



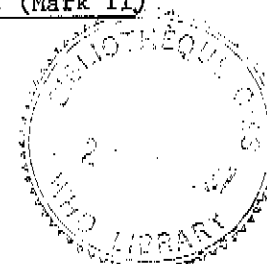
*Antimalarials - pharm  
proc. fac. drug off.*

8815

IN VITRO MICRO-TEST (MARK II) FOR THE ASSESSMENT OF THE RESPONSE OF PLASMODIUM FALCIPARUM TO CHLOROQUINE, QUININE, SULFADOXINE/PYRIMETHAMINE AND AMODIAQUINE

Instructions for use of the in vitro micro-test kit (Mark II) and for completing the record form

CONTENTS



	<u>Page</u>
PART A. INSTRUCTIONS FOR USE OF THE <u>IN VITRO</u> MICRO-TEST KIT (MARK II) .....	3
1. Contents of the micro-test kit .....	3
1.1 Micro-test kit A (basic kit) .....	3
1.2 Micro-test kit B (replenishment) .....	4
2. Important notes .....	4
3. Layout of micro-culture plates .....	5
3.1 Chloroquine (CHL) .....	5
3.2 Mefloquine (MEF) .....	6
3.3 Quinine (QNN) .....	6
3.4 Sulfadoxine/pyrimethamine (SDX/PYR) .....	6
3.5 Amodiaquine (AMO) .....	7
4. Procedures .....	7
4.1 Pre-evaluation of test subjects .....	7
4.2 Preparation of the growth medium .....	7
4.3 Performance of the Mark II micro-test .....	8
5. Examination of the post-culture blood slide .....	10
5.1 Background .....	10
5.2 Counting procedure for the CHL, MEF, QNN and AMO post-culture thick films ....	10
5.3 Counting procedure for the SDX/PYR post-culture thick films .....	10
6. Interpretation and reporting of test results .....	11
6.1 CHL, MEF, QNN and AMO tests .....	11
6.2 SDX/PYR tests .....	12

This document is not issued to the general public, and all rights are reserved by the World Health Organization (WHO). The document may not be reviewed, abstracted, quoted, reproduced or translated, in part or in whole, without the prior written permission of WHO. No part of this document may be stored in a retrieval system or transmitted in any form or by any means - electronic, mechanical or other without the prior written permission of WHO.

The views expressed in documents by named authors are solely the responsibility of those authors.

Ce document n'est pas destiné à être distribué au grand public et tous les droits y afférents sont réservés par l'Organisation mondiale de la Santé (OMS). Il ne peut être commenté, résumé, cité, reproduit ou traduit, partiellement ou en totalité, sans une autorisation préalable écrite de l'OMS. Aucune partie ne doit être chargée dans un système de recherche documentaire ou diffusée sous quelque forme ou par quelque moyen que ce soit - électronique, mécanique, ou autre - sans une autorisation préalable écrite de l'OMS.

Les opinions exprimées dans les documents par des auteurs cités nommément n'engagent que lesdits auteurs.

	<u>Page</u>
PART B. INSTRUCTIONS FOR COMPLETING THE RECORD FORM FOR <u>IN VITRO</u> MICRO-TEST (MARK II) RESULTS .....	13
1. Introduction .....	13
2. General remarks regarding completion of the record form .....	13
3. Completion of record form, section by section .....	14
3.1 Section A: COUNTRY AND PLACE OF TEST .....	14
3.2 Section B: COUNTRY AND PLACE INFECTION PROBABLY CONTRACTED .....	14
3.3 Section C: BLOOD TAKEN .....	15
3.4 Section D: INCUBATION .....	15
3.5 Section E: PATIENT .....	15
3.6 Section F: REASON FOR SCREENING .....	15
3.7 Section G: SAMPLE .....	15
3.8 Section H: DRUG TAKEN DURING LAST TWO WEEKS .....	15
3.9 Section I: PRE-CULTURE SLIDE EXAMINATION .....	16
3.10 Section J: RESULT OF MACRO-TEST .....	16
3.11 Section K: RESULTS OF MICRO-TEST .....	16
3.12 Section L: SLIDE CHECKING .....	17
3.13 Section M: MOVEMENTS OF PATIENT .....	17
3.14 Section N: CONCLUSION .....	17
Form for the Mark II micro-test "Response of <u>P. falciparum</u> to chloroquine, mefloquine, quinine, SDX/PYR and amodiaquine ( <u>in vitro</u> test)" .....	18
Fig. 1 .....	19
Annex 1. List of countries or areas by Region .....	20

PART A. INSTRUCTIONS FOR USE OF THE IN VITRO MICRO-TEST KIT (MARK II)

1. CONTENTS OF THE MICRO-TEST KIT

1.1 Micro-test kit A (basic kit)

Quantity

Tissue culture plate, 12 x 8 wells, predosed with:	12
chloroquine: 1 - 64 pmol per well	
mefloquine: 2 - 128 pmol per well	
quinine: 4 - 256 pmol per well	
sulfadoxine/pyrimethamine (SDX/PYR):	
10 - 10 000 pmol SDX : 0.125 - 125 pmol PYR per well	
amodiaquine: 0.25 -16 pmol per well	
NOTE: 12 plates in any combination of 5 types of plates as required.	
Eppendorf pipette, 50 µl	1
Eppendorf pipette tip, sterile	100
Stock bottle containing RPMI 1640 LPLF, sterile, 100 ml	2
Flask containing lyophilized L-glutamine, sterile, for 10 ml	20
Tube, Falcon, presscap, 6 ml	100
Syringe, sterile, disposable, 20 ml	20
Needle, 1 1/2" x 20 gauge, sterile	20
Microscope slide, frosted edge (box of 72 or 50)	2
Scalpel holder	1
Scalpel blade	5
Forceps	1
Aluminium foil (roll of 30 m x 30 cm)	1
Capillary tube, Heparin treated, sterile, 100 µl (tube of 20)	5
Rack, plastic covered wire	1
Label, round (sheet of 77)	2
Alcohol swabs	50
Pipette, sterile, 1 ml	20
Capillary tube, untreated, non-sterile, 50 µl	500
Mouthpiece for capillary tube	2
Bulb for capillary tube	2
Autolet pricker	1
Lancet for autolet	100
Platform for autolet	100
Glass writing pencil	1
Candle, pure paraffin	2
Buffer for staining vial with powder sufficient for 1 litre solution	5
Romanovsky stain A, plastic dropping bottle, 60 ml	1
Romanovsky stain B, plastic dropping bottle, 60 ml	1
Curved staining plate	1
Instructions for use of the Mark II micro-test kit	3
Sets of record forms for the Mark II micro-test kit	50
Photographs of pre- and post-culture parasites ( <u>Plasmodium falciparum</u> )	4
Grease, silicone, for candle jar (tube)	1
Gloves, protective, rewashable, size medium/large (20 pair each)	40

1.2 Micro-test kit B (replenishment)

Quantity

Tissue culture plate, 12 x 8 wells, predosed with:	6
chloroquine: 1 - 64 pmol per well	
mefloquine: 2 - 128 pmol per well	
quinine: 4 - 256 pmol per well	
sulfadoxine/pyrimethamine:	
10 - 10 000 pmol SDX : 0.125 - 125 pmol PYR per well	
amodiaquine: 0.25 -16 pmol per well	
NOTE: 6 plates in any combination of 5 types of plates as required.	
Eppendorf pipette tip, sterile	50
Stock bottle containing RPMI 1640 LPLF, sterile, 100 ml	1
Flask containing lyophilized L-glutamine, sterile, for 10 ml	10
Tube, Falcon, presscap, 6 ml	50
Syringe, sterile, disposable, 20 ml	10
Needle, 1 1/2" x 20 gauge, sterile	10
Microscope slide, frosted edge (box of 72 or 50)	1
Scalpel blade	2
Capillary tube, Heparin treated, sterile, 100 µl (tube of 20)	3
Label, round (sheet of 77)	1
Alcohol swabs	25
Pipette, sterile, 1 ml	10
Capillary tube, untreated, non-sterile, 50 µl	250
Bulb for capillary tube	2
Lancet for autolet	50
Platform for autolet	50
Glass writing pencil	1
Candle, pure paraffin	2
Buffer for staining vial with powder sufficient for 1 litre solution	2
Romanovsky stain A, plastic dropping bottle, 60 ml	1
Romanovsky stain B, plastic dropping bottle, 60 ml	1
Instructions for use of the Mark II micro-test kit	1
Sets of record forms for the Mark II micro-test kit	25
Gloves, protective, washable, size medium/large (10 pair each)	20

2. IMPORTANT NOTES

2.1 The material supplied with this micro-test kit and which is used to handle human blood is all disposable.

Because of the possible danger which this material can present to uninformed persons great care must be taken with its proper disposal after use.

The correct procedures are detailed in the document "Biosafety in in vivo and in vitro studies of human malaria" by David Payne (WHO/MAL/83.1000).

---

IN NO CIRCUMSTANCES SHOULD DISPOSABLE MATERIAL BE RE-USED.

---

2.2 In the selection of material used in the WHO standard (Mark II) in vitro micro-test kits every effort has been made to specify material that has a long - if not indefinite - shelf-life at normal ambient temperatures.

However, some components of the kit have limited shelf-lives which are considerably reduced even at normal ambient temperature ranges. Accordingly, the following two items should always be stored at +4°C (normal refrigerator temperature) and transported whenever possible at this temperature:

- RPMI 1640 LPLF liquid medium (2 years shelf-life);
- L-glutamine vials (2 years shelf-life).

Whenever possible the predosed test plates (3 years shelf-life) should also be stored at +4°C.

---

DO NOT PUT ANY OF THE COMPONENTS IN THE DEEP FREEZER.

---

2.3 The most convenient way of transporting sensitive material is in a thermos or cool box with appropriate quantities of cooling blocks. If ice is used, it should be sealed in waterproof bags.

Always separate the test material from direct contact with the coolant.

2.4 On receipt of the micro-test kits from the supplier, the recipient should immediately separate the components with a limited shelf-life and store them appropriately.

### 3. LAYOUT OF MICRO-CULTURE PLATES

#### 3.1 Chloroquine (CHL)

---

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
A	0	0	0	0	0	0	0	0	0	0	0	0	pmol/well
B	1												
C	2												
D	4												
E	8												
F	16												
G	32												
H	64												

---

Well A is the control.

Wells B - H represent a chloroquine concentration line based on a geometrical progression of 2<sup>0</sup>; 2<sup>1</sup>; 2<sup>2</sup>; 2<sup>3</sup>; 2<sup>4</sup>; 2<sup>5</sup>; and 2<sup>6</sup>.

3.2 Mefloquine (MEF)

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
A	0	0	0	0	0	0	0	0	0	0	0	0	pmol/well
B	2												
C	4												
D	8												
E	16												
F	32												
G	64												
H	128												

Well A is the control.

Wells B - H represent a mefloquine concentration line based on a geometrical progression of  $2^1$ ;  $2^2$ ;  $2^3$ ;  $2^4$ ;  $2^5$ ;  $2^6$ ; and  $2^7$ .

3.3 Quinine (QNN)

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
A	0	0	0	0	0	0	0	0	0	0	0	0	pmol/well
B	4												
C	8												
D	16												
E	32												
F	64												
G	128												
H	256												

Well A is the control.

Wells B - H represent a quinine concentration line based on a geometrical progression of  $2^2$ ;  $2^3$ ;  $2^4$ ;  $2^5$ ;  $2^6$ ;  $2^7$ ; and  $2^8$ .

3.4 Sulfadoxine/pyrimethamine (SDX/PYR)

Ratio 80:1. Dose shown for SDX.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
A	0	0	0	0	0	0	0	0	0	0	0	0	pmol/well
B	10												
C	30												
D	100												
E	300												
F	1 000												
G	3 000												
H	10 000												

Well A is the control.

Wells B - H represent a SDX/PYR concentration line based on a geometrical progression with the factors 3 and 3.33 (alternating) and the base 10.

### 3.5 Amodiaquine (AMO)

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	
A	0	0	0	0	0	0	0	0	0	0	0	0	pmol/well
B	0.25												
C	0.50												
D	1.00												
E	2.00												
F	4.00												
G	8.00												
H	16.00												

Well A is the control.

Wells B - H represent an amodiaquine concentration line based on a geometrical progression of  $2^{-2}$ ;  $2^{-1}$ ;  $2^0$ ;  $2^1$ ;  $2^2$ ;  $2^3$ ; and  $2^4$ .

## 4. PROCEDURES

### 4.1 Pre-evaluation of test subjects

4.1.1 Persons who have received quinine within the last 7 days, 4-aminoquinolines within the last 14 days, pyrimethamine and/or sulfonamides within the last 28 days, or mefloquine within the last 63 days should be excluded from the test. It is hoped that urine tests currently under development will improve the specificity and sensitivity of the testing procedures for the common antimalarial drugs. It is to be noted that the standard Dill-Glazko test as used for 4-aminoquinolines also gives a positive result with mefloquine and quinine. However, in both cases the colour produced is more orange than red (cognac colour) and will only be found when the patient has taken these drugs within the last few hours or is still in the course of taking treatment.

4.1.2 Written or oral consent, as appropriate, should be obtained from all test subjects from whom blood samples are taken.

4.1.3 Thick and thin blood films are taken from persons suspected of malaria and stained with Giemsa or another reliable Romanovsky stain. Patients who have single infections of *P. falciparum* and asexual parasitaemias in excess of 1000 parasites, but less than 80 000 parasites, are considered suitable for testing. These preselected patients should be subjected to a urine test for 4-aminoquinolines and sulfadoxine and those with positive results should be excluded.

4.1.4 Bio-data and other relevant data on each patient are recorded on the WHO form for the Mark II micro-test "Response of *P. falciparum* to chloroquine, mefloquine, quinine, SDX/PYR and amodiaquine (in vitro test)" supplies of which, along with detailed instructions, are provided with each micro-test kit, and the appropriate parts of the form compiled as the test proceeds. A sample of the form is attached.

### 4.2 Preparation of the growth medium

(a) Take from the test kit, or refrigerated storage, the following items:

- 1 bottle of 100 ml RPMI 1640 LPLF liquid medium;
- 1 or more vials L-glutamine (one vial per 20 test lines);
- Alcohol swabs (for sterilizing the rubber seals of the liquid medium bottle and L-glutamine vial(s));
- 1 syringe, 20 ml;
- 2 needles for above;
- 1 or more Falcon tubes, 6 ml;
- 1 plastic tube rack;
- 1 or more pipettes, graduated, sterile, 1 ml (1 pipette for each L-glutamine vial).

(b) Take the 20 ml syringe and 2 needles being careful to preserve sterility since there is no filtration phase in this test. Wipe clean the rubber seal of the LPLF liquid medium stock bottle with an alcohol swab, let dry and break the seal with one needle. Mount the other needle on the 20 ml syringe.

(c) Withdraw 10 ml of LPLF liquid medium from the 100 ml stock bottle. Recap the needle and place the syringe in a safe place.

(d) Remove the seal-breaking needle and after wiping clean the rubber seal of the L-glutamine vial with an alcohol swab and letting dry again, use the same needle to break the seal of the L-glutamine vial.

(e) Uncap the needle of the 20 ml syringe and introduce the 10 ml of LPLF liquid medium into the L-glutamine vial. Gently shake to dissolve the L-glutamine. Remove and recap the seal-breaking needle. Recap also the 20 ml syringe needle and keep both in a safe place for possible later use (see paragraph (g)).

(f) Remove the aluminium cap and rubber seal from the L-glutamine vial (being careful to preserve sterility), and using a 1 ml graduated pipette put 0.9 ml of L-glutamine plus LPLF medium into as many 6 ml Falcon presscap tubes as there are patients to be tested. Label the tubes with the serial numbers of the patients to be tested and store upright in tube rack.

(g) If additional tubes are required, the same preparatory procedures can be followed with the same syringes and needles previously used.

(h) Return the partially used LPLF liquid medium 100 ml stock bottle to cold storage and discard the used syringes and needles in accordance with the document "Biosafety in in vivo and in vitro studies of human malaria" by David Payne (WHO/MAL/83.1000).

(i) The 6 ml Falcon tubes containing 0.9 ml LPLF liquid medium can be stored in a refrigerator for up to 48 hours before use or can be transported in the field in a thermos containing ice cubes or coolant blocks. Ensure, however, that the tubes do not come into direct contact with the ice blocks and that they do not become inverted or immersed in water since this could result in contamination during incubation.

NOTE: When it is not feasible to carry out the in vitro test in the field or when it is necessary to transport the blood of patient(s) over a long distance, the individual patient's blood should be placed in the LPLF medium (see paragraph 4.3.1) and transported in a thermos with ice cubes or coolant blocks. It is imperative that the tubes should not come into direct contact with the coolant since shock lysis (spontaneous rupture of the red cells) may occur. This can be avoided if the tubes are wrapped in cottonwool or plastic foam. The most efficient coolant is wet ice: equal volumes of crushed ice and water. When this technique is used, care must be taken to protect the tubes from direct contact with the water which may cause contamination. If the tubes are first sealed in a plastic bag and then wrapped in cottonwool or plastic foam, this normally prevents any problem of contamination from the wet ice.

#### 4.3 Performance of the Mark II micro-test

4.3.1 With the Autolet apparatus prick the finger of the patient and withdraw 100  $\mu$ l blood into a sterile heparinized capillary tube. Transfer the blood quickly into a 6 ml plastic Falcon tube containing 0.9 ml LPLF liquid medium (see section 4.2 (f)) and duly label it with the serial number of the test subject. Press the cap firmly into place and gently agitate the tube to mix the blood and medium. The transfer of the blood from the heparinized capillary tube to the 6 ml medium tube is made possible by means of the small black 1 ml rubber bulb supplied with the kit. The bulb is slipped onto the distal end of 100  $\mu$ l capillary before the tube is used to collect the blood, and, by sealing the hole in the end of the bulb with a finger tip and applying slight pressure on the bulb, the blood in the capillary tube will be expressed into the medium tube without difficulty.

4.3.2 Prepare the pre-culture thick and thin films.

4.3.3 The blood/medium mixture is stable for several hours and the tubes can be carried in the breast pocket to maintain the contents at approximately body temperature. If transportation delays in excess of 4 hours are foreseen, the wet ice technique described in the NOTE at the end of section 4.2 should be followed. Ambient temperatures in excess of 40°C will destroy the parasites.

4.3.4 The plastic sealing strips on the required number of test wells of the appropriate test plate(s) are removed by first cutting along the appropriate rows with a scalpel and then lifting off the required area of plastic with forceps, taking care not to contaminate the wells.

4.3.5 All the wells of the appropriate row (one row for each test subject) are dosed with 50 µl of the blood/medium mixture (1:9) using the 50 µl Eppendorf pipette and a disposable sterile tip as provided with the kit. Dosing is always done starting with the control well (A) and following an increasing order of concentration ending at well H. It is most important that the blood/medium mixture in the 6 ml tube be agitated from time to time to ensure that the blood is kept in suspension and thus evenly distributed to all the wells. The sterile disposable tip is then removed and discarded.

4.3.6 A new sterile disposable tip is fitted to the Eppendorf pipette and the next row is set up in exactly the same way and so on until all the samples have been aliquoted onto the plate(s).

4.3.7 Place the lid on the microplate and with a glass marking pen write in the details of each test subject over the appropriate row of the plate.

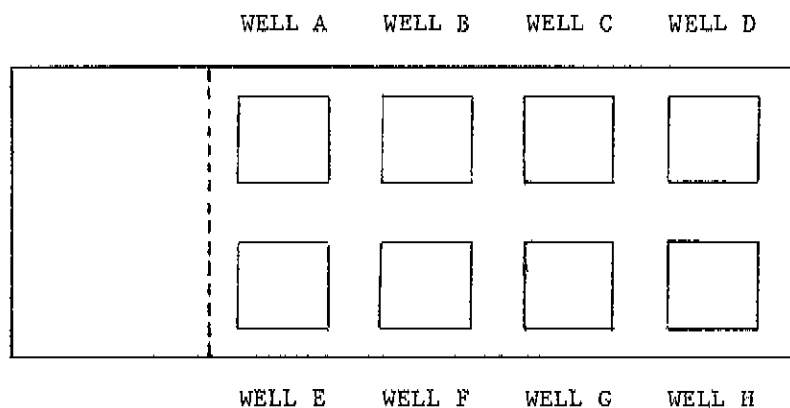
4.3.8 Shake the plate gently so that the drug deposit in the well is completely dissolved.

4.3.9 Take the candle jar from the incubator (set to give an internal temperature in the candle jar of 37.5°C; prewarming for at least one hour is most important) and load it with the plates to be incubated. Light two candles (only the pure paraffin candles as supplied with the kit should be used) and locate one on each side of the stacked plates. Do not put the candles on top of the plates. Replace the candle jar lid making a good seal but with the exhaust cock in the open position. When the second candle is at the point of extinction close the exhaust cock.

4.3.10 Place the candle jar in the incubator noting the time.

4.3.11 Incubate at 37.5°C (+ 0.5°C) for 24-30 hours (depending on the development stage of the trophozoites in the pre-culture slide). Experience to date indicates that isolates which have not produced schizonts within 30 hours are stressed by factors which invalidate the test procedure. By far the most common reason for delayed maturation of schizonts is the previous intake by the test subject of antimalarial drugs.

4.3.12 After incubation the contents of the test wells are harvested by removal of the supernatant with 50 ul capillary tubes (and the black rubber suction bulb) and the red blood cells deposited on the flat bottom of the wells are transferred to a clean microscope slide to form a series of thick films as detailed in the format below:



A fresh capillary tube must be used for each thick film.

4.3.13 The resultant thick films must be carefully dried before staining otherwise they will spontaneously detach from the slide. If air-dried, 24-48 hours are normally required, but this period can be reduced by drying in an incubator set at 37.5°C (30 minutes) or with a hair dryer. In this event care should be taken not to overheat the films and thus auto-fix them.

Some workers report highly satisfactory results with acetone treated slides. The thick film is air-dried until visibly dry, usually within 30 minutes. Then the slide is dipped into pure acetone, air-dried and stained as described below.

4.3.14 The thick films are stained for 30 minutes in a Giemsa stain of superior quality at a dilution of 1% (v/v) in water of pH 7.2. Great care must be exercised in handling the stained films until they are completely dry.

Drying can be performed in the air, in an incubator (37.5°C) or with a hair drier, but in the latter case care must be taken to avoid overheating and degradation of the stain.

## 5. Examination of the post-culture blood slide

### 5.1 Background

It is most important to realize that the schizont counting procedure for SDX/PYR is different from that for the other four drugs (CHL, MEF, QNN and AMO) in the Mark II micro-test system.

### 5.2 Counting procedure for the CHL, MEF, QNN and AMO post-culture thick films

The basis for the count is:

---

NUMBER OF SCHIZONTS WITH THREE OR MORE NUCLEI OUT OF A TOTAL OF 200 ASEXUAL PARASITES (i.e. SCHIZONTS AND TROPHOZOITES).

---

For an acceptable test, schizont maturation must be 10% or more (i.e. 20 schizonts with three or more nuclei per 200 asexual parasites). This count can then be expressed as a percentage of the control as in the following example:

CONTROL	DRUG UNDER TEST	
Number of schizonts, i.e. parasites with 3 or more nuclei per 200 parasites after incubation.	Number of schizonts. To be repeated for each test well.	
Control	No. of schizonts per 200 parasites after incubation	% of schizonts relative to control samples (controls = 100%)
98	49	$\frac{49}{98} \times 100 = 50\%$

### 5.3 Counting procedure for the SDX/PYR post-culture thick films

In the WHO standard micro-test the action of SDX/PYR on the developing schizont is not so clear cut as that of CHL, MEF, QNN or AMO. Schizonts with three nuclei or more develop even when the drug is effective although these nuclei are often not well defined and the schizont may appear abnormally developed. Accordingly, to establish the "breakpoint" or "end point" of sensitivity/resistance, is more difficult and the rate of "schizonts with three nuclei or more" cannot be used to define schizont growth as is the case for CHL, MEF, QNN and AMO.

To overcome this difficulty, and to avoid confusion in distinguishing normal from abnormal nuclei, the threshold of schizont growth has been changed to one of eight or more normal nuclei. The reason for this is that schizonts which develop to the stage of eight nuclei or more in the presence of SDX/PYR are usually not abnormal, and thus indicate normal growth (maturation).

Bearing in mind the foregoing, the counting of SDX/PYR thick films is based on three criteria:

- (a) presence of trophozoites;
- (b) presence of schizonts with 3-7 nuclei, and abnormal schizonts;
- (c) presence of normal schizonts with 8 nuclei or more.

SDX/PYR comparative asexual parasite counts are therefore slightly more complex than those of the standard WHO micro-test for CHL, MEF, QNN and AMO but with some practice are relatively easily mastered. The procedure for SDX/PYR is almost identical to that for the four other drugs, with the exception that it is necessary to enumerate schizonts with eight or more normal nuclei against the total asexual parasite count of 200.

## 6. INTERPRETATION AND REPORTING OF TEST RESULTS

When the results of tests become available, they should be immediately entered into the WHO form "Response of P. falciparum to chloroquine, mefloquine, quinine, SDX/PYR and amodiaquine" which has been specifically revised for the Mark II in vitro micro-test. The results of this test cannot be entered into the first series of in vitro test forms as the drug concentrations are different.

When the completed form is sent to the appropriate Regional Office the data will be computer-analysed and in due course will be included in the periodic report of the Global Monitoring Programme. However, the investigator can make his or her own analysis of the data either in the simplified form of a table or, by using the TI 59 programmable calculator, as a regression line based on probit analysis of logdose/response test from 3-8 points assay (see the document "Evaluation of in vitro tests for drug sensitivity in Plasmodium falciparum: probit analysis of logdose/response test from 3-8 points assay" by B. Grab & W. H. Wernsdorfer, WHO/MAL/83.990). The results provided by this latter treatment closely parallel those achieved by the computer of the Global Monitoring Programme.

### 6.1 CHL, MEF, QNN and AMO tests

Provided that

- (a) there is satisfactory growth in the control (i.e. 20 or more schizonts with three or more nuclei in 200 asexual parasites);
- (b) the original isolate did not contain more than 80 000 parasites per microlitre of blood, and
- (c) the infection was indeed P. falciparum alone, then:

Test drug	Satisfactory response	Indication of resistance
	Complete schizont inhibition at	Schizont growth at
Chloroquine	4 pmol or less	8 pmol or more
Mefloquine	(*)	64 pmol or more
Quinine	128 pmol or less	256 pmol or more
Amodiaquine	2 pmol or less	4 pmol or more

(\*) Determination of critical concentration pending (on the basis of comparative in vivo and in vitro tests).

More meaningful data are obtained when a series of tests (ideally 30 or more, but at a minimum 10) are grouped.

These grouped data are simply achieved by adding up the schizont counts for the control and each concentration and then dividing by the number of tests so as to obtain mean schizont counts. These are transformed into the percentage of schizonts relative to the control samples as described previously. This represents schizont maturation. If this figure is deducted from 100, the remainder is the percentage of schizont maturation inhibition.

The table below gives an example of this for a series of 10 acceptable chloroquine tests:

Test number	Schizont counts per test well of:							
	K	1 pmol	2 pmol	4 pmol	8 pmol	16 pmol	32 pmol	64 pmol
1	190	164	81	43	11	2	0	0
2	112	64	32	0	0			
3	178	179	160	138	112	90	40	0
4	196	192	190	186	110	65	23	0
5	49	53	21	11	2	0	0	
6	34	32	30	21	6	1	0	0
7	200	191	111	65	38	11	3	0
8	29	3	1	0	0			
9	128	64	30	16	2	0	0	
10	55	53	47	26	12	0	0	
Total schizonts	1 171	995	703	506	293	169	66	0
Mean number of schizonts	117	100	70	51	29	17	7	0
Percentage of control	100.0	85.5	59.8	43.6	24.8	14.5	6.0	0
Percentage of maturation		85.5	59.8	43.6	24.8	14.5	6.0	0
Percentage of inhibition		14.5	40.2	56.4	75.2	85.5	94.0	100.0

Obviously there is still significant schizont maturation in the wells containing 16 and 32 pmol (and three out of ten isolates show schizont maturation at 32 pmol). As already shown in the first table of this section 6.1, schizont maturation in the 8 pmol well is indicative of resistance. Eight of the above ten isolates showed schizont maturation at this well. This series demonstrates a status of manifest resistance.

The principal advantage of this system is the comparability of quantitative results, both between different geographical areas and between series from the same area at different times (longitudinal studies). Such monitoring will provide advance warning of important reductions of drug sensitivity, and enable the health authorities to devise drug policies which may delay the advent of resistance.

## 6.2 SDX/PYR tests

The thick film counts will produce data as shown in the following tabulated example:

Parameter	Well							
	A	B	C	D	E	F	G	H
Schizont with 8 or more normal nuclei	55	56	47	8	0	0	0	0
Other schizonts and rings	145	144	153	192	200	200	200	200
% schizont maturation (% of control)		100	85	15	0	0	0	0
% schizont inhibition		0	15	85	100	100	100	100

NOTE: Well A = control; well B = the lowest concentration of SDX/PYR;  
well H = the highest concentration of SDX/PYR.

The "breakpoint" in this example is therefore between well D (SDX 1000 pmol/PYR 12.5 pmol per well) and well E (SDX 3000 pmol/PYR 37.5 pmol per well). Data so far available indicate that the 90% inhibition level of the schizonts with eight or more nuclei reflects more accurately the true "breakpoint" and this point can be readily ascertained by means of a simple graph as demonstrated in the attached Fig. 1, using the data given above.

PART B. INSTRUCTIONS FOR COMPLETING THE RECORD FORM FOR  
IN VITRO MICRO-TEST (MARK II) RESULTS

1. INTRODUCTION

As with the original, the purpose of this revised record form (see attached sample) is to allow, through the cooperation of as many centres as possible, computer-assisted monitoring of the in vitro response of Plasmodium falciparum to antimalarial drugs. The range of test drugs has been expanded and now comprises chloroquine, mefloquine, quinine, sulfadoxine/pyrimethamine (SDX/PYR) and amodiaquine. The record form was developed in consultation with many investigators from many countries who were actually involved in the development and utilization of the in vitro tests. Therefore, it reflects a compromise with respect to the selection of the items of information to be retained for processing. Investigators may, very legitimately, wish to collect additional information, for which the form makes no provision. In those cases it is hoped that this form will still be used, and that the additional information will be recorded separately.

The form is automatically produced in four copies including the original. They are for: (1) the investigator; (2) WHO/MAP, Geneva; (3) the Regional Office; and (4) an authority or institution at the national level. The computer processing will, at least initially, be done by WHO, Geneva.

2. GENERAL REMARKS REGARDING COMPLETION OF THE RECORD FORM

The record form is completed by the investigator at the time the test is performed (see introductory paragraph to section 3). The record is designed as a primary record, i.e. for direct recording of observations, without any intermediate or provisional record from which the information would have to be copied (as this would mean additional work and risks of error).

The information to be put into this record falls into two categories:

- (a) numerical information for processing by computer; this information is put into the data columns, numbered from 1 to 201; part of that information is directly numerical (e.g. the number of schizonts in 200 asexual parasites at a given concentration of chloroquine); the rest of that information is not directly numerical but becomes numerical through the use of a predetermined numerical code (e.g. "reason for screening": eight "reasons" are listed and numbered; the number corresponding to the actual "reason" will be put in column 46);
- (b) information (verbal or numerical) that will not be processed by computer; but part of this information appears also in coded form in the previous category (e.g. the probable country of infection is given in words and in code); the rest is additional information which may be useful to the investigator, but is not required for computer processing (e.g. the name of the patient). Some investigators will collect and record still other information (see section 1).

### 3. COMPLETION OF THE RECORD FORM, SECTION BY SECTION

The form is arranged by sections, designated by a letter: A - I and K - N; the former section J (macro-test) has been deleted from this form. Each data space, called a column, has an identification number. A record is completed for persons found eligible by screening; the results of screening are not put onto this record.

- Sections A - H are completed at the time of blood collection, i.e. collection of the blood for culture and for the pre-culture slide (distinct from the earlier slide collected for screening).
- Section I is completed at the time of examination of the pre-culture slide.
- Sections K and L are completed at the time of examination of the slides from the tests.
- Section M is completed for cases in which resistance has been detected by the tests.
- Section N is completed when the investigator interprets the test results (this interpretation may make use of graphs, which remain with the investigator).

#### 3.1 Section A: COUNTRY AND PLACE OF TEST

States the place where the test is performed and identifies the institution and person (investigator) performing the test. Obviously, in some countries, more than one institution may be conducting tests and there may be several investigators. Space for an UNCODED NUMBER is provided for the investigator's convenience.

In the coded part, columns 1-4 are for a serial number for the test, unique within the country (except if more than 9999 tests have been performed in one country; in that case numbering starts again with 0001, 0002, etc.). Columns 5-7 and 8-9 are, respectively, for the code letters of the country (each country has a standard ISO Letter Code: see Annex 1) and the number of the institution (each country is expected to assign code numbers to its relevant institutions). The letters of the ISO Country Code may vary from those of the WHO Country Code which was previously used, but has now been replaced by the ISO Code.

In order to keep the test serial numbers (columns 1-4) unique within the country, different series of numbers should be attributed to the different institutions performing the test in the same country. This attribution should be made by mutual agreement between the responsible government official and the investigators (e.g. numbers 1 to 500 to institution 01, numbers 501 to 1 000 to institution 02).

#### 3.2 Section B: COUNTRY AND PLACE INFECTION PROBABLY CONTRACTED

In some places the person being tested may have contracted the infection in a different place from where the test was made, and may even have contracted it in another country. It is very important to identify the place of infection if this is at all possible. To enable analysts to identify the area with some certainty, the geographical location (coordinates) of the area of probable infection is required (this can either be read directly off a map, or represented by the nearest place recorded in the list of place names in the back of an atlas, or in official gazettes and postal union reference books).

Columns 10-12 are for the three letters of the ISO Country Code (see Annex 1) and 13-14 for the Province/State (each country is expected to assign code numbers to its primary subdivisions, such as states, provinces or territories). A list of the standard ISO Country Code abbreviations for each country is annexed to these instructions. The letters may vary from those previously used in the WHO Country Code.

The geographical location is coded as follows: columns 15 and 20 are used to give the nominal compass bearing (North or South for latitude, East or West for longitude) and columns 16-19 and 21-25 are for the actual map readings in degrees and minutes. It is important to note that numbers are written in columns 15 and 20 and not letters.

### 3.3 Section C: BLOOD TAKEN

Records the details of when the test was performed. The day, month and year (last two digits) are entered in columns 26-27, 28-29 and 30-31 respectively. The time, using the 24-hour clock, is written into columns 32-35, e.g. 1 May 1987, 7.30 p.m. (19.30 hours using the 24-hour clock) will be coded as follows:

/0/1/    /0/5/    /8/7/    /1/9/    /3/0/

### 3.4 Section D: INCUBATION

Records the incubation time in hours and minutes (24-hour clock); the starting time in columns 36-39; the termination time in columns 40-43. The duration of the incubation time is given in the space provided (hours only) but, as this is not required for the computer, it is not coded. The maximum time acceptable for a micro-test based on schizont maturation is 30 hours (see Part A of this document for further information).

### 3.5 Section E: PATIENT

The name and sex of the patient is entered in the space provided and is uncoded; the age, in years, is coded in columns 44-45 (if the patient is less than one year of age, two zeros are entered).

### 3.6 Section F: REASON FOR SCREENING

It is relevant to know the reason why a particular patient is chosen for screening, and this is indicated by selecting one of the explanations numbered 1 to 8 and entering it into column 46. Code 8 includes all reasons not listed in 1 to 7.

### 3.7 Section G: SAMPLE

It is also important to know what is the source from where the patient is drawn and this is shown by selecting one of the items coded 1 to 7, and entering it in column 47. Code 7 includes all sources not listed in 1 to 6.

### 3.8 Section H: DRUG TAKEN DURING LAST TWO WEEKS

As a rule, the test should be performed only on persons who have taken no antimalarial drugs during the last two weeks. Sometimes, however, it will not be possible to follow this rule. Hence this section. The answer to the question, "Did you take any antimalarial treatment during the last two weeks?", is coded in column 48 (1 = yes; 2 = no; 3 = do not know). If the answer is yes, the name of the drug, as stated by the person, is recorded in the space provided, and a code number is entered in column 49, according to the following code system:

<u>1</u>	4-aminoquinolines	<u>6</u>	pyrimethamine/sulfone
<u>2</u>	proguanil	<u>7</u>	antibiotics
<u>3</u>	pyrimethamine	<u>8</u>	other
<u>4</u>	quinine	<u>9</u>	unknown
<u>5</u>	pyrimethamine/sulfonamide		

If a patient has taken more than one separate drug, record the most recent.

Columns 50 to 53 provide space for urine tests for chloroquine (or amodiaquine), mefloquine, quinine and sulfadoxine. The result of the test is entered into the respective column, e.g. column 50 for chloroquine using 1 for positive, 2 for negative. If the result is doubtful 3 is entered, if the test is not done 4.

### 3.9 Section I: PRE-CULTURE SLIDE EXAMINATION

The examination of the pre-culture slide will involve the enumeration of asexual forms of P. falciparum and of leukocytes (WBC). The only information coded for computer processing is the actual total number of asexual P. falciparum counted, to be entered in columns 54-58, and the actual number of leukocytes (WBC) counted, to be entered in columns 59-62. In addition, space is provided for information which may be of importance to the investigator, but will not be processed by computer, namely the breakdown of P. falciparum rings actually counted into small, medium and large, and the number of asexual P. falciparum per mm<sup>3</sup> of blood.

### 3.10 Section J: RESULT OF MACRO-TEST

In view of the almost total replacement of this test by the micro-test, it has been excluded from the revised form. Macro-test results can still be recorded on the original form while stocks last.

### 3.11 Section K: RESULTS OF MICRO-TEST

Records the results of the schizont counts in the control and seven test wells. Each result has three columns allotted to it and there are separate rows of boxes for chloroquine, mefloquine, quinine, SDX/PYR and amodiaquine results, columns 63-86, 90-113, 117-140, 144-167 and 171-194 respectively. The batch numbers of the test plates are entered in columns 87-89, 114-116, 141-143, 168-170 and 195-197 respectively. The batch number of the RPMI 1640 LPLF medium is entered in boxes 198-199.

Three kinds of entry are required:

- figures denoting the actual schizont count when growth occurs;
- zeros when counts are made and no schizont growth is seen, and then zero is assumed after two consecutive zero counts have been recorded;
- dashes if the reading is impossible, e.g. if a post-culture thick blood film is lost or heavily contaminated.

Below are three examples demonstrating this in the micro-test result column of the form:

Example 1

**K RESULT OF MICRO-TEST**

CHLOROQUINE p mol/well →	Control	1	2	4	8	16	32	64
SCHIZONT./200 paras. →	141	152	151	132	067	015	004	000
	108 %	107 %	94 %	48 %	11 %	3 %	0 %	

Test plate batch No.

This is a resistant infection with schizont growth recorded in all concentrations except 64 pmol.

(Please note that a control count which exceeds 200 is impossible since the control represents the number of schizonts in a total count of asexual parasites - trophozoites and schizonts - of 200.)

Example 2

**K RESULT OF MICRO-TEST**

CHLOROQUINE p mol/well →	Control	1	2	4	8	16	32	64
SCHIZONT./200 paras. →	141	122	051	000	000	000	000	000
	87 %	36 %	0 %	0 %	0 %	0 %	0 %	0 %

Test plate batch No.

This is a sensitive infection with growth up to and including 2 pmol. Thereafter pmol concentrations 4 and 8 were read and found negative. In accordance with the rule of "two consecutive zeros" the count stopped here, but as the remaining three concentrations (16, 32 and 64 pmol) were tested, zeros are entered since we extrapolate that they would be zero if read.

Example 3

**K RESULT OF MICRO-TEST**

CHLOROQUINE p mol/well →	Control	1	2	4	8	16	32	64
SCHIZONT./200 paras. →	098	105	096	- - -	090	- - -	043	016
	107 %	98 %	- %	92 %	- %	44 %	16 %	

Test plate batch No.

A resistant infection with two thick films lost during the staining process - concentrations 4 and 16 pmol.

3.12 Section L: SLIDE CHECKING

It indicates whether slides have been sent to a reference centre for checking and the appropriate response (1 for yes, 2 for no) is entered in column 200.

3.13 Section M: MOVEMENTS OF PATIENT

Here a short summary of the patient's travels over the past 12 months is required. This is not coded by the investigator and will be coded by the computer processing unit at WHO/Headquarters, Geneva.

3.14 Section N: CONCLUSION

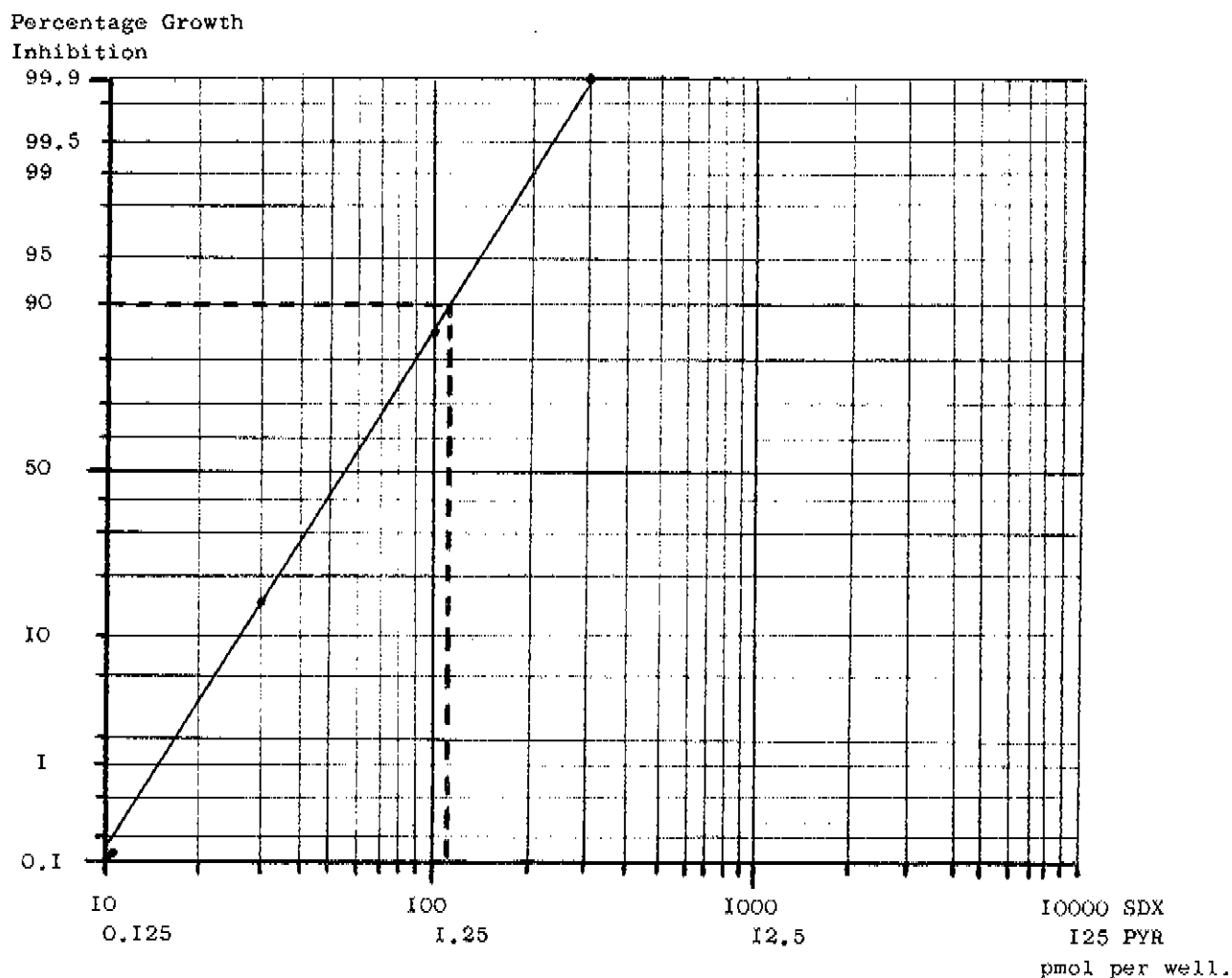
Here the investigator records his own conclusions about the case. This information is not coded.

## RESPONSE OF P.FALCIPARUM TO CHLOROQUINE, MEFLOROQUINE, QUININE, SDX/PYR AND AMODIAQUINE (IN VITRO-TEST)

(Please ensure that the drug concentrations of the test plates used match those given below in Section K) Revision 1987

<b>A COUNTRY AND PLACE OF TEST</b>		Serial No.: 1 <input style="width: 40px;" type="text"/>
Institution _____	City/Town _____	Country Code: 5 <input style="width: 40px;" type="text"/>
Investigator _____	Country _____	Institution: 8 <input style="width: 40px;" type="text"/>
Province/State _____	District/County _____	
<b>B COUNTRY AND PLACE INFECTION PROBABLY CONTRACTED</b>		Country Code: 10 <input style="width: 40px;" type="text"/> Prov Code: <input style="width: 40px;" type="text"/>
Country _____	Prov/State _____	Lat. in box 15 N = 1 S = 2 <input style="width: 40px;" type="text"/> <input style="width: 40px;" type="text"/>
District/County _____	Locality _____	Long. in box 20 E = 1 W = 2 <input style="width: 40px;" type="text"/> <input style="width: 40px;" type="text"/>
<b>C DATE AND TIME BLOOD TAKEN</b>		<input style="width: 40px;" type="text"/> <input style="width: 40px;" type="text"/> <input style="width: 40px;" type="text"/> <input style="width: 40px;" type="text"/> <input style="width: 40px;" type="text"/>
<b>D INCUBATION TIME</b>		Date _____ Duration (hours) _____
<b>E PATIENT</b>		Sex: MF <input type="checkbox"/> Less than 1 year = 00 <input type="checkbox"/> Age: <input style="width: 40px;" type="text"/>
<b>F REASON FOR SCREENING</b>		48 <input style="width: 40px;" type="text"/>
<input type="checkbox"/> 1 = Resist. or suspected resist. case	<input type="checkbox"/> 2 = Collateral case of resist. or suspect. resist. case	<input type="checkbox"/> 3 = Resist. in area of origin
<input type="checkbox"/> 4 = Resist. in area of orig. (abroad)	<input type="checkbox"/> 5 = Resist. in adjacent area	<input type="checkbox"/> 6 = Resist. in other rel. area
<input type="checkbox"/> 7 = Routine monitoring	<input type="checkbox"/> 8 = Other _____	
<b>G SAMPLE</b>		47 <input style="width: 40px;" type="text"/>
<input type="checkbox"/> 1 = General pop	<input type="checkbox"/> 2 = School	<input type="checkbox"/> 3 = Labour force
<input type="checkbox"/> 4 = Outpatient	<input type="checkbox"/> 5 = Inpatient	<input type="checkbox"/> 6 = Migrant labour
<input type="checkbox"/> 7 = Other _____		
<b>H DRUG TAKEN DURING LAST 2 WEEKS</b>	HISTORY: Any antimal. drug taken? <input type="checkbox"/> 1 = Yes <input type="checkbox"/> 2 = No <input type="checkbox"/> 3 = ? (box 48)	
	If "Yes" specify drug(s): _____ (box 49) <input style="width: 40px;" type="text"/>	
	URINE-TEST <input type="checkbox"/> 1 = pos. <input type="checkbox"/> 2 = neg.	<input type="checkbox"/> 3 = Doubtful <input type="checkbox"/> 4 = Not done
<b>I PRE-CULTURE SLIDE EXAM.</b>		49 <input style="width: 40px;" type="text"/> 50 <input style="width: 40px;" type="text"/>
No. asexual P.f. per mm <sup>3</sup> blood: _____		ASEXUAL P. FALCIPARUM small medium large No. counted: _____
<b>K RESULT OF MICRO-TEST</b>		54 <input style="width: 40px;" type="text"/> 59 <input style="width: 40px;" type="text"/>
CHLOROQUINE p mol/well SCHIZONT./200 paras. →		Test plate batch No. 87 <input style="width: 40px;" type="text"/>
Control	1 2 4 8 16 32 64	
_____ %	_____ %	_____ %
MEFLOROQUINE p mol/well SCHIZONT./200 paras. →		114 <input style="width: 40px;" type="text"/>
Control	2 4 8 16 32 64 128	
_____ %	_____ %	_____ %
QUININE p mol/well SCHIZONT./200 paras. →		141 <input style="width: 40px;" type="text"/>
Control	4 8 16 32 64 128 256	
_____ %	_____ %	_____ %
SDX/PYR p mol/well SCHIZONT with 8 or more normal nuclei /200 paras. →		168 <input style="width: 40px;" type="text"/>
Control	10 (50%) 20 (50%) 100 (50%) 200 (50%) 1000 (50%) 3000 (50%) 10000 (50%)	
_____ %	_____ %	_____ %
AMODIAQUINE p mol/well SCHIZONT./200 paras. →		195 <input style="width: 40px;" type="text"/>
Control	0.32 0.64 1 2 4 8 16	
_____ %	_____ %	_____ %
		198 <input style="width: 40px;" type="text"/>
RPMI1640 LPLF medium BATCH No.		
<b>L Were the slides referred for checking ?</b> yes = <input type="checkbox"/> 1 no = <input type="checkbox"/> 2		200 <input style="width: 40px;" type="text"/>
<b>M Has the patient travelled and where (during the last 12 months)?</b>		201 <input style="width: 40px;" type="text"/>
<b>N Conclusion:</b>		

FIG. 1



Interpretation.

Reading off the vertical scale at 90% growth inhibition the effective drug concentration is the combination of SDX/PYR or 110 pmol per well of SDX and 1.4 pmol per well of PYR.

## ANNEX 1

## LIST OF COUNTRIES OR AREAS BY REGION

This list is produced in accordance with ISO\* Code for the representation of names of countries, second edition, 1981-05-15.

<u>Country or area</u>	<u>Code</u>	<u>Country or area</u>	<u>Code</u>
AFRICA			
Algeria	DZA	Malawi	MWI
Angola	AGO	Mali	MLI
Benin	BEN	Mauritania	MRT
Botswana	BWA	Mauritius	MUS
Burkina Faso	HVO	Mozambique	MOZ
Burundi	BDI	Namibia	NAM
Cameroon	CMR	Niger	NER
Cape Verde	CPV	Nigeria	NGA
Central African Republic	CAF	Réunion	REU
Chad	TCD	Rwanda	RWA
Comoros	COM	St Helena	SHN
Congo	COG	Sao Tome and Principe	STP
Côte d'Ivoire	CIV	Senegal	SEN
Equatorial Guinea	GNQ	Seychelles	SYC
Ethiopia	ETH	Sierra Leone	SLE
Gabon	GAB	South Africa	ZAF
Gambia	GMB	Swaziland	SWZ
Ghana	GHA	Togo	TGO
Guinea	GIN	Uganda	UGA
Guinea-Bissau	GNB	United Republic of Tanzania	TZA
Kenya	KEN	Zaire	ZAR
Lesotho	LSO	Zambia	ZMB
Liberia	LBR	Zimbabwe	ZWE
Madagascar	MDG		
THE AMERICAS			
Antigua and Barbuda	ATG	Guyana	GUY
Argentina	ARG	Haiti	HTI
Bahamas	BHS	Honduras	HND
Barbados	BRB	Jamaica	JAM
Belize	BLZ	Martinique	MTQ
Bermuda	BMU	Mexico	MEX
Bolivia	BOL	Montserrat	MSR
Brazil	BRA	Netherland Antilles	ANT
British Virgin Islands	VGB	Nicaragua	NIC
Canada	CAN	Panama	PAN
Cayman Islands	CYM	Paraguay	PRY
Chile	CHL	Peru	PER
Colombia	COL	Puerto Rico	PRI
Costa Rica	CRI	St Christopher and Nevis	KNA
Cuba	CUB	St Lucia	LCA
Dominica	DMA	St Vincent	VCT
Dominican Republic	DOM	Suriname	SUR
Ecuador	ECU	Trinidad and Tobago	TTO
El Salvador	SLV	Turks and Caicos Islands	TCA
French Guiana	GUF	United States of America	USA
Grenada	GRD	Uruguay	URY
Guadeloupe	GLP	Venezuela	VEN
Guatemala	GTM	Virgin Islands (United States)	VIR

\* International Organization for Standardization.

<u>Country or area</u>	<u>Code</u>	<u>Country or area</u>	<u>Code</u>
EASTERN MEDITERRANEAN			
Afghanistan	AFG	Morocco	MAR
Bahrain	BHR	Oman	OMN
Cyprus	CYP	Pakistan	PAK
Democratic Yemen	YMD	Qatar	QAT
Djibouti	DJI	Saudi Arabia	SAU
Egypt	EGY	Somalia	SOM
Iran, Islamic Republic of	IRN	Sudan	SDN
Iraq	IRQ	Syrian Arab Republic	SYR
Jordan	JOR	Tunisia	TUN
Kuwait	KWT	United Arab Emirates	ARE
Lebanon	LBN	Yemen	YEM
Libyan Arab Jamahiriya	LYB		
EUROPE			
Albania	ALB	Malta	MLT
Austria	AUT	Monaco	MCO
Belgium	BEL	Netherlands	NLD
Bulgaria	BGR	Norway	NOR
Byelorussian Soviet Socialist Republic	BYS	Poland	POL
Czechoslovakia	CSK	Portugal	PRT
Denmark	DKN	Romania	ROM
Finland	FIN	San Marino	SMR
France	FRA	Spain	ESP
German Democratic Rep.	DDR	Sweden	SWE
Germany, Federal Rep.	DEU	Switzerland	CHE
Greece	GRC	Turkey	TUR
Hungary	HUN	Ukrainian Soviet Socialist Republic	UKR
Iceland	ISL	Union of Soviet Socialist Republics	SUN
Ireland	IRL	United Kingdom of Great Britain and Northern Ireland	GBR
Israel	ISR	Yugoslavia	YUG
Italy	ITA		
Luxembourg	LUX		
SOUTH-EAST ASIA			
Bangladesh	BGD	India	IND
Bhutan	BTN	Indonesia	IDN
Burma	BUR	Maldives	MDV
Democratic People's Republic of Korea	PRK	Nepal	NPL
		Sri Lanka	LKA
		Thailand	THA
WESTERN PACIFIC			
American Samoa	AMS	New Zealand	NZL
Australia	AUS	Niue	NIU
Brunei Darussalam	BRN	Papua New Guinea	PNG
China	CHN	Philippines	PHL
Cook Islands	COK	Republic of Korea	KOR
Democratic Kampuchea	KHM	Samoa	WSM
Fiji	FJI	Singapore	SGP
French Polynesia	PYF	Socialist Republic of Viet Nam	VNM
Guam	GUM	Solomon Islands	SLB
Hong Kong	HKG	Tokelau	TKL
Japan	JPN	Tonga	TON
Kiribati	KIR	Trust Territory of the Pacific Islands	PCI
Lao People's Democratic Republic	LAO	Tuvalu	TUV
Macao	MAC	Vanuatu	VUT
Malaysia	MYS	Wallis and Futuna	WLF
New Caledonia	NCL		