World Health Organization
Communicable Disease Control,
Prevention and Eradication
WHO Pesticide Evaluation Scheme (WHOPES)

WHO/CDS/WHOPES/GCDPP/2005.11

GUIDELINES

FOR LABORATORY AND FIELD TESTING OF LONG-LASTING INSECTICIDAL MOSQUITO NETS

World Health Organization

WHO/CDS/WHOPES/GCDPP/2005.11

GUIDELINES FOR LABORATORY AND FIELD TESTING OF LONG-LASTING INSECTICIDAL MOSQUITO NETS

COMMUNICABLE DISEASE CONTROL,
PREVENTION AND ERADICATION
WHO PESTICIDE EVALUATION SCHEME (WHOPES)

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

All reasonable precautions have been taken by the World Health Organization to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either express or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall the World Health Organization be liable for damages arising from its use.

Preparation of this document has been funded by the Global Collaboration for Development of Pesticides for Public Health (GCDPP).

CONTENTS

1. INTRODUCTION

The purpose of this document is to provide specific and standardized procedures and guidelines for testing long-lasting insecticidal mosquito nets (LNs) for personal protection and malaria control. It is intended to harmonize the testing procedures carried out to generate data for registration and labelling of such products by national authorities.

An LN is a factory-treated mosquito net expected to retain its biological activity for a minimum number of standard World Health Organization (WHO) washes and a minimum period of time under field conditions. Currently, an LN would be expected to retain biological activity for at least 20 standard WHO washes under laboratory conditions and 3 years of recommended use under field conditions, as defined in these guidelines. The guidelines do not include the testing/evaluation of products for long-lasting post-factory treatment of mosquito nets, which will be subject to separate WHO guidelines, or of the LNs that may use insecticides not currently recommended by WHO for such application.[1] Rather, they reflect the current state of knowledge on LN technology and will be subject to revision as more information becomes available.

The guidelines were reviewed and recommended by the WHO Pesticide Evaluation Scheme (WHOPES) Informal Consultation on the development of guidelines for testing/evaluation of long-lasting insecticidal mosquito nets, held at WHO headquarters in Geneva, Switzerland, on 4–7 April 2005.[2]

---

[1] http://whqlibdoc.who.int/hq/2003/WHO_CDS_WHOPES_2002.5_Rev.1.pdf (accessed 20 April 2005).

[2] The report will be available at: http://www.who.int/whopes/gcdpp/publications/en/

The document includes laboratory, small- and large-scale field studies to determine the efficacy and operational acceptability of an LN, 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,[3] 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]

Phase	Type of study	Activities
Phase I	Laboratory	• Regeneration of insecticide and wash resistance • Efficacy
Phase II	Small-scale field trials	• Wash resistance • Efficacy and impact on vector behaviour • Safety observations
Phase III	Large-scale field trials	• Long-lasting efficacy • Community acceptance • Safety observations

[3] http://whqlibdoc.who.int/hq/2004/WHO_PCS_04.1.pdf (accessed 20 April 2005).
[4] http://www.who.int/malaria/vectorcontrol.html

2. LABORATORY TESTING (PHASE I)

The objectives of the laboratory testing are to determine the efficacy and wash resistance of an LN and to study the dynamics of the insecticide on the fibre. The aim of these experiments is not to simulate washing that would be experienced under field conditions but rather to provide a consistent, repeatable protocol that would allow for comparisons between different laboratories and different LN products.

The test includes:

- determination of the period of time required for full regeneration of the LN after washing;
- determination of the efficacy and wash resistance of the LN against susceptible vector species.

A certificate of chemical analysis must be provided by the manufacturer to ensure that the concentration of the active ingredient is within +25% of the declared content.

2.1 Regeneration and wash resistance

2.1.1 Regeneration time

In order to determine the time period required for regeneration of an LN after standard washing and holding at 30 °C, bioassays (as outlined below) 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 for 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. Details of standard washing (see 2.1.3) and bioassays (see 2.2.1) are provided below.

2.1.2 Wash resistance

The resistance of an LN 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 knock down (KD) versus number of washes. The number of washes providing mortality and/or KD above the cut-off point (more than 80% mortality after 24 hours and/or above 95% KD after 60 minutes post-exposure) is reported. If an LN falls below the cut-off point, the study should continue until 20 washes are reached; a tunnel test (see 2.2.2) should then be conducted.

2.1.3 WHO washing procedure

Net samples (25 cm x 25 cm) will be individually introduced into 1-l beakers containing 0.5 l deionized water, with 2 g/l soap[5] (pH 10–11) added just before and fully dissolved. Beakers will be

[5] Currently, "Savon de Marseille" is recommended as the standard soap. Further standardization, including the use of products recommended by the International Organization for Standardization, or other standard products, is necessary.

immediately introduced into a water bath at 30 °C and shaken for 10 minutes at 155 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 are dried at room temperature and stored at 30 °C in the dark between washes.

2.2 Efficacy

2.2.1 Bioassays

Five susceptible,[6] non-blood fed, 2–5-day old *Anopheles*[7] (species to be stated in the test report) mosquitoes will be exposed to netting materials (25 cm x 25 cm) for 3 minutes, under standard WHO cones[8] (*Figure 1*), after which they are held for 24 hours with access to sugar solution. KD is measured after 60 minutes post-exposure and mortality after 24 hours. At least 50 mosquitoes on each net (10 replicates) and samples from four different nets should be tested. Results should be reported for each net tested along with the pooled results (5 x 10 x 4 = 200 mosquitoes). Mosquitoes exposed to untreated nets are used as controls. Bioassays will be carried out at 25 $\pm$ 2 °C and 75 $\pm$ 10% RH.

Nets washed at least 20 times that cause $\geq$80% mortality and/or $\geq$95% KD meet the criteria to undergo Phase II testing.

---

[6] Susceptibility should be confirmed at least twice per year using standard WHO susceptibility test kits.

[7] Caution should be exercised in comparing results obtained using different *Anopheles* species.

[8] WHO tube tests may be used pending determination of appropriate and equivalent cut-off values.

If coloured LNs are marketed, their bioavailability curves should be compared with those of white nets and, if significantly different, should be considered a separate product requiring full testing and evaluation.

2.2.2 Tunnel tests

The efficacy of treated nets can be underestimated if judged only based on standard cone bioassays. This is specially the case with insecticides that have a high excito-repellent effect, such as permethrin and etofenprox. Therefore, the efficacy (mortality and blood feeding inhibition) of LNs washed 20 times or more that no longer meet the criteria of standard cone bioassays will be studied in the laboratory, by releasing non-blood fed female anopheline mosquitoes, aged 5–8 days, in a tunnel (square section 25 cm x 25 cm) made of glass, 60 cm length (*Figure 2*). At each end of the tunnel, a 25-cm square cage is fitted (extension) and covered with polyester netting. At one third of the length, a disposable cardboard frame is placed with the treated netting sample. The surface of netting "available" to mosquitoes is 400 cm^2 (20 cm x 20 cm), with nine holes each 1 cm in diameter: one hole is located at the centre of the square; the other eight are equidistant and located at 5 cm from the border.

In the shorter section of the tunnel, a bait (e.g. guinea-pig for *An. gambiae*) is placed, unable to move. In the cage at the end of the longer section of the tunnel, 100 females are introduced at 18:00. Females are free 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 ± 2 °C and 80% ± 10% relative humidity 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 (24-hour) mortalities of mosquitoes from the two sections of the tunnel.

Nets washed at least 20 times that cause mortality ≥80% and or blood-feeding inhibition ≥90% in tunnel tests meet the criteria to undergo Phase II testing.

2.2.3 Supplementary tests

Chemical assays. Chemical assays of the total insecticide content of the netting (following the methodology recommended by the manufacturer) before and after wash resistance studies will support better interpretation of the results.

Other assays, such as the median time to knock-down test, may provide useful supplementary information on the bioavailability of the insecticide on the LN after washing. However, caution should be exercised in comparing different insecticide products.

Figure 1. Cone bioassay on mosquito nets (courtesy of Dr Vincent Corbel, Institut de Recherche pour le Développement (IRD), Montpellier, France).

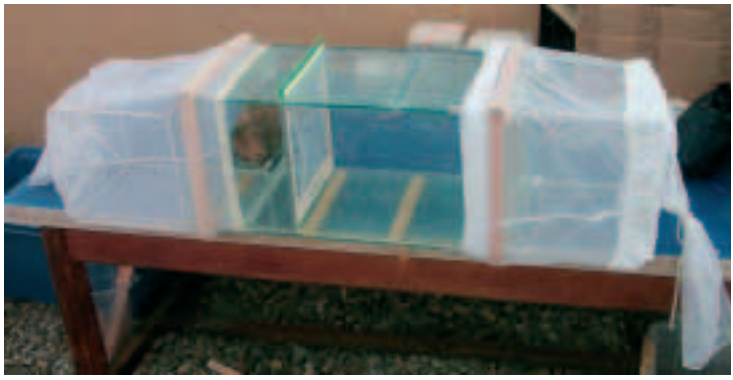

Figure 2. A tunnel made of glass for the study of the efficacy of insecticide-treated mosquito nets (courtesy of Dr Vincent Corbel, Institut de Recherche pour le Développement (IRD), Montpellier, France).

3. SMALL-SCALE FIELD TRIALS (PHASE II)

The efficacy of LNs in terms of blood-feeding inhibition, deterrence, induced exophily and mortality will be studied in experimental huts (*Figure 3*) using susceptible, free-flying, wild mosquitoes.

The small-scale field studies include:

- determination of the efficacy of washed and unwashed LNs and their impact on the behaviour of susceptible wild mosquitoes (anophelines and, where possible, culicines);
- recording the perceived side-effects of the LN among users.

3.1 Efficacy and impact on mosquito behaviour

The impact of washed and unwashed LNs on the blood-feeding inhibition of susceptible (confirmed by WHO susceptibility tests), free-flying, wild mosquitoes (anophelines and, where possible, culicines), their tendency to be repelled or driven out of houses and their mortality will be assessed using experimental huts (fitted with entry slots to prevent escape of mosquitoes), exit traps and/or screened veranda; and with mechanisms for excluding ants and other scavengers that might carry off dead mosquitoes from the huts during the night.

Untreated nets will be used as a negative control. A net conventionally treated with the same insecticide using the WHO-recommended concentration and formulation(s) that is washed to just before exhaustion should also be used as a positive

control. The point of exhaustion should be determined at the field site by washing the conventionally treated net using the Phase II protocol. WHO cone bioassays are performed after each wash. The last wash for which the net still causes ≥80% mortality or ≥95% KD is considered to be the number of washes required before exhaustion.

The following treatment arms should be tested:

- Unwashed LN
- LN washed 20 times
- LN washed according to manufacturer's claim (or maximum washes determined in Phase I)
- Polyester, conventionally treated net washed under Phase II conditions until just before exhaustion (see previous paragraph)
- Polyester, conventionally treated net washed 20 times
- Untreated net (use of the same fabric and mesh size as the test LN is preferred).

The nets are washed according to a protocol adapted from the standard WHO washing procedure used in Phase I, and on cycles equal to the number of days necessary for the full regeneration of the insecticide (bioavailability) established in Phase I. Nets are washed in 10 l of water (preferably well-water or de-chlorinated water with a maximum hardness of 5 dh) and manual agitation for 10 minutes at approximately 20 rotations per minute. Nets will be thoroughly rinsed twice in fresh water and dried horizontally in the shade. The nets will be stored at ambient temperature between washes. Washing should be carefully planned to ensure that all material is ready at the same time.

Each week, the treatment arms are rotated among the huts according to a Latin square scheme (*Annex 1*). Five nets are used per treatment arm and each net is tested one night during the week. At the end of the week, the huts are carefully cleaned and aired to remove potential contamination. The trial should continue for a multiple of 6 weeks to ensure complete rotation through the huts. In most cases, 12 weeks should be long enough to obtain sufficient numbers of mosquitoes for adequate statistical analysis. Before use in the study, each net (including control) should have a total of six holes (4 cm x 4 cm) cut in the sides using sharp scissors to simulate the conditions of a torn net (two holes on each large side and one on each small side).

Two additional LNs from the same production batch should be obtained from industry so that after random allocation to experimental huts, pieces of the netting from the two LNs can be sent for chemical residue analysis. Furthermore, two additional nets are treated conventionally, so that samples of such nets can also be sent for chemical analysis, in order to confirm the initial concentration of the insecticide on mosquito nets. For this purpose, four samples (30 cm^2 x 30 cm^2) are cut along a diagonal across the roof and three samples along a diagonal across each side, using sharp scissors. The samples are 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 each net.

Ethical clearance should be obtained from the appropriate institutions and authorities before starting the study.

Adult volunteers sleep under the nets, and mosquitoes are collected the next morning. Informed consent should be obtained from all volunteers participating in the study. Effective chemoprophylaxis

should be provided where appropriate, and volunteers should be medically supervised. Sleepers are rotated randomly among huts each night of the study. They shall enter the hut at dusk and remain inside until dawn. In the morning, dead mosquitoes are collected from the floor of the hut as well as from the exit traps and inside the nets; resting mosquitoes are collected using aspirators from inside the net and from the walls and roof of the hut and exit traps. Mosquitoes are scored by location as dead or alive and as fed or unfed. Live mosquitoes should be placed in small cups and provided with access to sugar solution for 24 hours to assess delayed mortality.

The primary outcomes measured in experimental huts are: the deterrence (reduction in hut entry relative to the control huts fitted with untreated nets); induced exophily (the proportion of mosquitoes that exit early and are found in exit traps); blood-feeding inhibition (the reduction in blood feeding compared with that in the control huts); and immediate and delayed mortality (the proportion of mosquitoes that are killed). The first three of these outcomes are indicators of personal protection, and benefit individual users. The fact that blood-seeking females are killed is also important because community-wide use of treated nets can, in some circumstances, produce a "mass effect", i.e. a reduction in the density of infective mosquitoes in the area and, consequently, protection of the whole community, including those not using treated nets.

For statistical analysis, the number of mosquitoes entering the huts, the proportion of mosquitoes that exit early, the proportion that are killed within the hut and the proportion that successfully blood feed should be compared by species with the hut as the repeat unit.

The primary criteria in evaluating an LN in experimental huts should be blood-feeding inhibition and mortality in the prominent malaria vectors at the study site. An LN washed 20 times or more should perform equal to or better than a conventionally treated net washed until just before exhaustion. The maximum number of washes to be claimed by a manufacturer will be the one the LN can withstand under Phase II (experimental huts).

3.2 Perceived side-effects

The sleepers in the huts should be questioned about perceived adverse or beneficial side-effects of the LNs and of the conventionally treated and untreated nets.

***Note*:** In view of the long-term studies that may be required to fully test or evaluate an LN product, *interim recommendations* on its use for malaria prevention and control may be given subject to the following: use of WHO-recommended insecticides in making the LN; satisfactory completion of laboratory and small-scale field testing; and confirmation that after at least 20 standard WHO washes the LN performs equal to or better than a conventionally treated net washed until just before exhaustion. It is assumed that in such circumstances the available information on the performance of the conventionally treated nets will assist in anticipating the performance of the LN product in operational settings.

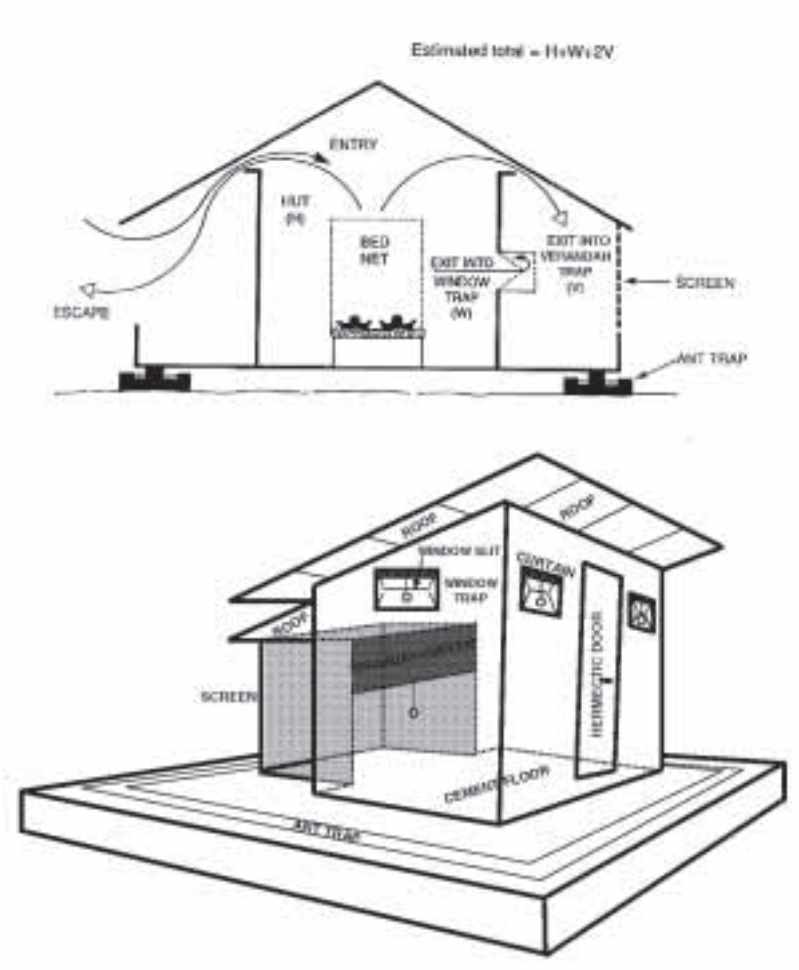

Figure 3. Design of two experimental huts commonly used in west and east Africa (top: United Republic of Tanzania, courtesy of Professor C.F. Curtis, London School of Hygiene and Tropical Medicine; bottom: west Africa, courtesy of Dr J.M. Hougard, Institut de Recherche pour le Développement (IRD), Benin).

4. LARGE-SCALE FIELD TRIALS (PHASE III)

The efficacy, longevity and fabric integrity as well as the community acceptance of an LN will be studied in household randomized trials lasting at least 3 years and in comparison with conventionally treated nets, using the same insecticide, at the WHO recommended dose (highest dose of the range). Conventionally treated nets will be followed for up to 1 year or until biological activity, as measured in bioassays, declines to a level that is significantly lower than that observed in the LN.[9] The efficacy will be determined through regular bioassays and chemical residue analysis at the start and completion of the study.

Ethical clearance should be obtained from the appropriate institutions and authorities before starting the study.

Statistical power to detect changes over time in the performance of the LN should be calculated separately for the outcome variables; the maximum number of samples required shall be used for the calculation of the number of households to be included in each arm of the study (LNs versus conventionally treated nets). In most studies, 30 nets sampled every 6 months is adequate. In calculating the total number of nets required for the study, consideration should be given to possible losses and to nets that need replacing during both the sampling programmes and the study period.

Several communities should preferably be used, and in each community the households shall randomly be allocated to each arm of the study. Baseline information on acceptability, net preferences

[9] LNs will be provided to study participants once their conventionally treated nets have been removed from the study.

(e.g. size, colour, shape) and washing habits should be collected in each community in advance of the study. To distinguish net types, each net should be given a unique code (using an indelible marker or sewing a label on each net) and a net master list developed for later identification and for random selection during follow-ups.

Samples of the consignment of LNs, as well as samples of conventionally treated nets (for each treatment), should be subject to chemical assays 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 to facilitate interpretation of the bioassay data. For this purpose, four samples (30 cm^2 x 30 cm^2) 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. A total of 30 mosquito nets will, however, be samples at the end of the study, in order to account for the higher variability in insecticide content expected on nets used under field conditions.

Nets are randomly drawn from the net master list by the principal investigator and used by the research team for collection of samples for cone bioassay, at the start of the project and every 6 months thereafter. Each household sampled for bioassays will also be interviewed to assess perceived adverse or beneficial side-effects, net utilization patterns (including early morning observations), method and number of washes (questionnaire to be developed and adapted to the local context) and physical integrity of the net (size and number of holes). The household will be provided with a new net and removed from the study.

To reliably measure washing frequency in the community, another net within the sampled households or a net in a neighbouring household will be marked with a water soluble marker. The net will be revisited 1 month later to check the water soluble mark.

Susceptible, non-blood fed, 2–5-day old laboratory-bred *Anopheles* (species to be stated in the report) mosquitoes should preferably be used for cone bioassay, following the instructions provided in section 2.1.1. The bioassay will be carried out on a sample (25 cm x 25 cm) that will be cut from the middle of one larger side of the net. Wild anopheline mosquitoes may be used only if their full susceptibility to the insecticide has been confirmed. The species and blood-fed/non-blood fed condition of mosquitoes should be reported.

If, at the end of 3 years, at least 80% of nets meet the cut-off criteria for either the WHO cone bioassay test or the tunnel test, then the product meets the definition for an LN.

ANNEX 1. Latin square rotation scheme for testing/evaluation of six different treatment arms in experimental huts

		Treatment rotation					
Week	**Day**	**Hut A**	**Hut B**	**Hut C**	**Hut D**	**Hut E**	**Hut F**
1	1	1	2	3	4	5	6
	2	1	2	3	4	5	6
	3	1	2	3	4	5	6
	4	1	2	3	4	5	6
	5	1	2	3	4	5	6
2	8	2	3	4	5	6	1
	9	2	3	4	5	6	1
	10	2	3	4	5	6	1
	11	2	3	4	5	6	1
	12	2	3	4	5	6	1
3	15	3	4	5	6	1	2
	16	3	4	5	6	1	2
	17	3	4	5	6	1	2
	18	3	4	5	6	1	2
	19	3	4	5	6	1	2
4	22	4	5	6	1	2	3
	23	4	5	6	1	2	3
	24	4	5	6	1	2	3
	25	4	5	6	1	2	3
	26	4	5	6	1	2	3
5	29	5	6	1	2	3	4
	30	5	6	1	2	3	4
	31	5	6	1	2	3	4
	32	5	6	1	2	3	4
	33	5	6	1	2	3	4
6	36	6	1	2	3	4	5
	37	6	1	2	3	4	5
	38	6	1	2	3	4	5
	39	6	1	2	3	4	5
	40	6	1	2	3	4	5