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***IN VITRO* MICRO-TEST (MARK III) FOR THE ASSESSMENT
OF THE RESPONSE OF *Plasmodium falciparum* TO
CHLOROQUINE, MEFLOROQUINE, QUININE, AMODIAQUINE,
SULFADOXINE/PYRIMETHAMINE AND ARTEMISININ**

Instructions for use of the *in vitro* micro-test kit (Mark III)

World Health Organisation
Division of Control of Tropical Diseases

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Photograph of pre- and post-culture parasites (<i>Plasmodium falciparum</i>)	1
Grease, silicone, for candle jar (tube)	1
Gloves, protective, rewashable, size medium/large (5 pairs each), pairs	10

sensitivity *in vitro* can be an indicator of future therapeutic failure. Unlike the *in vivo* tests, the results of an *in vitro* test are not disturbed by on-going malaria transmission. The *in vitro* test may be carried out with several drugs

simultaneously and is independent of the patient's clinical condition. It also permits the monitoring of drug response with compounds for which an *in vivo* test is not yet available. *In vitro* tests are **not**, under normal circumstances, employed for the guidance of individual treatment. In the context of drugs with widely varying absorption (e.g. halofantrine) and in conjunction with *in vivo* tests, they permit the differentiation between true resistance and treatment failure due to pharmacokinetic factors.

2.2 Tissue culture plates predosed with halofantrine (range 0.015 - 15.0 pmol per well), pyrimethamine (range 0.125 - 125 pmol per well) and pyronaridine (range 0.1 - 6.4 pmol per well) are available on request, but not routinely provided as part of test kits A and B.

2.3 There are risks associated with taking human blood for the tests. Hepatitis B virus, human immunodeficiency virus (HIV) and other infectious pathogens can be transmitted by the repeated use of lancets, needles and other instruments. All materials supplied with this micro-test kit to handle human blood are disposable. They should be used **once** only and great care must be taken with its proper disposal after use. The correct procedures are detailed in document WHO/MAL/83.1000, Rev.1, *Biosafety in in vivo and in vitro studies of human malaria*, by D. Payne.

**UNDER NO CIRCUMSTANCES
SHOULD DISPOSABLE MATERIAL BE RE-USED**

2.4 In the selection of material used in the WHO standard (Mark III) *in vitro* micro-test kits, every effort has been made to specify material that has a long shelf-life at normal ambient temperatures. **However, the RPMI 1640 LPLF liquid medium and the artemisinin-dosed plates (ART) have a limited shelf-life which is considerably reduced when the material is stored at normal ambient temperature.** Therefore, these items should always be stored at +4°C (normal refrigerator temperature) and transported at this temperature.

2.5 Additionally and whenever possible, the other pre-dosed plates (CHL, MEF, QNN, HAL, AMO, PYR, SDX/PYR, PND) should also be stored at +4°C.

**DO NOT PUT ANY OF THE COMPONENTS OF THE TEST KIT
IN THE DEEP FREEZER**

2.6 The most convenient way of transporting temperature-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 PROTECT TEST MATERIAL FROM
DIRECT CONTACT WITH COOLANT**

2.7 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

Well	(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 chloroquine concentrations of 0.2; 0.4; 0.8; 1.6; 3.2; 6.4 and 12.8 μmol per l blood. The concentrations are expressed in μmol / l blood as the malaria parasite shows selective uptake of chloroquine.

3.2 Mefloquine (MEF)

Well	(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 mefloquine concentrations of 0.4; 0.8; 1.6; 3.2; 6.4; 12.8 and 25.6 μmol per l blood. The concentrations are expressed in μmol / l blood as the malaria parasite shows selective uptake of mefloquine.

3.3 Quinine

Well	(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												
C	4												
D	8												
E	16												
F	32												
G	128												
H	256												

Well A is the control.

Wells B - H represent quinine concentrations of 0.08; 0.16; 0.32; 0.64; 1.28; 2.56 and 5.12 μmol per l blood-medium mixture (BMM). The concentrations are expressed in μmol / l BMM as the malaria parasite does not show selective uptake of quinine.

3.4 Halofantrine

(available on request)

Well	(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	0.015												
C	0.05												
D	0.15												
E	.50												
F	1.50												
G	5.00												
H	15.00												

Well A is the control

Wells B - H represent halofantrine concentrations of 0.3; 1.0; 3.0; 10.0; 30.0; 100.0 and 300.0 nmol per l BMM. The concentrations are expressed in nmol / l BMM since the malaria parasite does not seem to show selective uptake of halofantrine.

3.5 Amodiaquine

Well	(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 amodiaquine concentrations of 0.05; 0.1; 0.2; 0.4; 0.8; 1.6 and 3.2 μmol per 1 blood. The concentrations are expressed in μmol / 1 blood as the malaria parasite shows selective uptake of amodiaquine.

3.6 Artemisinin

Well	(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	0.15												
C	0.5												
D	1.5												
E	5.0												
F	15.0												
G	50.0												
H	150.0												

Well A is the control.

Wells B - H represent artemisinin concentrations of 3; 10; 30; 100; 300; 1000 and 3000 nmol per 1 BMM. The concentrations are expressed in nmol / 1 BMM as the malaria parasite does not show selective uptake of artemisinin.

3.7 Sulfadoxine / pyrimethamine

Ratio 80 : 1. Dose shown for sulfadoxine.

Well	(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 sulfadoxine concentrations of 0.2; 0.6; 2.0; 6.0; 20.0; 60.0 and 200.0 μmol per l BMM. The corresponding concentrations of pyrimethamine in wells B - H are 0.0025; 0.0075; 0.025; 0.075; 0.25; 0.75 and 2.50 μmol per l BMM. The concentrations are expressed in μmol / l BMM as malaria parasites show no selective uptake of sulfadoxine and pyrimethamine

3.8 Pyrimethamine

(available on request)

Well	(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	0.125												
C	0.375												
D	1.25												
E	3.75												
F	12.5												
G	37.5												
H	125												

Well A is the control.

Wells B - H represent pyrimethamine concentrations of 2.5; 7.5; 25; 75; 250; 750 and 2500 nmol per l BMM. The concentrations are expressed in nmol / l BMM as the malaria parasite does not show selective uptake of pyrimethamine.

3.9 Pyronaridine

(available on request)

Well	(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	0.1												
C	0.2												
D	0.4												
E	0.8												
F	1.6												
G	3.2												
H	6.4												

Well A is the control.

Wells B - H represent pyronaridine concentrations of 2; 4; 8; 16; 32; 64 and 128 nmol per 1 BMM. Since it is not yet known whether malaria parasites take up pyronaridine selectively, the drug concentrations are **provisionally** expressed in nmol / 1 BMM.

4. Procedures

4.1 Pre-evaluation of Test Subjects

4.1.1 All subjects should be questioned with respect to their recent history of antimalarial drug use.

Persons who have received quinine, artemisinin or artemisinin derivatives 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 56 days should be excluded from the test. Most urine tests give relatively reliable results only for a short period after drug intake, but rarely cover the indicated time-spans. In cases of doubt, the micro-test may be performed, but it would be helpful to take a blood sample and have it analysed for residual drug(s).

4.1.2 Written or oral consent, as appropriate, should be taken from all subjects whose blood is to be taken for the test. [NB: all patients with symptomatic malaria should be treated with the appropriate drug immediately after blood sampling for the micro-test. In all cases, patient care must take priority over carrying out the test.]

4.1.3 Thick and thin blood films should be taken from persons suspected of malaria, and stained with Giemsa or another reliable Romanowsky stain. Patients who have mono-infections with *Plasmodium falciparum* and asexual parasitaemias in excess of 1 000 parasites, but less than 80 000 parasites per μ l blood, are considered suitable for testing. The preselected patients should be subjected to a urine

test for 4-aminoquinolines and sulfonamides (and tests for other drugs, if available), and those with positive readings should be excluded.

4.1.4 Biodata and pre-incubation parasite density should be entered in the appropriate test record form (see Annex II for example). The parts of the form relating to schizont counts are filled in after the test has been read.

4.2 Preparation of the growth medium

- (i) Take the following items from the test kit or cold storage, as appropriate:
 - C 1 bottle of 20 ml RPMI 1640 LPLF liquid medium;
 - C Alcohol swab (for sterilizing the rubber seal of the medium bottle);
 - C 1 sterile syringe, 5 ml;
 - C 2 sterile injection needles for above;
 - C Number of sterile Falcon tubes, 6 ml, as required (1 per isolate to be tested); mark the tubes with the patient number and the volume of medium required;
 - C 1 plastic tube rack (to be used for the Falcon tubes).

- (ii) Calculate the volume of liquid LPLF medium which will be required for the number of planned tests. Each 100 µl blood sample (isolate) requires 0.9 ml of RPMI 1640 LPLF medium to make a total of 1 ml blood-medium-mixture (BMM). This volume is sufficient for a duplicate series (i.e. two columns of 8 wells) of the same drug or two tests of different drugs (one column of each drug). If more than two drugs are to be tested (or duplicate tests to be conducted with more than one drug), then it will be necessary to increase the amount of RPMI 1640 LPLF liquid medium (and that of the blood sample) accordingly. For example, if 4 drugs (e.g. CHL, QNN, MEF and ART) are to be tested in single tests, then medium and blood samples will be required for 4 columns of 8 wells each: that is 2 x 100 µl blood and 2 x 0.9 ml liquid medium which is 200 µl blood and 1.8 ml of RPMI 1640 LPLF liquid medium. If duplicated tests are required for the 4 drugs, then the corresponding amounts would be 4 x 100 µl blood and 4 x 0.9 ml medium.

- (iii) Take the 5 ml syringe and the two needles, being careful to preserve sterility since there is no filtration phase in this test. Remove the central cap shield of the RPMI 1640 LPLF liquid medium bottle, baring the central part of the rubber seal. Wipe the rubber seal of the medium bottle clean with an alcohol swab, let it dry and then insert one needle. Mount the other needle on the 5 ml syringe, pierce the rubber seal of the medium bottle and gently withdraw the required volume of RPMI 1640 LPLF liquid medium specific for the Falcon tube to be dosed. Remove the syringe, fill the medium into the scheduled Falcon tube, and recap Falcon tube. Re-insert the syringe on the needle and withdraw the stipulated volume of medium for the next Falcon tube, fill the medium into the Falcon tube, and again, recap the tube. This procedure is repeated until all scheduled Falcon tubes have been dosed, followed by the removal and safe disposal of the needles from the medium bottle. If there is any medium left, the bottle should be stored in the refrigerator again (at 4°C) and should be used up within one

week.

NOTE: The 6 ml Falcon tubes containing 0.9 ml (or multiples) of RPMI 1640 LPLF liquid medium can be stored in a refrigerator (4°C) for up to 48 hours before use or can be transported in the field in a thermos container containing ice cubes or coolant blocks. Do 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 the contamination of the BMM.

4.3 Performance of the MARK III micro-test

4.3.1 Disinfect the skin site of blood sampling (finger of children and adults, big toe of young infants) with an alcohol swab and let the site dry. With the Autolet apparatus or a sterile, disposable haemolancet, prick the finger or big toe and withdraw 100 µl blood into a sterile heparinized capillary tube. Transfer the blood quickly into the duly labelled 6 ml plastic Falcon tube containing an appropriate quantity of RPMI 1640 LPLF liquid medium (see section 4.2.b.). If more than two test lines are to be run, further capillary tubes need to be filled with blood and emptied into the medium. After the blood has been added to the medium, press the cap of the Falcon tube firmly into place and gently shake the tube to mix blood and medium. The transfer of the blood from the heparinized capillary tube to the medium tube is facilitated by means of the small black rubber bulb supplied with the kit. The bulb is slipped onto the distal end of the 100 µl capillary tube before it is used to collect the blood. By sealing the hole in the end of the rubber bulb with a fingertip 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 BMM is stable for several hours, and the tubes can be carried in a 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 Before dosing, the test plates should be allowed to acquire ambient temperature while they are still in their wrappings in order to avoid the formation of moisture which could lead to contamination. When the test plates have acquired ambient temperature, they should be removed from the wrappings. The plastic sealing strips on the required number of test series of the appropriate plate(s) are removed by first cutting along the appropriate columns with the scalpel and then lifting off the required area of plastic with a forceps, taking care not to contaminate the wells.

4.3.5 All the wells of the appropriate column are dosed with 50 µl of the BMM (1:9) using the 50 µl fixed volume Eppendorf pipette and a disposable sterile tip as provided with the test kit. Dosing is **always** done starting with the control well (A) and following an increasing order of concentrations, ending with well H. It is extremely important that the BMM in the Falcon tube be shaken 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 column is set up in exactly the same way, and so on until all the scheduled wells have been dosed.

4.3.7 Place the lid on the microplate and, with a glass pen, write the details of each test over the appropriate column of the plate.

4.3.8 Shake the plate **gently**, without lifting it from the laboratory bench, so that the drug deposits in the wells are completely dissolved.

4.3.9 Take the candle jar from the incubator (set to give an internal temperature in the candle jar of 37.51C; a prewarming of at least one hour is 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 place one on each side of the stacked plates. **Do not** put the candles on top of the plates. Replace the candle jar lid, being careful to seal it well, leaving the exhaust cock in the open position. When the second candle is about to go out, close the exhaust cock.

4.3.10 Place the candle jar in the incubator set at 37.51C, noting the time.

4.3.11 Incubate at 37.51C (" 0.51C) for 24-30 hours, depending on the development stage of the trophozoites in the preculture slide. Experience to date indicates that isolates which have not produced schizonts within 30 hours are influenced by factors which invalidate the test procedure. By far the most common reason for delayed maturation is the previous intake of antimalarial drugs by the test subject.

4.3.12 After incubation, the contents of the test wells are ~~harvested~~ by removing the supernatant with an Eppendorf pipette, 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:

WELL A	WELL B	WELL C	WELL D
G	G	G	G
WELL E	WELL F	WELL G	WELL H
G	G	G	G

4.3.13 The same Eppendorf tip can be used if the ~~harvest~~ starts with well H and proceeds, well by well, up to well A (which is the last to be harvested). The Eppendorf tip is then discarded before starting the harvest of a new series, proceeding again from well H to A.

NOTE: If a different harvesting sequence is chosen, the Eppendorf tip must be changed after every well.

4.3.14 The resultant thick films must be carefully dried before staining, as they will otherwise spontaneously detach from the slide. If air-dried, 24-48 hours are normally required, but this period can be reduced (and the growth of contaminants avoided) by drying the films in an incubator set at 37.51C for 30 minutes or with a hair dryer. If the latter method is used, care should be taken not to overheat the films, as this would result in autofixation.

4.6.15 Some workers report highly satisfactory results from acetone-treated slides. The thick films are air-dried until visibly dry, which usually takes 30 minutes. The slide is then dipped into pure acetone, air-dried and stained as described below.

4.3.16 The thick films are stained for 30 minutes in a Giemsa stain (reliable brand) at a dilution of 1% (v/v) in buffered water of pH 6.8. Great care must be exercised in handling the stained films until they are completely dry.

4.3.17 Drying can be performed by air, in an incubator (37.51) or with a hair dryer, but again, in this 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 PYR and SDX/PYR is different from that for the other drugs (CHL, MEF, QNN, AMO, ART, HAL, PND)** in the Mark III micro-test system.

5.2 Counting procedure for the CHL, MEF, QNN, AMO, ART, HAL and PND tests

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 in the control (well A) must be 10% or more (i.e. 20 schizonts with three or more nuclei per 200 asexual parasites). The counts read in the drug wells can then be expressed as a percentage of the control as in the following example:

CONTROL	DRUG UNDER TEST To be repeated for each test well	
	No. of schizonts per 200 parasites after	% of schizonts relative to control samples
Number of schizonts, i.e. parasites with 3 or more nuclei, per 200 parasites after		

incubation	incubation	(control = 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 PYR or SDX/PYR on the developing schizont is not as clear cut as that of the drugs mentioned in section 5.2, since these drugs may affect nuclear division. 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. Thus, it is more difficult to establish the >breakpoint= or >end point= of sensitivity/resistance, and the presence of schizonts with three nuclei or more cannot be used to define schizont formation as is the case for CHL, MEF, QNN, AMO, ART, HAL and PND.

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 PYR or SDX/PYR are usually not abnormal, and thus indicate normal growth (maturation). The criterion for PYR and SDX/PYR is schizonts of 8 nuclei or more.**

NOTE: parasites with a large number of minute chromatin corpuscles scattered within a swollen mass of cytoplasm are not to be counted as schizonts. Such bodies may typically occur under the impact of PYR or SDX/PYR. (These do not produce merozoites.)

PYR and SDX/PYR comparative asexual parasite counts are therefore slightly more complex than those of the standard WHO micro-test for CHL, MEF, QNN, AMO, ART, HAL and PND, but with some practice can be mastered relatively easily. The procedure for PYR and SDX/PYR is almost identical to that for the seven other drugs, except 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

For data analysis, the investigator may opt for a simple evaluation from a table showing the original data and those derived after standardizing for control growth, or for a more elaborate, computer-adapted procedure [Wernsdorfer & Wernsdorfer (1995). The evaluation of *in vitro* tests for the assessment of drug response in *Plasmodium falciparum*. Mitt.Österr.Ges.Trop.Med.Paras. 17: 221-228], which provides a wide array of parameters, including those required for a graphic display of results, and for parallel or longitudinal comparison of drug response data. Diskettes of this programme are available on request from the Malaria Unit, Division of Control of Tropical Diseases, World Health Organization, CH-1211 Geneva, Switzerland.

6.1 CHL, MEF, QNN, AMO, ART, HAL and PND Tests

The definition of resistance *in vitro* has been derived from the *in vivo* drug response in non-immune patients. In such patients, there is generally a good relationship between *in vivo* and *in vitro* response with well-absorbed drugs, e.g. CHL, AMO, MEF, QNN, in the absence of vomiting or

diarrhoea. With poorly or variably absorbed drugs (e.g. HAL), pharmacokinetic factors may produce drug failure *in vivo* in the presence of sensitive malaria parasites. The

indication of *in vitro* resistance in the table below is correlated to *in vivo* response in non-immune patients.

Provided that:

- C there is satisfactory growth in the control (i.e. 20 or more schizonts with three or more nuclei in 200 parasites);
- C the original isolate did not contain > 80 000 parasites / μ l blood; and
- C the infection was indeed *P.falciparum* alone.

then:

Test Drug	Satisfactory response	Indication of resistance
	Complete schizont inhibition at	Schizont formation at
Chloroquine	4 pmol or less	8 pmol or more
Mefloquine	16 pmol or less	32 pmol or more
Quinine	128 pmol or less	256 pmol or more
Amodiaquine	2 pmol or less	4 pmol or more
Halofantrine	1.5 pmol or less*	5 pmol or more*
Artemisinin	**	**
Pyronaridine	**	**

* Provisional figures

** 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 a minimum of 10) are carried out at the same time and place and are grouped. These grouped data are achieved by listing, as a first step, the test results as they were read. Subsequently, the readings obtained in the drug wells of each series are transformed into percentages of the control (control = 100%) and listed to the first decimal. The standardized data are added up, column by column, and the sums divided by \bar{n} (the number of tests) in order to calculate the mean percentage of schizont maturation at each drug concentration. Finally, the degree of inhibition of schizont maturation (in %) is calculated by subtracting the percentage of schizont maturation from 100. In the table opposite, the procedure is shown using a series of 10 acceptable chloroquine tests.

The procedure is simplified when using the computer programme mentioned in Section 6. After entering study area, year, drug and its concentrations in nmol or μ mol / l (blood or BMM as appropriate), the schizont counts are entered as they were read for each isolate between wells A and H. Standardization for schizont maturation in the control wells is part of the programme which yields practically every

important regression parameter. The Probit Calculus Sheet for the above series is given as an example in Annex I. The series shows a good fit of the observed data points to the regression line (chi-square for heterogeneity = 0.1992 at maximum permissible 11.1).

Schizont counts (per 200 parasites) in well								
Test Number	A	B	C	D	E	F	G	H
	Control	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	0	0	0
3	178	179	160	138	111	67	34	0
4	196	192	190	186	110	65	23	0
5	49	53	21	11	2	0	0	0
6	34	32	30	21	6	1	0	0
7	200	191	111	65	38	11	3	0
8	29	13	3	0	0	0	0	0
9	128	64	30	16	2	0	0	0
10	55	53	47	26	12	0	0	0
As Percentages of Control:								
1	100.0	86.3	42.6	22.6	5.8	1.1	0.0	0.0
2	100.0	57.1	28.6	0.0	0.0	0.0	0.0	0.0
3	100.0	100.6	89.9	77.5	62.4	37.6	19.1	0.0
4	100.0	98.0	96.9	94.9	56.1	33.2	11.7	0.0
5	100.01	08.2	42.9	22.5	1.0	0.0	0.0	0.0
6	100.0	94.1	88.2	61.8	17.6	2.9	0.0	0.0
7	100.0	95.5	55.5	32.5	19.0	5.5	1.5	0.0
8	100.0	44.8	10.3	0.0	0.0	0.0	0.0	0.0
9	100.0	50.0	23.4	12.5	1.6	0.0	0.0	0.0
10	100.0	96.4	85.5	47.3	21.6	0.0	0.0	0.0
TOTAL	1000.0	831.0	563.8	371.6	185.3	80.3	32.3	0.0
Total / n = % maturation (mean)	100.0	83.10	56.38	37.16	18.53	8.03	3.23	0.00
% inhibition	(0)	16.90	43.62	62.84	81.47	91.97	96.77	10.0

In the above example, there is obviously still significant schizont maturation in the wells containing 8 and 16 pmol chloroquine, and three out of ten isolates show schizont maturation even at 32 pmol. As indicated in the first table of this section, schizont maturation in the 8 pmol well is a sign of resistance. Eight of the ten isolates showed schizont maturation at this well. This series reflects a status of manifest resistance as also evident from the EC-data in Annex I.

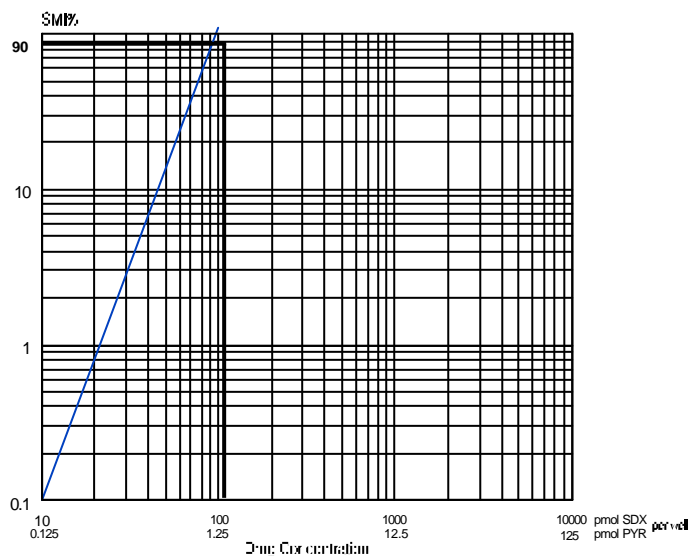
The principal advantage of this system is the comparability of quantitative results, both between different geographical areas and between series from the same area at different times (longitudinal studies).

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
Schizonts 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
% inhibition of schizont maturation		0	15	85	100	100	100	100

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



The \triangleright breakpoint \triangleleft in this example is therefore between well D (SDX 100 pmol / PYR 1.25 pmol per well) and well E (SDX 300 pmol / PYR 3.75 pmol per well). Data so far available indicate that the 90% inhibition level, related to schizonts of eight or more normal nuclei, reflects more accurately the true \triangleright breakpoint \triangleleft , which can be readily ascertained by means of a simple diagram as shown in the figure, using the data given above.

Procedure and interpretation:

The observed data are entered according to drug concentration (x-axis) and inhibition (y-axis). A line of the best optical fit is drawn. Reading this line off the vertical scale at 90% inhibition yields the approximate IC-90. Here the IC-90 for the combination of SDX/PYR shows approx. 110 pmol SDX and 1.4 pmol PYR per well. This corresponds to concentrations of 2.2 μ mol SDX and 0.028 μ mol PYR per litre blood-medium mixture (BMM)

ANNEX I

Probit calculus Sheet

Study Area: Sampelia
Year: 1997
Drug: Cholorquine

A	B	C	D	E	F	G	H
Control SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ	Conc. SCHIZ
0	0.2	0.4	0.8	1.6	3.2	6.4	12.8
190	164	81	43	11	2	0	0
112	64	32	0	0	0	0	0
178	179	160	138	111	67	34	0
196	192	190	186	110	65	23	0
49	53	21	11	2	0	0	0
34	32	30	21	6	1	0	0
200	191	111	65	38	11	3	0
29	13	3	0	0	0	0	0
128	64	30	16	2	0	0	0
55	53	47	26	12	0	0	0

Drug Concentration	SMI%
0.2	16.90
0.4	43.61
0.8	62.85
1.6	81.16
3.2	91.97
6.4	96.77
12.8	100.00

n =	10	S =	3.3797	f_s	1.7017
a =	5.4749	A =	1.4803	f_{EC-50}	1.7047
b =	0.8166	K =	7	f_{EC-90}	2.3940
r =	0.9949	N =	40	f_{EC-95}	2.7915
χ^2	0.1992	R =	64	f_{EC-99}	3.8898

EC	Mean	95% Confidence Levels	
		Lower	Higher
EC ₁	0.0324	0.0083	0.1260
EC ₁₆	0.1654	0.0970	0.2820
EC ₅₀	0.5590	0.3279	0.9529
EC ₈₄	1.8893	1.1083	3.2206
EC ₉₀	2.6852	1.1217	6.4285
EC ₉₅	4.1898	1.5009	11.6958
EC ₉₉	9.6508	2.4810	37.5400

ANNEX II