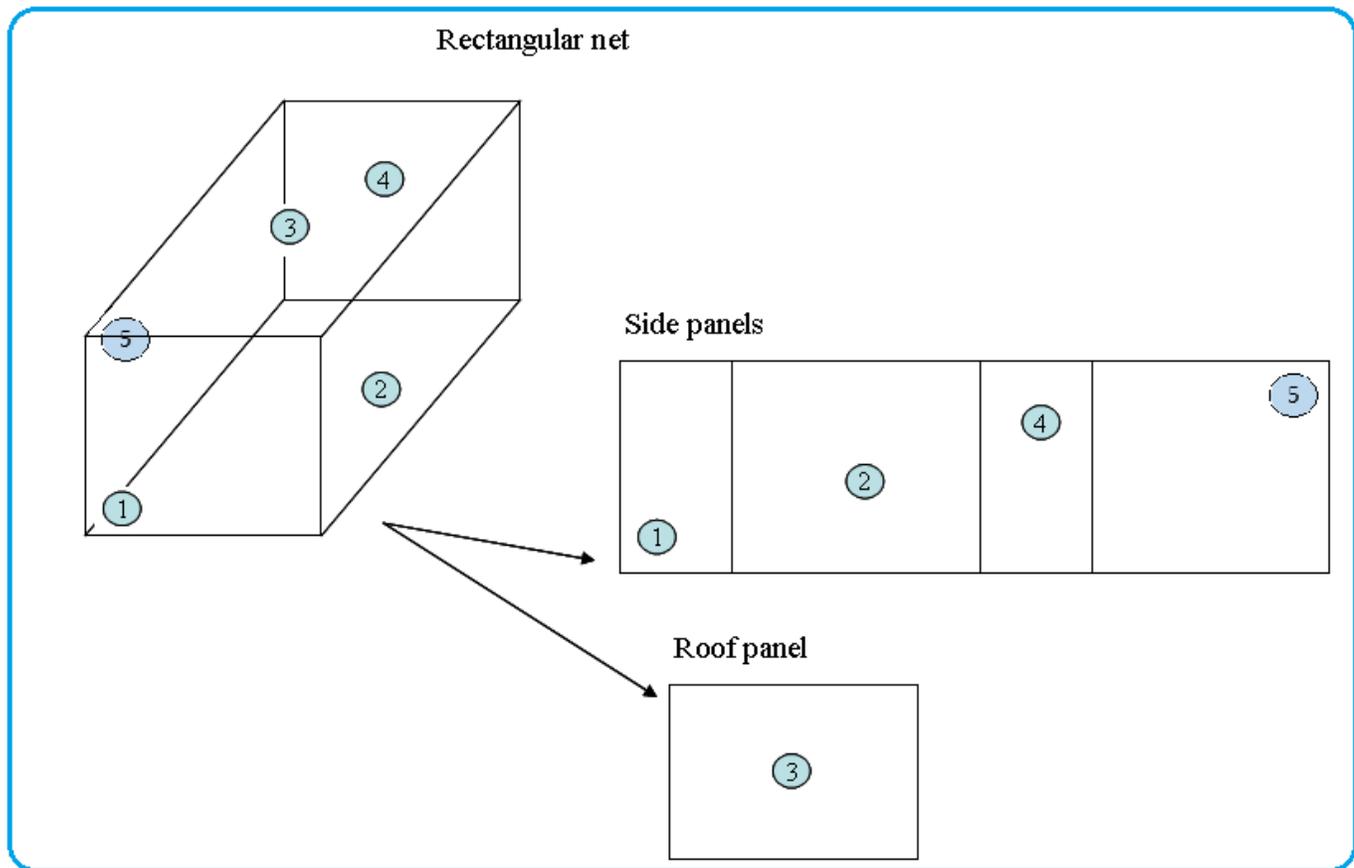
Name and signature of interviewer -----

Date ____/____/____

Annex 2

Sub- sampling procedure for biological assays and chemical analysis

Sub- sampling of the net using standard WHO cones will be performed at the locations outlined in the diagram below:



Side panels bio-cone test and Tunnel test sampling places

Holes classification:

- size 1: smaller than a thumb (0.5–2 cm),
- size 2: larger than a thumb but smaller than a fist (2–10cm),
- size 3: larger than a fist but smaller than a head (10–25 cm) and
- size 4: larger than a head (> 25 cm).



Fig 2. A tunnel made of glass for the study of the efficacy of insecticide-treated mosquito nets



Annex 3

Informed consent Form

Informed consent Form for _____
Name of principal Investigator _____
Name of organization _____
Name of project _____

This Informed consent Form has two parts:

Information sheet (to share information about the study with you)
Certification of consent (for signatures if you choose to participate)
You will be given a copy of the full Information consent Form

Part I: Information sheet

Introduction

I am X, Working for the Y organization I am doing research on the disease malaria, which is very common in this country and in this region. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in research. Before you decide, you can talk to anyone you feel comfortable with about the research.

This consent form may contain words that you do not understand, please ask me to stop as we through the information and I will take time to explain. If you have questions later, you can ask them or me or of another researcher.

Purpose of the research

Malaria is making many people sick in your community, we want to find ways to stop this from happening, we would like to investigate the performance of a long lasting insecticide treated net under conditions of normal use in your community and believe that you can help us by telling us about how you use your net. We want to learn about how long the net is effective under different conditions so that we know when the net should be replaced and how the net should be taken care of.

Type of Research, Intervention

This research will involve your participation in a brief questionnaire and testing of your net over a maximum of 12 month period.

Participant selection

You are being invited to take part in this research because we feel that you experience as a mother/ head of the household/ as a responsible citizen can contribute much to our understanding and knowledge of mosquito net use.

Voluntary participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. The choice that you make will have no bearing on your job or on any work-related evaluations or reports. You may change your mind later and stop participating even if you agreed earlier.

Procedures

We are asking you to help us learn more about mosquito net use in your community. We are inviting you to take part in this research project. If you accept you will be asked to fill out a survey which will be read to and you can say out loud the answer you want me to write down.

If you do not wish to answer any of the questions included in the survey you may skip them and move on to the next question (Describe how the survey will be distributed and collected) the information recorded is confidential, and no one else except (name of persons (S) with access to the information) will have access to your survey.

Risks and Discomforts

We do not wish for you to feel uncomfortable talking same the topic you do not have to answer any question or take part in the discussion/ interview/ survey if you feel the questions (S) are too personal or if talking about them makes you uncomfortable.

Benefits

There will be no direct benefit to you, but your participation is likely to help us find out more about how to take care of these mosquito nets so that they last longer.

Incentives

You will not be provided any incentive to take part in the research. However, we will give you a new long lasting mosquito net at the end of the study.

Confidentiality

The research being done in the community may draw attention and if you participate you may be asked questions by other peoples in the community. We will not be sharing information about you to anyone outside of the research team the information that we collect from this research project will be kept private. Any information about you will have a number on it instead of your name. Only the researchers will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given to anyone except (name who will have access to the information).

Sharing the Results

The knowledge that we get from this research will be shared with you and your community before it is made widely available to the public. There will be small meetings in the community and these will be announced.

Right to Refuse or withdraw

You do not have to take part in this research if you do not wish to do so and choosing to participate will not affect your job or job-related evaluations in any way you may stop participating in the questionnaire at any time that you wish.

Who to contact

If you have any questions, you can ask them now or later If you wish to ask questions later, you may contact any of the following (name, address/telephone number/ e-mail)

This proposal has been reviewed and approved by (name of the local authority)

Part II: Certificate of consent

I have been invited to participate in research about mosquito net use. I understand that I will Participate I a questionnaire and net testing. I have been informed that there are no risks involved. I am aware that there may be no benefit to my personal and that I will receive a new long lasting mosquito net at the end of the study. I have been given the name and address of a researcher who can be easily contacted.

I have read the foregoing information. Or it has been read to me. I have had the opportunity to ask questions about it and questions. I consent volunteered to be a participate in this study and understand that I have a right to withdraw from the study at any time.

Print name of participant-----

Signature of participant-----

Date-----

Day /month/ year

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has given the name of the witness

Print name of witness-----

AND Thumb print of participants

Signature of witness-----

Date-----

Day/ month/ year

I have accurately read or witnessed the accurate reading of the form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given freely.

Print name of researcher-----

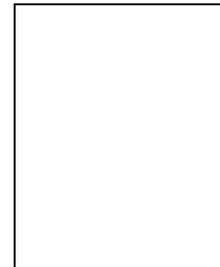
Signature of researcher-----

Date-----

Day/ month/year

A copy of this informed Consent Form has provided to the participate _____

(Initialed by the researcher) _____ **DATE** _____



consent

consent