

Towards Quality Testing of Malaria Rapid Diagnostic Tests: Evidence and Methods

Manila, Philippines
20 - 22 July 2004

Geneva, Switzerland
28 February - 2 March 2006

Kisumu, Kenya
22 - 23 June 2006



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TOWARDS QUALITY TESTING OF MALARIA RAPID DIAGNOSTIC TESTS: EVIDENCE AND METHODS

Developed from the proceedings of the:

**WHO Informal Consultation on Development and Methods for
Testing Malaria Rapid Diagnostic Tests
Geneva, Switzerland
28 February - 2 March 2006**

and

**Consultation of the malaria specimen bank steering group
Kisumu, Kenya
22 – 23 June 2006**

REPORT FROM PROCEEDINGS OF THE
WHO INFORMAL CONSULTATION ON DEVELOPMENT OF METHODS FOR
TESTING MALARIA RAPID DIAGNOSTIC TESTS
and the
CONSULTATION OF THE MALARIA SPECIMEN BANK STEERING GROUP

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NOTE

The views expressed in this report are those of participants in the WHO Informal Consultations on development of methods for testing malaria rapid diagnostic tests, Manila 2004, Geneva 2006, and Kisumu 2006, and do not necessarily reflect the policies of the Organization.

This report updates the plan and recommendations for laboratory methods for quality assurance of malaria rapid diagnostic tests presented in the report of the Informal Consultation on Laboratory Methods for Quality Assurance of Malaria Rapid Diagnostic Tests, Manila, 20–22 July 2004. World Health Organization Regional Office for the Western Pacific, Manila (2004).

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CONTENTS

	<u>Page</u>
1. INTRODUCTION	1
1.1 Background.....	1
1.2 Objectives of the 2006 consultation	2
1.3 WHO aims for laboratory testing methods for malaria RDTs.....	2
2. SUMMARY OF RECENT DEVELOPMENT ACTIVITY	4
2.1 RDT sensitivity and predictive values	4
2.2 Relationship between antigen concentration and parasite density.....	4
2.3 Antigen stability	5
2.4 The effect of genetic diversity on RDT sensitivity.....	5
2.5 Other issues relating to laboratory SOPs.....	6
2.6 Other RDT QA development.....	6
3. PRODUCTION OF GOOD PANELS	7
3.1 Principles for developing methods to produce panels	7
3.2 Principles for determining contents of panels	7
3.3 Detection of antigen	9
4. ASSESSMENT OF RDT STABILITY	10
4.1 Early outcomes of heat stability trial.....	10
5. GENERAL REQUIREMENTS FOR TESTING MALARIA RDTs.....	10
5.1 Setting standards for sensitivity.....	11
5.2 When an RDT should be tested	11
5.3 Definition of a product and lot	13
5.4 RDT specifications to be assessed in an evaluation scheme	14
5.5 Management of an RDT testing scheme.....	14
5.6 Other specifications necessary for RDT procurement.....	15
6. DETAILED FUNCTION OF PROPOSED LABORATORY-BASED MALARIA RDT ASSESSMENT SCHEME.....	16
6.1 Development of product testing programme	16
6.2 Product testing entry criteria	16
6.3 Product testing criteria.....	17
6.5 Composition of panels.....	19
6.5.1 Product-Testing Panel	19
6.5.2 Lot testing.....	19
6.5.3 Panels available to manufacturers	20
6.6 Quality control testing in remote areas.....	20
7. FURTHER DEVELOPMENT OF TESTING METHODS AND PANELS	22
ANNEX 1 Further development required.....	23
ANNEX 2 Provisional timeline for malaria RDT product testing and development	24
ANNEX 3 Draft terms of reference for malaria specimen collection and RDT lot quality assurance testing sites	25
ANNEX 4: Draft terms of reference for malaria specimen banks	27
ANNEX 5: Informal consultation on development of methods for testing malaria rapid diagnostic tests, Geneva, 28 February–2 March 2006	28

ANNEX 6:	Consultation of malaria specimen bank steering group, Kisumu, Kenya, 22–23 June, 2006	38
REFERENCES	44

EXECUTIVE SUMMARY

An initiative to develop quality assurance (QA) methods for malaria rapid diagnostic tests (RDTs), coordinated by the WHO Regional Office for the Western Pacific, WHO Roll Back Malaria Department (Global Malaria Programme) and the UNICEF – UNDP - World Bank – WHO Special Programme for Research and Training in Tropical Diseases (TDR), has been in place since 2002 (WHO 2003). Malaria RDTs, based on lateral flow technology, are the only practical way of providing such diagnosis on a wide scale in most endemic areas, as microscopy is difficult to sustain outside major centres. However, wide variations in RDT product quality underscore the need for a product testing and QA scheme that will ensure the suitability of RDTs for wide-scale use. A previous consultation in Manila in 2004 outlined a development plan for quality assurance for RDTs, including specimen banks and product testing, lot testing and methods for use at a village health worker level.

This document updates recommendations from a follow-up informal consultation on the development of methods for testing malaria RDTs held in Geneva, Switzerland from 28 February to 2 March 2006 and in Kisumu, Kenya, from 22 to 23 June 2006. Substantial changes and updates to the 2004 report findings and recommendations are in blue print throughout this document. New annexes detail the updates. The Methods Manual of Standard Operating Procedures (SOPs) referred to in this report has been updated to Version 4 (WHO 2006a).

Testing of RDT stability and sensitivity has been continuing on a limited scale within a laboratory network coordinated by WHO, based on SOPs developed as part of the malaria RDT QA initiative. Limited testing of new products submitted by manufacturers has also been conducted by laboratories participating in the network. Data on the extent of variation in target antigen structure and the influence of this variation on RDT sensitivity, on stability of RDTs and stored parasite panels, and procedures for panel preparation and characterization, were discussed during both the 2004 and 2006 consultations. Further research will assist in the planning and prioritization of the design and operation of RDT quality assessment. The 2006 consultations reaffirmed the importance of maintaining an emphasis on laboratory-based, rather than field-based assessments of RDT accuracy and stability. Recommendations were made for RDT specifications for the lower limit of detection of parasites (LLD), the composition of panels, and requirements for the global product testing scheme, including the maintenance and expansion of post-purchase product and lot monitoring, in addition to screening and consent procedures.

The strategy to develop a product testing and quality assurance programme, which includes the ongoing development of the malaria parasite specimen bank, was further refined, as were the roles and terms of reference for various institutions in the collaborative network. The strategy will serve as a basis for:

- a product testing programme to guide procurement and set standards for manufacturers
- methods for quality assurance of product lots after procurement
- availability of panels for use in product testing and improvement by developers

Maintaining the timetable in this report will depend on securing adequate funding. Improved quality of diagnosis will increase the value of substantial funds already committed to malaria case management through improved targeting of antimalarial drugs.

List of Abbreviations

Ab	Antibody
ACT	Artemisinin-based combination therapy
Ag	Antigen
AMI	Army Malaria Institute (Queensland, Australia)
CDC	Centers for Disease Control and Prevention (Atlanta, United States of America)
CNM	National Center for Parasitology, Entomology and Malaria Control, Cambodia
DRD	Diagnostics Research and Development (TDR/PRD)
EHT	Essential Health Technologies (WHO)
ELISA	Enzyme-linked immunosorbent assay
GMP	Global Malaria Programme
IPC	Institut Pasteur de Cambodge (Phnom Penh, Cambodia)
IPB	Institut Pasteur de Bangui (Bangui, Central African Republic)
HIV	Human immunodeficiency virus
HRP2	Histidine-rich protein II
HTDL	Hospital for Tropical Diseases (University College of London Hospitals, London, United Kingdom of Great Britain and Ireland)
IHRDC	Ifakara Health Research and Development Centre, Tanzania
KEMRI	Kenya Medical Research Institute, Nairobi and Kisumu, Kenya.
LLD	Lower limit of detection
Mab	Monoclonal antibody
pLDH	Parasite lactate dehydrogenase
RBM	Roll Back Malaria (WHO)
RDT	Rapid diagnostic test
RITM	Research Institute for Tropical Medicine (Department of Health, the Philippines)
PRD	Product Research and Development (TDR)
QA	Quality assurance
QC	Quality control
PCR	Polymerase chain reaction
SDI	Sexually Transmitted Disease Diagnostics Initiative (TDR/PRD/DRD)
SOP	Standard operating procedure
TDR	UNICEF-UNDP-World Bank-WHO Special Programme for Research and Training in Tropical Diseases
UL	University of Lagos, Nigeria
UP	University of the Philippines
WHO	World Health Organization

1. INTRODUCTION

1.1 Background

Parasite-based (aetiological) diagnosis of malaria is becoming increasingly important as rising resistance of malaria parasites to less expensive drugs has led to rising use of artemisinin-based combination therapy (ACT) and other higher-cost drug combinations. Malaria rapid diagnostic tests (RDTs), which detect parasite-specific antigen through lateral flow immunochromatography, have great potential to fill the need for testing in remote areas where good microscopy cannot be maintained. The success of RDTs in improving the targeting of drug therapy, and their acceptance in malaria management by remote health workers and patients, will depend on demonstrating the reliability and accuracy of the tests through quality assurance (QA) programmes.

It is estimated that about 25 million RDTs were procured globally in 2005. The number and range of commercially available products have also rapidly increased, while regulatory procedures for diagnostics remain limited in many malaria-endemic countries. Methods to accurately monitor the quality of tests are difficult to implement and maintain. The sensitivity of available tests can vary widely, as can susceptibility to deterioration due to environmental exposure after manufacture, particularly in tropical conditions. There is an urgent need to provide clear guidance to countries and UN agencies on the quality of available RDTs and their appropriateness for various settings.

Quality assurance of malaria RDTs was addressed in WHO informal consultations in 1999 and 2003, and discussed in detail in the reports of those meetings (WHO 2000; WHO 2003). The consultation in 2003 outlined a plan for collection of wild-type parasites for laboratory-based QA, and an outline for multicentre field trials which could form the basis for a laboratory-based QA scheme and define the attributes of various products to guide procurement. The consultation also recommended further investigation of methods for quality control (QC) of RDTs in remote locations and making panels of parasites available to manufacturers to facilitate internal QA and development.

During 2003 and 2004, WHO, through the Regional Office for the Western Pacific, the Roll Back Malaria (RBM) Department of WHO Headquarters and the UNICEF-UNDP-World Bank-WHO Special Programme for Research and Training in Tropical Diseases (TDR), continued to develop a methods manual in collaboration with a number of institutions and developers containing standard operating procedures (SOPs) and methods for QA of malaria RDTs. This work raised a number of research questions and challenges to the development of a sound RDT testing scheme. The rapid expansion and development of commercially available products (over 30 now exist), the high cost of field trials and emerging data on target antigens have created a shift in focus towards laboratory-based testing rather than field studies as a means of product quality assessment. The increasing costs of malaria treatment and continued variation in product performance in published trials (Gaye et al. 1998; Ricci et al. 2000; Iqbal et al. 2001; Rubio et al. 2001; Coleman et al. 2002; Craig et al. 2002; Huong et al. 2002; Mason et al. 2002; Kolaczinski et al. 2004) has lent an increased urgency to the need for accurate, evidence-based information on the performance of commercially available RDTs. In view of this, a small informal consultation of experts associated with current development work on laboratory methods for malaria RDT quality assurance was convened in Manila, the Philippines, from 20 to 22 July 2004 with the objectives of:

- reviewing progress and problems in the development of RDT testing techniques;
- developing closer collaboration between participating institutions;

- planning for laboratory-based development necessary to provide a functioning system for testing the accuracy and stability of malaria RDTs;
- outlining an appropriate laboratory-based system suitable for WHO to adopt to test/prequalify malaria RDTs. [3]

The 2004 consultation reaffirmed the importance of continuing to develop malaria RDT testing methods but with renewed focus on laboratory-based, as opposed to, field-based testing, and methods for QA of malaria RDTs. Participants reviewed progress on laboratory-based testing methods and designed the laboratory component of a proposed global product testing programme. A consensus was reached that a bank of well-characterized specimens would be useful for producing panels for product evaluation and for quality control and assurance. The outcomes of the 2004 informal consultation are detailed in the previous report (WHO 2003). The 2004 outcomes also provide the basis for the document, modified through the follow-up informal consultation held in Geneva, Switzerland, in 2006. The 2006 consultation tackled issues unresolved following the 2004 consultation which are important in ensuring that product testing and quality control testing results are relevant to working environments. Continuing questions regarding the overall public health and cost benefits including action on negative results and the effect on treatment-seeking compliance can only be fully addressed when reliable, quality assured RDTs are in place.

1.2 Objectives of the 2006 consultation

With the goal of beginning a global product testing scheme by the end of 2006, this consultation aimed to:

- review progress on product testing and QC methods since the 2004 meeting;
- determine further research and capacity strengthening necessary to improve product testing and enhance sustainability;
- finalize the structure of the RDT testing programme and harmonize protocols and methods;
- define the function and contents of the specimen banks;
- identify key institutions, individuals and collaborations that will perform activities.

1.3 WHO aims for laboratory testing methods for malaria RDTs

Quality assurance for RDTs includes all processes for ensuring and maintaining a high quality of diagnostic performance, from the manufacture of individual components of devices to their use and interpretation by the end user (WHO 2003). These various aspects of QA are being addressed by the WHO Western Pacific Regional Office, TDR and WHO Global Malaria Programme (GMP) (Figure 1). The development of laboratory methods for testing RDTs, the subject of the 2004 and 2006 consultations, aims to test RDT accuracy and stability in a repeatable, transparent manner in order to:

- provide advice to countries on appropriate RDT procurement;
- maintain a public list of relative characteristics of commercially available products to guide purchase or recommendation by Malaria Medicines and Supplies Services and other malaria control programmes and funding agencies;
- provide SOPs and other support to member countries to monitor RDT accuracy centrally and peripherally;
- make panels available for internal QA and testing by manufacturers and developers.

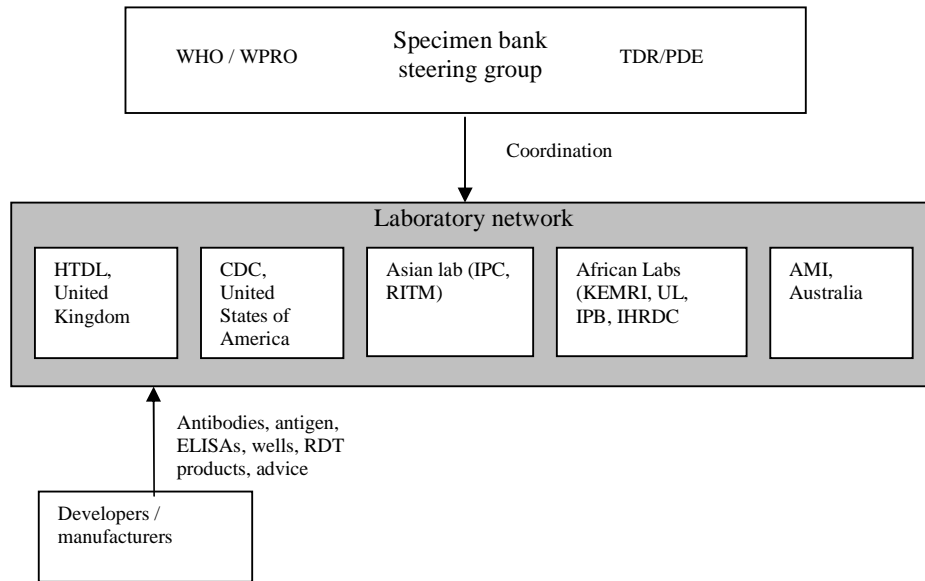


Figure 1: Current arrangements for the development of laboratory methods RDT QA, specimen bank and product testing.

2. SUMMARY OF RECENT DEVELOPMENT ACTIVITY

Methods for collecting, diluting and preserving wild-type parasites, assembling evaluation panels and using these panels for testing RDTs have been further developed since the 2003 and 2004 consultations (WHO 2003), and are detailed in SOPs developed for specimen collection and preservation, and RDT testing (WHO 2006b). For a global and repeatable testing system to be operational, further clarification of the effect of antigen variation, the relationship between antigen concentration and parasite density and other issues concerning the use of parasites and recombinant antigen, is necessary. Considerable progress has been made in clarifying these issues, with some results indicating a need to modify previous recommendations.

2.1 RDT sensitivity and predictive values

Previous WHO consultations concluded that 95% sensitivity at 100 parasites/ μ L of blood is appropriate (WHO 2000; WHO 2003). In this context, sensitivity is equivalent to the “lower limit of detection” (LLD) of the RDT. The 2004 consultation [3] concluded that this target should be revised, and a more appropriate target would rely on parasite antigen concentration (see below). Tests that detect histidine-rich protein II (HRP2) were noted to have a lower LLD (i.e. they are more sensitive) than RDTs detecting parasite lactate dehydrogenase (pLDH), though this varied somewhat between commercial products. As noted below (2.4), HRP2 genetic diversity also has an effect on RDT sensitivity. Aldolase LLD was variable between testing laboratories and products. As aldolase is coded by a single copy gene, available protein may be less, explaining why aldolase-detecting test lines on RDTs are frequently less sensitive than RDT test lines using other targets. Apart from product quality, the LLD may be affected by parasite stage, duration of infection, the amount of antigen released by the parasites (this varies between antigens and imposes an absolute threshold for LLD), variation in the structure of some antigens, and host immunity. A parasite density of 100 parasites/ μ L appears to be close to the absolute limit of detection for some antigens in the lateral flow assay formats presently in use and may not always be attainable.

2.2 Relationship between antigen concentration and parasite density

At a given parasite density, the antigen concentration will depend on:

- the total parasite load (sequestered and circulating parasites: affects *Plasmodium falciparum* only);
- the developmental stage of parasites;
- variation in antigen expression
- the accumulation of persistent antigens (e.g. HRP2) with duration of infection;
- antigen expression by the parasite;
- the persistence of antigen after elimination of parasites.

In parasite panels, the relationship may be further influenced by:

- technique (including microscopy accuracy, dilution accuracy and mixing)
- possible variation in donor blood
- possible effects of preservatives or anticoagulants

Measurement of the relationship is further influenced by the technique enzyme-linked immunosorbent assay (ELISA) used for quantification of antigen, and the affinity of monoclonal antibodies (Mabs) in the ELISA kit to the antigen of the particular parasite (isolate or strain).

Significant variation in antigen detection in parasite dilutions of 100 parasites/ μL was noted at the consultation. Likely causes are natural variation in antigen concentration due to the reasons listed above, as investigation of possible flaws in technique indicated that such flaws had not occurred. The SOPs had previously been modified to use dilutions of 200 parasites/ μL as an interim baseline for testing RDT sensitivity in view of this (WHO 2006b). As provisional criteria for WHO acceptance, the present consultation recommends the use of 200 parasite/ μL samples as a basis for assessing adequate RDT sensitivity, with <10% false positives among “non-clean” negative samples and <5% false positives among “clean” negative samples. The former recommendation of “95% sensitivity at about 100 parasite / μL ” remains the general recommendation for RDT sensitivity, but variation in antigen concentration to parasite density, and antigen variation, necessitates the use of a higher parasite density for testing purposes.

Assessment of antigen concentration using quantitative ELISA on a limited number of parasite samples indicates a wider variation in the antigen-parasite relationship with HRP2 than pLDH. Extensive testing and modification of HRP2 and pLDH ELISA since the 2004 consultation is continuing and it is expected that reliable ELISA will be available for assessment of antigen concentration versus parasite density ratios before mid-2006. Aldolase ELISA testing is close to completion at Centers for Disease Control and Prevention (CDC) Atlanta, United States of America.

2.3 Antigen stability

Antigen stability data presented at the consultation indicated greater stability of antigen in blood samples than the SOPs had allowed. Dilutions of 200 parasites/ μL maintained at room temperature (20°C – 25°C) for 60 days remained positive to both HRP2 and pLDH RDTs at, by visual comparison, similar test-line intensity. Beyond this period, HRP2 remained detectable, but pLDH was reduced. It was noted that pLDH persisted at detectable levels in samples stored at 4°C for 12 months. Further clarification of these data using ELISA is necessary, but it appears that storage at 4°C for two to three days during preparation prior to freezing samples will not significantly impair panel quality. Research presented on the heat stability of new products confirmed that HRP2-based RDTs tested were generally more stable at higher temperatures than the pLDH-based RDTs, but that this is not universal.

During long-term storage, panels of *P. falciparum* collected 20 months earlier, diluted to 100 parasites/ μL , and stored at -70°C, still produced stable positive reactions on quality-assured pLDH and HRP2 tests, indicating that parasite antigens will not degrade for months or years under such conditions.

2.4 The effect of genetic diversity on RDT sensitivity

Evidence presented to the 2004 consultation indicated an extensive variation in HRP2 structure from *P. falciparum* isolates within and between countries from which these parasites had been examined, and that this variation is likely to influence the sensitivity of HRP2-detecting RDTs at parasite densities below 500 parasites/ μL . HRP2 variation, or at least the range of variation, may vary geographically, but the 2004 consultation noted that more studies were needed to reach this conclusion. Some HRP2-detecting RDTs are likely to cross-react with other HRP2-like antigens, and this cross-reactivity may modulate the effect of HRP2 variation on RDT sensitivity.

Evidence presented to the 2006 consultation indicated that genetic variation, which had been determined by sequencing, can affect the sensitivity of RDTs detecting HRP2. Certain repeat patterns of parasite genetic diversity in HRP2 antigens were predictive of a significantly lower RDT detection sensitivity. Predictions based on samples of two types of repeat patterns from the Asia Pacific region,

indicate that <84% of *P.falciparum* parasites were likely to be detected at densities <250 parasites/ μ L, a finding consistent with reports from Asia of variation in performance of RDTs. While the study showed no indication of poor sensitivity from the isolates from Africa or South America, this result may be more indicative of the small number of isolates examined from those regions than of RDT sensitivity and low variation.

The target epitopes of most existing anti-HRP2 Mabs have yet to be defined, but work on identifying the targets of a number of Mabs is underway. Establishing the target epitopes and mapping these epitopes on a range of falciparum malaria isolates from different regions will provide useful guidance concerning the potential for existing Mabs, or combinations of Mabs, to be used in RDTs. It is also necessary to determine the appropriateness of parasite samples and isolates, and use of recombinant HRP2, for assessment of RDT sensitivity.

The extent of variation in pLDH and parasite aldolase is less well defined than HRP2, but appears to be much lower – with preliminary results showing all strains of the same species of *Plasmodium* to have identical amino acid sequences. Evidence presented at the 2004 consultation suggests that various isomers exist in some non-falciparum parasites that may potentially influence the sensitivity of pLDH-detecting RDTs. Further investigation is underway at Institut Pasteur de Cambodge, Cambodia (IPC) and the Army Medical Institute, Enoggera, Queensland, Australia (AMI).

As ELISAs for detection of target antigens generally rely on Mabs, variation in antigen structure and resultant variation in affinity of Mabs for antigen will affect the results of quantitative ELISAs, and will impact on characterization of the antigen concentration in QC panels. African samples are likely to be multi-strain, and so less affected by antigen variation. HRP2 characterization of these samples may be less important than for those from Asia.

Further samples should be obtained from the major geographic regions of malaria endemicity to determine the extent of global antigen variation. Thus the samples should be relatively evenly distributed and come from the major geographic divisions, including South-East Asia, Papua New Guinea, Oceania, South Asia, East Africa, West Africa, Southern Africa and Central and South America.

2.5 Other issues relating to laboratory SOPs

While further data on antigen concentration are being accumulated, interim measures to determine appropriate lower dilutions for RDT testing are needed. These include serial dilution and testing against a quality-assured RDT, to determine the dilution at which this RDT produces a weak, but clear positive result, if 200 parasites/ μ L dilutions do not produce a positive test line.

A consistently available supply of fresh, parasite-free Type O blood for dilution of blood containing parasites has been a problem. Assessment of the appropriateness of stored blood (e.g. blood-bank discards) and of the effect of various anticoagulants (e.g. citrate, heparin, EDTA) is needed.

Use of preservatives (antimicrobial agents) may be necessary if preparation times from blood extraction to freezing are extended.

2.6 Other RDT QA development

A summary of work was presented to the consultation on the development and use of positive control wells and temperature monitors for remote-area QA, cool storage using evaporative coolers and assessment of blood transfer methods.

Vaccine vial monitors may offer an inexpensive way of flagging exposure of RDTs to potentially damaging temperatures. One commercially available vaccine vial monitor is showing potential for operating within a useful range for commercially available RDTs, and other products will be investigated. Variation in temperature tolerance of RDTs makes selection of suitable monitors problematic, and development of new monitors specific for products would be expensive.

A study of field use of HRP2-positive control wells and stability of HRP2 and pLDH wells began in 2004 in Cambodia and the Philippines. Results should be available around mid-2006, and the results should allow refinement of the wells. It is expected the wells will be introduced into the field in some Asian countries on a wide scale in 2007 with WHO assistance. National Bioproducts Institute in South Africa is investigating packaging methods for improving stability and including aldolase.

Densitometers suitable for reading malaria RDT test-line intensity have been assessed, and provide a method to increase consistency of results for laboratory assessment of RDTs by eliminating subjective interpretation and observer variability.

3. PRODUCTION OF GOOD PANELS

3.1 Principles for developing methods to produce panels

To develop testing panels which reliably predict the accuracy of RDTs in the field it will be necessary to clearly characterize the range of antigen variation in wild parasites globally (particularly with HRP2 in falciparum malaria), determine the relationship of antigen concentration to parasite density, and then to clearly characterize the contents of samples in a panel, in terms of:

- antigen structure
- antigen concentration
- characteristics of blood¹
 - anticoagulants
 - preservatives
 - age
 - mixing

Reliable antigen capture assays are crucial to achieve this and to compare relative effects on RDTs of wild-type parasite specimens, parasites from culture, and recombinant antigens in blood. A drawback in using recombinant antigen is that when it is added to parasite-free blood it will only be present in extracellular fluid in the sample and therefore antigen release through effective lysis of cells and release of parasites by the buffer on the RDT will not be tested.

3.2 Principles for determining contents of panels

- QC panels used for testing malaria RDTs must contain antigen that is representative of the range of structural variation (epitope expression) encountered in the field.

¹Exclusion of blood-borne viruses is detailed in the Methods Manual (WHO 2006). The possibility of variation in the detection of parasites in HIV-positive and HIV-negative blood should be investigated. Microscopy methods are detailed in the Methods Manual. It is essential to determine which other characteristics of preserved blood affect RDT sensitivity, then characterize the panel accordingly. Work on this has been delayed until reliable ELISAs are available, but should commence soon.

- In at least part of the panel, the antigen must be at a concentration representative of the lowest level of malaria infections that a good malaria management programme would expect to detect and treat.
- The panel should also represent the range of antigen concentrations that are likely to be encountered, or at least predict the sensitivity of the RDT to this range.
- All four major human malaria parasite species should be represented.
- The substrate in which the antigen is held must mimic the action of fresh blood on the RDT as closely as possible.
- The panel must be stable and reproducible.

To achieve the above criteria, the panel for product testing should include a combination of

- recombinant antigen,
- cultured parasites, and
- wild-type parasites.

Inclusion of recombinant antigen allows the production of highly-reproducible serial dilutions for determination of LLD of RDTs.

Inclusion of cultured parasites:

- Allows identical samples containing whole, intracellular parasites to be produced indefinitely to allow standardization of testing across time
- Ensures HRP2 tertiary and quaternary structure is normal, which may not be the case with recombinant protein.

Based on recent evidence, the 2006 Geneva consultation recommends that in the development of panels, the following principles be adhered to:

- Recombinant antigen in serial dilutions to 50 parasite/ μ L equivalent, or below. This will be determined by the Specimen Bank Review Committee based on results of the 2006 antigen quantitation studies.
- Cultured parasites, with density decided according to antigen concentration.
- Wild-type parasites at 200 parasites/ μ L and high/natural density with the exclusion of outliers in terms of antigen concentration and HRP2 structure from the main panel. The exclusion criteria should be standardized by the Specimen Bank Review Group.
- The addition of 500 parasite μ L dilutions for non *P. falciparum* samples, and 1000 parasite/ μ L for HRP2 structurally-variant *P. falciparum* samples.
- Predominance of *P. falciparum* as the main variation between parasite samples in antigen available for binding is likely to occur due to HRP2 variation and to sequestration of parasites.
- Parasite-negative samples, including known causes of false-positive reactions and illnesses likely to be differential diagnoses of malaria.

With these characteristics in mind, it is necessary to determine the effects of antigen structure on RDT sensitivity, the relative antigen concentrations in different epidemiological situations and stages of infection, and stability of the panel.

All samples in a panel will need to be characterized² by:

- Microscopy;
- Polymerase chain reaction (PCR) for determination of species;
- HRP2 sequencing for determination of gene sequence of target antigen (species, ± antigen structure);
- Antigen quantitation (ELISA);
- Geographical origin.

Under the SOPs currently in use, the integrity of parasite QC panels is checked using quality-assured RDT that are stored at 4°C until used (WHO 2006b). The SOP will to be modified to specify measurement of antigen content by ELISA, when adequate reliability of ELISA has been demonstrated.

3.3 Detection of antigen

It is necessary to accurately determine the concentration of antigen in each QC sample (at least for samples with low parasite density) and the range of concentrations expected to correlate with a certain parasite density in the field. If the antigen is characterized in terms of structure (HRP2, and other antigens if necessary), the likely field sensitivity of an RDT could be predicted from results of testing using the characterized sample.

Standard curves of antigen concentration in lysed samples and parasite density will therefore need to be developed using antigen capture by ELISA for a range of isolates characterized geographically and clinically. For HRP2 at least, the antigenic structure of each isolate will also need to be known.

Four ELISAs have been under trial for quantitative analysis of parasite antigen, two for HRP2 and two for pLDH. One pLDH and one HRP2 ELISA perform consistently with a linear range of about 200–5000 parasites/μL.

An aldolase ELISA still needs to be developed, but progress has been held up due to delays in obtaining raw materials.

² Characterization (antigen quantitation and species-specific PCR) for African sites will generally be based at KEMRI, non-African sites at CDC. HRP2 sequencing will be performed by AMI. Some characterization may occur at collection sites.

4. ASSESSMENT OF RDT STABILITY

Assessment of RDT stability is a necessary component of any QA or RDT testing scheme, as degradation by heat and moisture is likely to be a significant factor in RDT failure in the field.

4.1 Early outcomes of heat stability trial

A heat stability trial of five commercial products was conducted in 2004 - 2005 in the Hospital for Tropical Diseases (HTDL), Centers for Disease Control and Prevention (CDC) and Research Institute for Tropical Medicine (RITM) prior to the consultation. Results indicated:

- a marked difference in temperature stability of products between the laboratories;
- significant test-to-test variation at borderline parasite density with at least two products;
- consistent difference in temperature stability between products detecting HRP2 compared with products detecting pLDH;
- significant difference in freeze-thaw stability between products detecting HRP2 compared with products detecting pLDH;
- failure of one product from outset, which was overcome when the cassette was dismantled, the lateral flow strip removed, and blood and buffer placement modified.

Identical RDT lots and blood samples were used, and identical evaluation protocols were followed. A difference in storage conditions for blood/parasite samples (liquid nitrogen prior to study and -80°C during the study, versus -20°C at all times) may explain the difference in stability between them, and this requires further investigation. Degradation during transport from manufacturers to testing sites is also possible.

The reasons for the difference in temperature stability between RDTs targeting different parasite antigens may relate to the stability of other RDT components and the stability of the Mabs used in the RDTs.

Further investigation to determine this would be useful, though not specifically necessary for the design of an RDT testing scheme. If the Mabs are less stable, more stable Mabs may be available that target these antigens.

Preliminary reports from a further stability study investigating decay curves of line intensity of various RDTs at different temperatures, undertaken by RITM and London School of Hygiene and Tropical Medicine LSHTM and WHO, were presented to the 2006 consultation. Early results indicate a wide variation in decay curves between products. This data suggests that predictions of shelf life from accelerated data are at least product specific and no generalized model can be developed to produce accurate predictions. The 2006 consultation therefore recommended a simplified stability component for the final product testing protocol (Annex 5).

5. GENERAL REQUIREMENTS FOR TESTING MALARIA RDTs

Requirements for testing RDTs are discussed in a previous publication (WHO 2003), and issues concerning assessment of RDT sensitivity are discussed above. The 2003 consultation (WHO 2003) recommended the use of wild-type parasites at high and low dilutions, and that recombinant antigen should be considered in future.

5.1 Setting standards for sensitivity

The LLD for RDTs recommended by the previous consultations was 100 parasites/ μ L (WHO 2000; WHO 2003). However, the evidence of variation in RDT sensitivity resulting from structural variation of the target antigen and the high variation in parasite density versus antigen concentration, discussed earlier, suggests that setting an LLD in terms of parasite density is not biologically credible. Evidence also suggests that an LLD of 100 parasites/ μ L may not be achievable with some target antigens. This has three major implications:

- (1) The LLD may be better defined in terms of antigen concentration, based on good data on the parasite/antigen relationship.
- (2) Panel constituents should include well characterized wild-type parasites from a wide range of geographical and epidemiological backgrounds, and include culture and recombinant antigen specimens that can be better standardized and controlled.
- (3) Rating of RDTs in terms of LLD against well-characterized samples is likely to be more useful than recording the ability of the RDT to detect an arbitrary low parasite density.

5.2 When an RDT should be tested

A testing scheme for RDTs should provide accurate information to guide procurement of RDTs and on the sensitivity, specificity and stability of RDTs in use. A number of models for RDT product testing schemes exist, including those for HIV, hepatitis B and C, and Chagas disease (WHO 2001a; WHO 2001b; WHO 2002; WHO 2004), and those for syphilis rapid tests (TDR 2003). Disease diagnostics schemes for hepatitis, HIV and Chagas disease rely on product testing in a single reference laboratory according to SOPs developed by WHO. Manufacturers pay for testing, and retesting is at the behest of the manufacturer. The scheme for syphilis diagnostics has relied on testing in eight laboratories in various regions, coordinated by two reference laboratories. Testing has been laboratory-based, and is now moving to a field-based phase. A tuberculosis specimen bank (TDR/PRD/DRD; TDR Diagnostics Research and Development) is also under development for use in future testing schemes.

Although malaria RDTs are likely to be used primarily in resource-poor, remote field situations, the consultation agreed that field testing was not ideal for a long-term product testing scheme due to the high cost of recruiting sufficient numbers of malaria patients, the high rate of product modification in the commercial sector and the difficulty in adequately characterizing the antigen content of blood samples. Evidence presented to the consultation demonstrated a variation from lot to lot in some products and structural problems affecting RDT sensitivity, including cassette design and characteristics of nitrocellulose membranes. This indicates that although it is necessary for guiding procurement, one-time product testing does not provide adequate confidence in product

quality and lot testing must also be carried out. Lot testing must be simple, inexpensive, standardized and repeatable and based on parasites derived from the region where the product will be used. In view of this, a two-tier system for product testing was proposed (Figure 2):

- (1) **Product testing** would be used in at least two central reference laboratories, using standard panels and SOPs to assess likely performance in the field. A product need be tested only once and should include samples from at least two lots.
- (2) **Lot testing** would be used after purchase at a regional level, using shorter panels of local parasite strains to ensure performance is equivalent to a published product's test results. These panels should also be included in central reference laboratory panels used for product testing.

It was also considered important that manufacturers and developers have access to well-characterized panels to aid product development and for internal QA procedures, and to allow manufacturers to ensure products are suitable for submission to an external product testing scheme.

The consensus of the consultation was that product testing should occur simultaneously in at least two laboratories, in view of the potential for testing results being affected by damage during transport and storage of RDTs, possible deterioration of panels, and variation in technique and interpretation. Models for RDTs for other diseases encourage manufacturers to visit testing laboratories and demonstrate testing techniques (WHO 2001a; WHO 2001b; WHO 2002; WHO 2004). Lot testing should be organized on a regional basis, with enough laboratories to ensure maintenance of standards. These laboratories should have access to locally representative parasites.

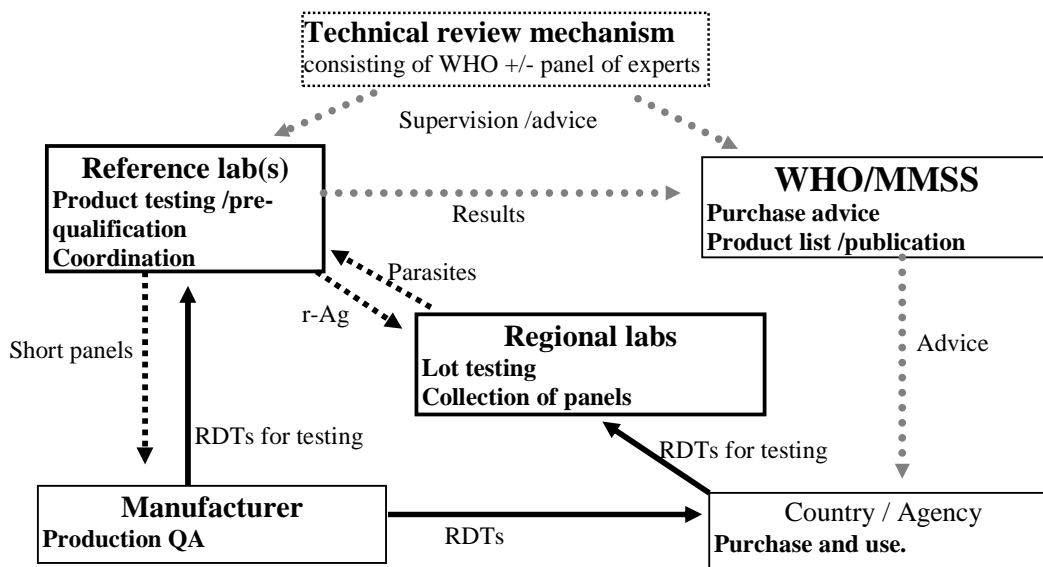


Figure 2: Potential way in which an RDT testing scheme, involving product testing, lot testing and provision of panels to developers and manufacturers, might be organized

5.3 Definition of a product and lot

It is necessary to clearly define the terms “product” and “lot” to implement the proposed testing scheme, as product testing results should be applied only to a specifically defined and labelled product, and lot testing results to a clearly defined and labelled lot. The consultation made the following recommendations:

- (1) **Lot.** The definition of a “lot” is the responsibility of the manufacturer. All manufacturers must have ISO 13485:2003 or US FDA 21 CFR 820 certification and an appropriate “lot” definition is must be compatible with this, although the strict definition is not clearly defined³.
- (2) **Product.** Defining a malaria RDT “product” for the purposes of a product testing and pre-qualification scheme is more difficult. However, it should be based on consistency in overall design and in the major constituents of the RDT that are likely to have a significant impact on RDT stability or accuracy. Assuming that evidence of equivalent performance can be provided, the following is recommended:⁴

³ See Annex 6.

⁴ The specification, “Nitrocellulose – A change in specifications of the nitrocellulose membrane should constitute a new product. A change in manufacturer should not.” has been removed from the definition of a product, as the 2006 consultation considered this unreasonably restrictive.

- (a) Similar but relabelled products from various manufacturers should generally be considered different products (see co-listing of products in section 6.1: Product testing or prequalification scheme.
- (b) Monoclonal antibodies – A change in target epitope, or of the species from which target antigen for Mab development is derived, should constitute a new product. A change in source (manufacturer) or modifying the amount of Mab used in a test would not constitute a new product if the Mab cell line originated from the same source.
- (c) Dye conjugate (signal reagent) – A change in specification or type of label (e.g. colloidal gold, latex particle or liposome) should constitute a new product, but a change in manufacturer/source should not.
- (d) Format – A change in assay presentation between, for example, a dipstick, cassette or card, constitutes a new product.
- (e) Buffer – A change in assay buffer constituents or pH does not constitute a new product.

(3) **Equivalence of performance.** Where changes made have the potential to significantly affect accuracy of the RDT, including changes in raw materials or components including Mabs, signal reagents, buffers, nitrocellulose membranes, or in cassette design, equivalence of performance data should be provided to the product testing coordinating body to demonstrate that the modified product has a performance equivalent to, or better than, that previously submitted to formal testing. As this is an activity that should be performed as part of routine internal QA by the manufacturer, demonstration and notification of equivalence should not result in additional costs or workload.

5.4 RDT specifications to be assessed in an evaluation scheme

The aspects of malaria RDT that determine its usefulness vary depending on the situation in which it is intended to be used. Rather than defining certain standards that a test should achieve to be “acceptable”, it is recommended that results of testing and prequalification of various aspects of RDT performance should be listed, together with a guide to interpretation. Models exist for RDT for other diseases (WHO 2001a; WHO 2001b; WHO 2002; TDR 2003; WHO 2004). The testing schedule is discussed in more detail in Section 6.3, and Table 1.

5.5 Management of an RDT testing scheme

Various issues pertaining to the management of product testing (Figure 2) were clarified by the 2006 consultation.

- Supervision and certification of laboratories where testing takes place. The 2006 consultation agreed to piloting an external laboratory quality assessment tool (EQA) which has been developed by WHO CSR Lyon and adapted for malaria laboratory assessment. The tool involves supervisory visits, proficiency testing/panel testing and blinded rechecking of positive/negative specimens. The EQA assesses against accepted norms and standards, in this case, the SOPs. While the process can be expensive and labour/time intensive, the benefits are seen to outweigh these challenges and support for this type of assessment will be sought. The EQA is to be piloted in March 2006 in the Cambodia and Philippines laboratory sites. WPRO will draft SOPs for product testing laboratories to cover coordination between product testing sites, training, refinement of SOPs, consistency, comparable equipment, calibration and test conditions, proficiency panels and record keeping. These SOPs will be sent to CDC and to Kenya Medial

Research Institute, Nairobi and Kisumu, Kenya KEMRI. It is most important that SOP be standardized between collection and testing centres, allowing for variations to suit local conditions that do not affect the quality of samples or of RDT testing.

- Mode and duration of publishing results. The 2006 consultation determined that all product testing results will be published on a website and in hard copy, after clearance by the Specimen Bank Review Committee. The results of each mode of testing (performance against panel, stability, ease of use) will be published, together with the standards recommended by WHO. Lot testing results will not be published but will be provided to the procurer requesting testing (which may be governments, NGO, institutions), to a database maintained by WHO and to the Specimen Bank Review Committee (SBRC). The Specimen Bank Review Committee may de-list products or any products that have been co-listed (see 6.1), if poor lot testing results are deemed to warrant this. Once tested, results will be listed until the product is withdrawn from commercial availability, or de-listing is recommended by the SBRC.
- Legal issues. There is potential for litigation by manufacturers or purchasers related to published test results. Letters of agreement regarding publication of results should be considered.
- Sustainability. This involves the allocation of costs for product and lot testing. To defray the costs of testing and publication, the payment of a testing fee is to be determined.
- Definition of a new product and notification of product modifications. Consensus was reached on the definition of a “new product” (see 5.3), while the definition of a “lot” is accepted as defined by ISO13485:2003 or US FDA 21 CFR 820.
- Monitoring of transport and storage of RDTs prior to testing. Electronic temperature monitors should be sent with products from manufacturers.
- Responsibility if RDTs fail the post-purchase lot testing procedure at regional laboratories. Procurers (sending institutions) should be notified immediately of failed lots, as specified in the Methods Manual (WHO 2006b). After confirmatory testing at a second institution confirms poor performance, a final report should be sent. Results should be sent to WHO and the sending institution, but not published openly by WHO or the testing centre (further dissemination of information and action on that lot is the responsibility of the institution that owns the lot). All results will be entered into a database held by WHO, and repeated failures brought to the attention of the specimen bank steering group. The committee can require the manufacturer to submit for repeat product testing to maintain listing of products.

Funding models include payment by manufacturers (WHO 2001a; WHO 2001b; WHO 2002; WHO 2004) and provision of RDTs free of charge, with external funding of evaluation (TDR 2003). In the long term, product testing at cost by manufacturers would appear more sustainable, while funding of lot testing may involve a combination of external funding and funding by purchasers as part of the QA budget of a diagnostic programme. Provision of panels to manufacturers may provide a means of supplementing programme costs.

5.6 Other specifications necessary for RDT procurement

An important part of QA for RDTs, in addition to testing, is appropriate purchasing. The 2003 WHO consultation on RDTs emphasized issues to consider, including evidence of the quality of the manufacturing process and evidence of stability (WHO 2003). Since then, discussions with manufacturers indicate that many have not achieved certification under the ISO standards scheme, but this should be achievable without significant cost implications. Requirements were discussed briefly

at the 2004 consultation, with consensus that a move to recommend purchase only from manufacturers with such certification is appropriate. ISO13485:2003 is specific for manufacture of medical devices. At the 2006 consultation, agreement was reached that requirements be tightened to ISO13485:2003 or US FDA 21 CFR 820, as a prerequisite to acceptance into the product testing programme.

6. DETAILED FUNCTION OF PROPOSED LABORATORY-BASED MALARIA RDT ASSESSMENT SCHEME

The 2004 consultation concentrated on the design of laboratory methods for testing RDTs. Aspects of supervision, organization, funding and RDT assessment – other than sensitivity, specificity and stability – were discussed more fully during the 2006 consultation with specific responsibilities assigned.

6.1 Development of product testing programme

The overall development of the product testing scheme will be coordinated by WHO/TDR and WHO/WPRO, as previously, in collaboration with WHO Global Malaria Programme and the WHO CSR office in Lyon. CSR Lyon will specifically assist in the development of external quality assurance by providing advice and assistance including, where possible, from their existing networks.

The present large expert consultation will be the last before the launch of the product testing programme. There will, however, be a small development review committee to oversee the technical and logistical aspects of programme development. The core membership of this committee will consist of WHO/TDR, WHO/WPRO (reporting back to WHO GMP and the Regions), the specimen banks (KEMRI and CDC, two collection sites (one African and one non-African on a rotating basis) and one non-WHO agency. According to technical needs, a non-core group of experts will be invited. Two further small meetings are planned for 2006. Once the specimen banks are assembled, the committee will oversee the use of samples to ensure access is within the terms of reference and will ensure the ethical approval of sample collection. The Steering Group's terms of reference will include oversight of specimen bank development and maintenance, overseeing the characterization of specimen banks samples and access to the specimen bank and oversight of product testing, and approving the publication of results.

6.2 Product testing entry criteria

Product testing will evaluate malaria RDT products against both well characterized panels and criteria identified in section five and in accordance with the latest version of the SOPs provided by WHO. Testing should occur independently at two or more reference laboratories and results consolidated for publication.

There are four conditions recommended for manufacturers to fulfil for acceptance of a product into the product testing programme:

1. Provide evidence of ISO 13485:2003 or US FDA 21 CFR 820
2. Provide an acceptable temperature stability protocol for internal QC testing
3. Commence the prescribed stability test (Annex 6)
4. Formally accept conditions for product testing and publication of results (Annex 5)

Further, entry should initially be restricted by product type:

1. No electricity required
2. Result read visually without need for further equipment
3. Operate with whole blood
4. Result within 30 minutes of placing blood
5. Detect antigen (rather than antibody)
6. Include a system control (control line)

The 2006 consultation recommended that a Product Specific Audit by a notified body be a criterion for acceptance for product testing. This will be further considered by WHO and the Steering Group to ensure it is harmonized with other WHO plans for diagnostic test prequalification for other diseases.

Co-listing of products

In cases where products with different names and “manufacturers” are produced on the same production line, a single product may be submitted for testing and products identical to this co-listed with the results. Such co-submission will require written application from the countries concerned and provision of evidence that the products are the same (Section 5.3). In the case of co-listed products, if a single listed product is deemed by the Specimen Bank Review Committee to warrant de-listing for poor performance on lot testing, all co-listed products will be de-listed and require re-application for product testing. Where products are submitted individually for testing, they will be deemed to be independently manufactured and delisting will involve the named product only.

Products should be submitted for testing by manufacturers at a charge to the manufacturer to be determined by WHO and the steering group. It was the consensus of the 2004 consultation that charging to cover costs is appropriate. Results will be published in hard copy and posted on the WHO website (see 5.5). This is similar to the model for evaluation of other RDTs (WHO 2001a; WHO 2001b; WHO 2002; TDR 2003; WHO 2004). Manufacturers will be encouraged to visit reference laboratories to demonstrate tests and discuss procedures.

Manufacturers should notify the WHO and coordinating body of equivalence of performance after changes in product format (section 5.3: Definition of a product and lot). This should then be noted on the website. Major product changes requiring re-testing should also be noted in the publication and on the website. WHO could then recommend that countries and agencies use the website and publication for guidance on purchasing.

6.3 Product testing criteria

Sensitivity and specificity⁵

Test sensitivity should be based on serial dilutions of recombinant antigen with down to and below an equivalent of 100 parasites/ μ L, with culture and wild-type parasites at 200 parasite/ μ L and

⁵ Note on predictive values: positive and negative predictive values are more relevant to field trials and less useful in laboratory settings due to their dependence on prevalence. If reported, it is useful to adjust predictive values to take into account the ratio of positive and negative samples in the panel.

higher dilutions. Sensitivity should also be tested against a panel of wild-type antigen variant parasites of approximately 200 parasites/ μL . Using serial dilutions of recombinant antigen, it is useful to report sensitivity in terms of lower limit of detection (LLD; see section 5.1: Setting standards for sensitivity). The specimen bank against which products will be tested is detailed in Table 1.

Specificity should be measured with negative blood samples, including samples known to have induced false/positive reactions with malaria RDTs and samples from non-parasitaemic cases with malaria-like symptoms from malaria endemic areas (see section 6.4: Composition of panels).

RDTs should be selected as follows:

- RDTs from two production lots are to be tested;
- two RDT from each lot are to be tested against each low-density panel sample, one against each high-density sample;
- each test is to be read by two prequalified technicians (shielded to the results of each other) and a densitometer;
- the lots are to be tested for the lower limit of detection (LLD) against serial dilutions of recombinant antigen (when available in future), and 200 parasites/ μL and natural density for wild-type samples;
- the sequence of specimen bank samples will be randomized for each product.
- Tubes containing aliquots for product testing will be re-labelled with stick-on labels over written labels over the tubes on removal from freezer with a pre-determined numbering system to ensure blinding.

Stability

Stability assessment should measure the stability of RDTs across the variables of time, temperature and humidity. Assessment during product testing should be performed on 2 lots, against a single bank samples at 200 parasite/ μL and 500 parasite/ μL designated for stability testing (~recombinant antigen) and consist of:

- 10 tests per lot, at 35°C and 75% humidity, for 2 months
- 10 tests per lot, at 45°C and 75% humidity, for 2 months
- 10 tests per lot, at 45°C and 75% humidity, for 6 months **if** $\geq 80\%$ positive after 45°C for 2 months

Stability assessment must also be carried out by the manufacturer on at least one lot, against a single bank samples at 200 parasite/ μL and 500 parasite/ μL supplied to manufacturer, and consist of:

- ≥ 10 tests per lot, at least every 3 months, at upper limit of manufacturer's specified storage range, until manufacturer's specified expiry date
- ≥ 10 tests per lot, at least every 3 months, at 35°C for 12 months.

As a precondition of product testing, the manufacturer's tests must have commenced before or at time of product testing, and as results become available, they are to be reported to WHO for publication on the website.

Ease of use

An overall scoring system for ease of use is not appropriate as interpretation would depend on the intended use of the test (e.g. hospital versus remote clinic). Each test should therefore be described, and characteristics of each test should be reported, including:

- The blood transfer method (with commentary on appropriateness of each)
- Number of timed steps
- Total time to obtain result
- Aspects of blood safety
- The quality of the instructions (pictorial versus text)

6.5 Composition of panels

Principle. Assess RDT sensitivity against common and unusual parasite isolates and predict sensitivity in different regions, and assess RDT specificity.

6.5.1 Product-Testing Panel

The principles on which the component of the specimen bank for product testing is based is detailed in Section 3.2. The bank components are outlined in Table 1.

6.5.2 Lot testing

Lot testing should use smaller panels and concentrate on ensuring adequate sensitivity and that stability during shelf life is within the manufacturer's specifications. Testing should occur after procurement on batches submitted by countries and other agencies to QC laboratories, preferably in the same region, and tested according to standard SOPs.⁶

The lots are to be tested against wild-type samples of 200 parasites/ μ L and a higher density. Research and development should aim to develop stable recombinant antigen or lyophilized samples for post-purchase lot testing.

The lot testing institutions will serve as sources of specimens containing wild-type parasites for the product testing reference laboratories, and reference laboratories will be available to provide confirmatory testing of failed RDTs, using identical panels.

A model for the lot testing scheme is operating in the Western Pacific Region, where two laboratories, RITM and Institut Pasteur de Cambodge (IPC), test RDTs from countries in the Region against a panel of specimens containing local parasites, store them at 28°C, and monitor sensitivity every three months through the product shelf life. Test reports are sent directly to WHO and the procuring country. There are no good models for sustainable funding for this activity. The Western Pacific Region pilot scheme is currently funded through WHO. The laboratories also test products on request for manufacturers. Data presented to the 2006 consultation illustrated that if lot testing is to be widely available the procedure needs to be simplified by developing stable samples, as the

⁶ WHO. Rapid Diagnostic Tests for Malaria: Methods Manual for Laboratory Quality Control Testing. Version 3 (World Health Organization - Regional Office for the Western Pacific, Manila, 2006).

laboratories are now running near capacity and developing new QC samples of cryo-preserved parasites is difficult in low endemic areas.

6.5.3 Panels available to manufacturers

Short panels should be available to manufacturers and developers, at a cost sufficient to cover their production and administration costs. Panels should consist of well characterized recombinant antigen and cultured parasites, and be identical to parts of the product testing panel. Provision of wild-type parasites to manufacturers and developers is not considered an appropriate part of the WHO scheme, as allowing commercial bodies access to patient blood samples may create potential legal complications and will limit access to parasites for the other arms of the QA scheme.

6.6 Quality control testing in remote areas

Positive control wells containing small amounts of recombinant or lyophilized antigen have potential for testing RDTs at a peripheral level, and potentially for lot testing after purchase. A study of a commercially available pLDH well, and an HRP2 well developed for the purpose, was presented to the 2006 consultation. Further work is required in refining the amount of antigen in the wells (to an equivalent of 200 parasites/ μ L), and to ensure the wells are sufficiently stable. The consultation recommended that this work be prioritized by WHO.

Table 1. Composition of malaria specimen bank and RDT testing panels

P: product testing panel. L: post-purchase lot testing panel. LM: lot testing panel available to manufacturers.

Sample type	Details	P	L	LM
Cultured <i>P. falciparum</i>	>20 isolates 200 parasite/uL equivalence, high parasite density HRP2, Pf pLDH, Pv pLDH, aldolase sequenced ^a	X	X ^c	X
Recombinant antigen ^b	Serial dilutions to below 100 parasite/μLequivalent HRP2: ~3 alleles (common and uncommon) Pf pLDH (1) Pv pLDH (1) aldolase: (1)	X	X ^c	X
Wild-type <i>P. falciparum</i> Total 100 common <i>P. falciparum</i> , plus HRP2 variants (variants at 200p/μ, 1000 p/μL, and natural dilution)	Natural density and 200 parasite/μL dilution Sites: South Asia South East Asia Pacific (2) Africa (3) South and Central America (2) 10-15 isolates per site Characterized by ELISA, PCR (species) Characterize HRP2 structure ^e	X	X ^c	
Wild-type <i>P. vivax</i>	20 isolates 200 and 500 parasite/μL, and high	X	X ^c	
? chimp <i>P. vivax</i>	5 isolates 200 and 500 parasite/μL, and high	X		?
Wild-type/chimp <i>P. ovale</i>	5+ isolates 200 and 500 parasite/μL, and high	X		?
Wild-type/chimp <i>P. malariae</i>	5+ isolates 200 and 500 parasite/μL, and high	X		?
Parasite-negative human blood	Anti-nuclear antibody (ANA) 5-10 RPR positive 5-10 Rheumatoid factor positive 5-10 Heterophile antibody positive 5-10 Anti-mouse antibody positive 5-10 Clean negatives (none of above) 50 Other tropical diseases ^e , including: Chagas disease dengue typhoid leishmaniasis	X		X ^c

a Use samples with wide range of epitope expression

b Common sequence

c Post-purchase lot testing underway in Asia currently uses local wild-type *P. falciparum* dilutions (and *P. vivax* for combination RDTs). In future, lyophilized cultured or recombinant antigen may be possible, allowing increased standardization and lower costs, identical to the manufacturer lot testing panel. A reduced parasite-negative panel contains “clean” negatives only.

d Need to characterize HRP2 structure will depend on further evidence of significance of variation.

e Tropical fevers that are common differential diagnoses of malaria.

7. FURTHER DEVELOPMENT OF TESTING METHODS AND PANELS

In order to develop well characterized panels of parasites for use in testing malaria RDTs further research and development is necessary in a number of areas, most of which is currently under way or planned within the existing laboratory network (Figure 3). Plans for collaboration between WHO, the network of institutions currently involved with WHO in development work, and other institutions, were developed in more detail during the consultation and are the subject of a separate document for internal circulation. The activities involved are outlined in Annex 1. A potential timeline for development is detailed in Annex 2. This will depend on adequacy and promptness of funding (Annex 3).

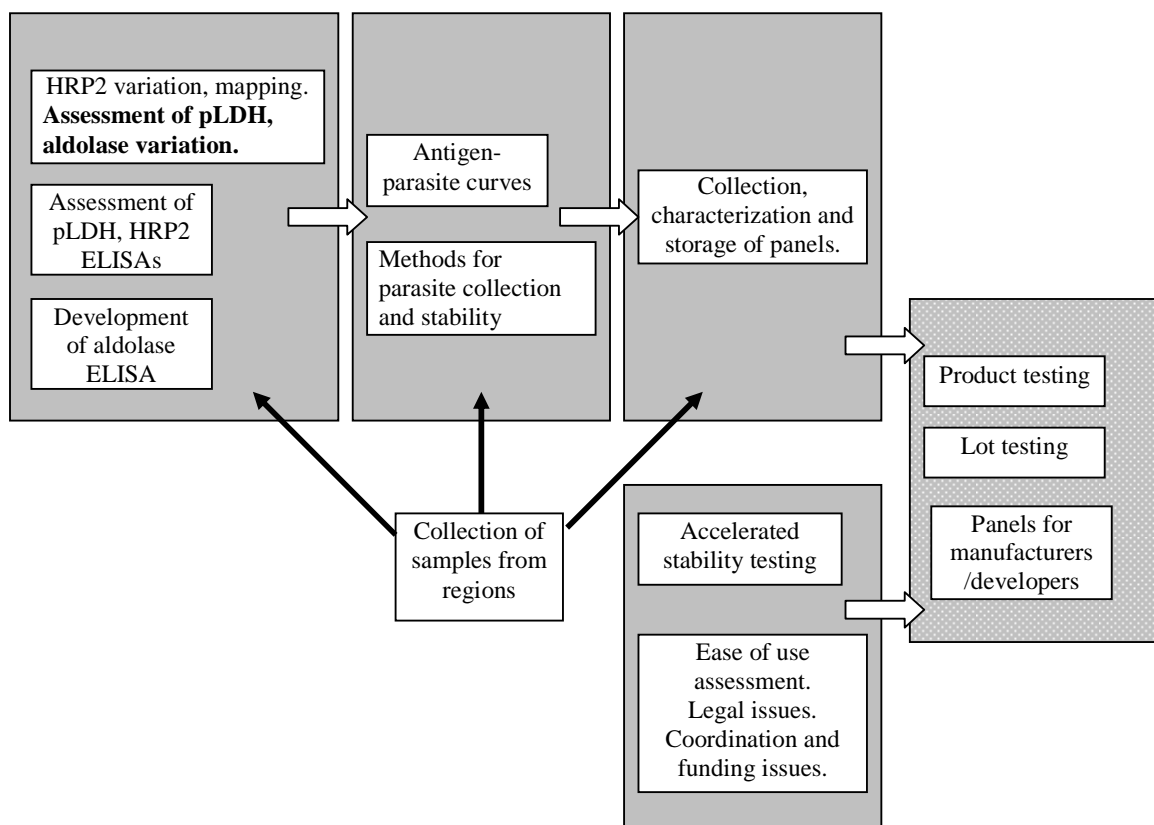


Figure 3. Outline of development work necessary for RDT testing scheme.

ANNEX 1 Further development required

Wild-type panel collection	
<i>Microscopy.</i>	<i>Compare EP, WCC, RCC methods Intra-slide, inter-slide, inter-reader consistency Inter-lab consistency</i>
Donor blood	Donor-donor variation Old vs. new blood Citrate: EDTA : Heparin
Mixing time	
Short-term antigen stability	Ag stability from collection to freezing of aliquots
Panel stability	
Ag stability when frozen	pLDH, HRP2, aldolase Stability in liquid N ₂ , -70°C, -20°C, 4°C,
Freeze-thaw effect on RDT	
Other panel issues	
Obtain & characterize recombinant Ag	HRP2, pLDH, aldolase
Variation of Ag with parasite stage	
Half-life of HRP2, pLDH, aldolase in-vivo	This is considered useful for understanding the relationship between parasite density and antigen concentration, but this is not essential to QA/product testing development
Ag concentration vs. parasite density	HRP2, pLDH, Aldolase MULTIPLE SITES WITH MICROSCOPICALLY AND CLINICALLY-CHARACTERIZED SAMPLES.
Ag distribution in blood	Ag activity in cells vs. plasma
<i>Ag. variation</i>	<i>HRP2 pLDH aldolase Multiple sites blood spot collection Characterization of culture lines</i>
RDT stability	
Stability of component parts	Will determine importance of product changes
<i>Temperature decay curves of whole RDTs, accelerated stability predictions</i>	<i>Test Expert opinion</i>
Assessment of packaging	

Italics: Nearing completion

ANNEX 3: Draft terms of reference for malaria specimen collection and RDT lot quality assurance testing sites

Criteria for specimen collection/lot QC testing sites for malaria rapid diagnostic test product evaluation (to be read in conjunction with the report of the 2004 Informal Consultation on Laboratory Methods for Quality Control Testing of Malaria Rapid Diagnostic Tests (WHO, 2004).

Background

As part of a WHO initiative to improve quality of malaria diagnosis based on RDT, WHO is coordinating a network of laboratories to collect and prepare parasite samples suitable for use in testing RDTs, and to test RDTs submitted by national malaria programmes and other bodies to ensure sufficient quality for use in the field. The network will include central reference laboratories/specimen banks where more extensive product testing will take place.

The specimen collection/product testing sites will collect and prepare samples of wild-type parasites according to standard operating procedures, characterizing by microscopy and screening for bloodborne viruses. Some of these samples will be transferred to the central specimen banks, where further characterization will take place. Some collection sites may perform further characterization, depending on pre-existing capacity.

The collection sites will also test locally procured lots of RDTs using retained specimens, according to the SOPs. In the future, it is expected that more standardized samples (possibly recombinant antigen or culture-based samples) will be used. Some sites may perform only one of the two functions, specimen collection or testing using external specimens.

Technical and competence requirements

1. Access to cases suitable to provide specimens, within reach of preparatory facility
2. Availability of prequalified expert microscopists
3. Characterization of panels (ELISA) (in collaboration with other laboratories)
4. Ship and receive international biological specimens
5. Receive, test, and store RDTs according to s
6. Storage (-80C) and archiving of specimens
7. Consistently monitor storage conditions
8. Prepare paperwork, summaries of testing/records and collate returned reports
9. Maintain electronic database of panels and results

Equipment and space requirements

1. Set of automatic pipettes, binocular microscope and centrifuge
2. ELISA incubator; ELISA reader, ELISA washer
3. pH meter (if Giemsa home-made)
4. Slide dryer, staining station
5. Thermometer; vortex mixer
6. Blood cell count analyser
7. Communication means (internet connexion)
8. Freezers (-80°C) with alarm and ensured power supply
9. Refrigeration
10. Incubators (1-2) dedicated to project
11. Bench space
12. ELISA readers and related devices ?
13. Computer access

Terms of reference

1. Follow agreed standard protocols
2. Maintain local specimen bank and database
3. Characterize specimens by parasite density, white cell count, red cell count, haemoglobin⁷
4. Screen for bloodborne viruses (HIV, Hepatitis B, hepatitis C)
5. Provide suitable specimens to reference banks
6. Test and monitor RDTs from purchased lots from countries
7. Test RDTs from field on request
8. Provide rapid feedback on results (<7 days)
9. Research to refine protocols, in collaboration with WHO and other laboratories
10. Collaborate with WHO in publication of results
11. Be overseen by institutional (ethics) review board, and external quality assurance (EQA) programme, including participation in an international malaria microscopy EQA programme

⁷ Further characterization may be requested in coordination with WHO and Specimen Bank site.

ANNEX 4: Draft terms of reference for malaria specimen banks

Criteria for specimen banks/product testing sites for malaria rapid diagnostic test product evaluation to be read in conjunction with the report of the 2004 Informal Consultation on Laboratory Methods for Quality Control Testing of Malaria Rapid Diagnostic Tests (WHO, 2004).

Background

WHO proposes to commence a product testing programme for malaria rapid diagnostic tests (RDT), based on testing of sensitivity and specificity against a standardized panel of parasite-positive and negative blood samples, and accelerated stability testing. Samples for the panels will be collected from sites in Asia, Africa and South America through institutions collecting samples and conducting post-purchase testing of RDT. Product testing will take place under coordination of WHO in two laboratories. These laboratories will receive and store samples, perform part of sample characterization, and test products submitted by manufacturers. They will maintain a database of samples, provide technical advice to regional RDT laboratories performing post-purchase testing and dispatch specimens where requested to these laboratories.

Technical competence requirements

1. Characterization of panels (ELISA) (in collaboration with other laboratories)
2. Ship and receive international biological specimens
3. Storage (-80°C) and archiving of specimens
4. Prepare paperwork, summaries of testing/record and collate returned reports.
5. Maintain electronic database of panels and results.
6. Oversee entire operation (ensure adequate specimens at central and regional banks, oversee transport of RDTs and panels)
7. Provide expert technical advice to regional laboratories
8. Provide *blinded* product testing and ease-of-use assessment

Equipment and space requirements

1. Freezers (-80°C) with alarm and ensured power supply.
2. Refrigeration (RDTs)
3. Incubators (2-3) dedicated to project
4. Bench space
5. Storage space for RDTs (temperature-controlled)
6. ELISA readers and related devices.
7. Computer access

Terms of reference

1. Receive specimens and maintain a specimen bank and associated sample database
2. Characterize samples with quantitative ELISA
3. Perform testing of malaria RDT on request from WHO, according to an agreed protocol, including:
 - a. sensitivity and specificity against an agreed panel
 - b. temperature stability testing
 - c. ease-of-use testing
4. Collaborate in establishing a protocol for product testing
5. Be overseen by an established IRB system and obtain timely IRB approval for activities
6. Work in collaborative way with regional laboratories and (other) specimen bank/product testing laboratories
7. Collaborate with WHO in publication of results through WHO website and publications
8. Provide technical advice to sub-regional RDT QA laboratories

ANNEX 5: Informal consultation on development of methods for testing malaria rapid diagnostic tests, Geneva, 28 February–2 March 2006

Summary of outcomes and recommendations

Product testing

Conditions for acceptance of product into product testing programme

1. ISO 13485:2003 or US FDA 21 CFR 820
2. Product specific audit by notified body⁸
3. Commencement of stability test (see below)
4. Formal acceptance of conditions for product testing and publication of results (see below)
5. Payment of a testing fee (to be determined) to defray costs of testing and publication

Co-listing of products

In cases where products with different names and “manufacturers” are produced on the same production line, a single product may be submitted for testing and products identical to this co-listed with the results. Such co-submission will require written application from the companies concerned and provision of evidence that the products are the same (Section 5.3 of main text). In the case of co-listed products, if a single co-listed product is deemed by the Specimen Bank Review Committee to warrant de-listing for poor performance on lot testing, all co-listed products will be de-listed and require re-application for product testing. Where products are submitted individually for testing, they will be deemed to be independently manufactured and delisting will involve only the product named.⁹

Parasite detection

Details: see product testing panel (Table 1 of main document)

Principles of panel:

- Serial dilutions of recombinant antigen to 50 parasite/μL equivalent or below.¹⁰
- Wild type and cultured parasites at 200 parasite/μL and high/natural density,¹¹ with exclusion of outliers in terms of antigen concentration and HRP2 structure from main panel.

⁸ Mechanism to be determined after discussion within WHO and the Specimen Bank Steering Group, to ensure harmonization with WHO policy on prequalification of other diagnostics. The product specific audit may be introduced at a later date as part of a wider prequalification process.

⁹ In cases where a product is de-listed for poor performance, the Specimen Bank Review Committee may recommend specific lot testing of other products believed to be the same as the de-listed product.

¹⁰ Equivalence range to be determined by Specimen Bank review Committee, according to results of 2006 antigen quantitation studies.

- Addition of 500 parasite/μL dilutions (to 200 parasite/μL samples) for non-*P. falciparum* samples, and 1000 parasite/μL for HRP2 structurally-variant *P. falciparum* samples.
- Parasite-negative samples.

Specimen bank sites and storage

Specimen bank and product testing sites will initially be KEMRI, Kisumu, Kenya, and CDC Atlanta, USA.

Storage of samples will be at -80°C, with back-up freezers at both sites.

Sample characterization:¹²

Microscopy

Antigen quantitation (ELISA)

PCR for species

HRP2 sequencing

Characterization (antigen quantitation and species specific PCR) for African sites will be based at KEMRI, non-African sites at CDC. HRP2 sequencing will be performed by AMI. Some characterization may occur at collection sites.

Principles of testing:

- Two production lots
- Two RDT from each lot against each panel sample
- Each test read by two prequalified technicians (one shielded to results of the other), and densitometer
- Test for lower limit of detection (LLD) against serial dilutions of recombinant antigen, and 200 parasites/μL for wild-type and culture-derived samples.
- Sequence of specimen bank samples will be randomized for each product.
- Both specimen banks will test both products initially. This may be modified later to testing at a single bank on review by the Specimen Bank Review Committee.

WHO acceptance criteria could be based around the following example, but need to be considered in the context of projected use of the RDTs:

- 95% detection of 200 parasite/μL samples
- ≤ 5% false positives among “non-clean” negative samples
- ≤ 2-3% false positives among “clean” negative samples

¹¹ Dilutions need to be standardized with lot testing panels by Specimen Bank Review Committee. Working Group 2 recommended 200 and 500 parasite/μL and natural density for wild-type *P. falciparum* again

¹² Exclusion of bloodborne viruses is detailed in methods manual. The possibility of variation in detection of parasites in HIV-positive and HIV-negative should be investigated. Microscopy methods are detailed in the Methods Manual.

Statistical properties of these criteria:

True performance of test %	Chance in % that 95/100 or more will be detected:
90	6
91	10
92	18
93	29
94	44
95	62
96	79
97	92
98	98.5
99	99.9

Therefore, a test with a true performance expected to be between 97% and 98% would achieve this level with 95% probability. It should be noted that the panel will contain specimens known to have a level of antigen within the limits and RDT is expected to detect, so performance should be high.

Stability testing

Stability assessment during product testing:

10 tests, at 35°C and 45°C for two months at 75% humidity, against a single bank sample at 200 parasite/ μL and potentially 500 parasite/ μL ¹³

Stability assessment by manufacturer¹⁴

The Geneva consultation recommended :

Minimum of 45°C at 75% relative humidity for 6 months, 35°C at 75% relative humidity for 12 months and real-time monitoring at room temperature, against a 200 parasite/ μL sample.¹⁵

Stability test must be commenced as precondition for product testing, and results reported to WHO and published on website as they become available. These recommendations are modified subsequently (Annex 6)

Ease-of-Use testing

- Blood collection and transfer
- Setting up test, complexity of test
- Quality of instructions
- Interpretation of test

Definition of “product” and “lot”

- “Product” definition is modified from the recommendations of the 2004 Manila consultation.

¹³ Use of a 500 parasite/ μL sample specifically for stability testing.

¹⁴ This will require an appropriate sample to be available to manufacturers, as part of the lot testing panel, and may be delayed until after the product testing programme has commenced. This may be in addition, or included in, requirements for ISO13485:2003 and US FDA 21 CFR 820 accreditation.

¹⁵ Stability protocol will be refined by the Specimen Bank Steering Group on discussion with experts and manufacturers.

- “Lot” definition is responsibility of the manufacturer, within the limits set by ISO13485:2003.¹⁶
- “Equivalence of Performance” is unchanged from the 2004 Manila consultation definition.

Publication

All product testing results will be published on a website and in hard copy, after clearance by the Specimen Bank Review Committee.

Results of each mode of testing (performance against panel, stability, ease of use) will be published, together with the standards recommended by WHO.

Lot testing results will not be published but will be provided to the procurer requesting testing, a database maintained by WHO, and the Specimen Bank Review Committee. The Specimen Bank Review Committee may de-list products if poor lot testing results are deemed to warrant this.

Duration of listing of product

Once tested, results will be listed until the product is withdrawn from commercial availability, or de-listing is recommended by the Specimen Bank Review Committee.

Standard operating procedures for specimen collection and lot testing in regional laboratories.

Regional laboratories will be required to collect parasite samples for the product testing panel of the specimen bank and for locally maintained lot testing panels.

The current use of cryo-preserved panels for lot testing is considered an interim measure while QC samples stable at 4°C and easily transportable are developed. A method of testing that could be economically implemented at a national programme level should be the eventual goal of lot testing development.

There may be an ongoing requirement for small numbers of cryo-preserved wild-type parasites for lot testing in specific areas where variant parasites are common.

Modifications to Standard Operating Procedures Methods Manual

Principles on which revisions are made:

SOPs should be standardized between collection and testing centres, allowing for variations to suit local conditions that do not affect the quality of the samples or of RDT testing. This will be reflected in Version 4 of the *Methods Manual*, to be developed before mid-2006.

Additional background:

Microscopy and dilutions:

Dilutions remain based on parasite density. Strict prequalification of microscopists and adherence to agreement criteria are essential. All laboratories should be enrolled in an international EQA scheme for malaria microscopy.

¹⁶ All manufacturers must have ISO13485:2003 or US FDA 21 CFR 820 certification, and an appropriate “lot” definition is therefore clearly defined in their manufacturing process.

Antigen quantitation as a basis for dilutions is impractical at present (may be appropriate when stability of antigens at 4°C is known). At present, antigen quantitation should be performed after dilution and aliquoting, and outliers be discarded.

Parasite density should be based on true white cell count (counting against 500 white cells). Working group recommended that Earle-Perez method be dropped from Methods Manual. This can be reviewed by Specimen Bank Review Committee when results of recent multisite comparison of parasite quantitation methods is completed.

Screening of samples in the field should be performed where possible in addition to RDT-based screening, to allow inclusion of antigen variants and uncommon species that react poorly with RDTs and may therefore be excluded¹⁷.

Of all blood films, 10% should be externally cross-checked (at other collection sites).

Bloodborne virus screening:

At sites with low HIV prevalence, the current Asian site method of delayed screening after QC sample preparation may continue. At high HIV prevalence sites, screening should occur before sample preparation using a rapid tests, with later laboratory confirmation. Whether screening is conducted before or after initial finger-prick screen for parasite density is for each site to decide, with reference to local policy.

Consent:

Consent forms used in Asia and developed for Africa are to be standardized to include essential elements required by ethics committees at all collection and use sites, and compatible with TDR and WHO ethics review board requirements (WHO 2006c).

Unresolved issues still to be decided by Specimen Bank Review Group

Format of ease-of-use testing.

Appropriate antigen concentration of samples, taking structural variation into account (this is a research question), and standardization of antigen quantitation by ELISA.

Details of coordination between specimen collection and product testing sites, training, refinement of SOPs calibration and test conditions, proficiency panels, record keeping and database maintenance.

Number of RDTs at testing intervals for lot testing, and number of QC samples used.

Use of high and low dilutions, or high, moderate and low dilutions, in lot testing.

Finalization of microscopy methods in SOPs, and use of same or separate slides for thick and thin films.

¹⁷ Microscopy screening included as optional as may not be possible at some remote Asian and American collection sites.

Finalization of standard operating procedures for maintenance of specimen bank and product testing, record keeping, and for coordination between product testing sites and collection sites. These will be drafted by WHO Regional Office for the Western Pacific.

Research priorities

1. Research priorities for specimen bank development and use

- ELISAs: Aldolase and HRP2 ELISA development to produce consistent affordable product (in progress), optimization of all ELISAs with proficiency panels, new Mabs and variants
- Use of lyophilised samples for ELISA EQA
- Short-term stability of target antigens at 4°C, to determine acceptable delay from blood extraction to cryopreservation
- HRP2 structural variant evaluation in South America and in Africa
- Conclusion of assessment of structural variation of Aldolase and pLDH
- Stage specificity for pLDH and aldolase production of *P. falciparum* (in progress), *P. vivax*, *P. ovale* and *P. malariae* and their gametocytes.
- Cases with false-negative HRP2-based tests at high parasite density – determine whether these are of significant frequency for samples to be included in panel

2. Research to further refine testing and improve sustainability

- Positive control well development: refinement of pLDH and HRP2 wells, and development of aldolase wells. Effects on RDT sensitivity of solute used for reconstitution of positive control wells
- Relevance (predictiveness) of accelerated stability testing to real-time RDT shelf-life
- Effect of anticoagulant on QC samples: EDTA (currently used) versus heparin, citrate
- Long-term antigen stability at -80°C versus -20°C storage, and in liquid nitrogen
- Relationship of available recombinant antigens to wild-type parasite antigen structure, and response of RDTs. (+/- new recombinant antigens for QC testing)
- Effect of HIV serology status on malaria RDTs.

3. Other research priorities, not directly related to RDT testing programme

- Better devices for blood collection
- Improved monoclonal antibodies (improved stability and sensitivity) and target antigens
- QPCR for stage-specific RNA (NASBA): identification of stage of antigen production etc.
- Algorithms for use in the field (particularly for management of non-malarial febrile illness)

Revised definitions of product and lot

See Section 5.3, main text.

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ANNEX 6: Consultation of malaria specimen bank steering group, Kisumu, Kenya, 22–23 June, 2006

Summary of outcomes and recommendations

(Updating and expanding recommendations of the informal consultation in Geneva, February-March 2006)

Summary

A malaria specimen bank and product testing programme for malaria rapid diagnostic tests is being developed by WHO. This programme, and associated research and development work, is a collaboration between a number of WHO units (including WHO Regional Office for the Western Pacific, Special Programme for Research and Training for Tropical Diseases, WHO/CSR Lyon office, WHO Global Malaria Programme, and a network of external institutions detailed in the main text of this report.

From June 22 to 23 2006, a meeting of the Specimen Bank Steering Group was convened in Kisumu, Kenya, to review progress since the previous consultation in Geneva between February and March 2006, including:

- progress on quantitative ELISAs and antigen quantitation
- results of studies of HRP2, pLDH and aldolase variation
- the external quality assurance (EQA) tool for collection sites and specimen bank
- draft standard operating procedures for the specimen banks
- progress on specimen collections and transfer.

The steering group meeting also reviewed outcomes and unresolved issues from the Geneva consultation.

The work of the KEMRI facility at Kisumu was discussed by Dr Sam Martin, Dr Juma Rashid and Dr John Waitumbi, and a tour of facilities for the proposed specimen bank was conducted.

This annex summarizes outcomes of the Kisumu meeting. Detailed outcomes will also be incorporated into the *Methods Manual for Laboratory Quality Control Testing of Malaria RDTs Version 4*, *The Standard Operating Procedures* under development for specimen banks and product testing, and the onsite evaluation tool under development for external quality assurance of specimen collection and RDT lot-testing sites.

Specimen bank characterization

Quantitative ELISAs: progress on testing and development

Further work at HTD has identified a commercially available HRP2 ELISA with good consistency and detection at low parasite densities to below 50 parasites/ μ L. This ELISA will be used for characterizing initial samples for the specimen bank providing an affordable consistent supply can be established.

Data available to date from HTD on the ratio of HRP2: parasite density indicates that variation is very wide on samples obtained predominantly from Asia. Testing of further samples is important to:

- determine the antigen content of each panel sample prior to adding it to the bank;
- allow removal of “outliers” with unusually high or low antigen concentration;
- define the common range of antigen concentration at 100 to 200 parasites/ μ L, to a standard for manufacturers to aim at, and for development of future lot-testing panels and positive control wells based on recombinant antigen.

No clear difference was established in HRP2 concentration between samples with predominance of early or late trophozoites. While a large variation in HRP2 expression is expected, accumulated HRP2 from previous parasite cycles and variable affinity of antibodies in the ELISA due to variation in HRP2 structure may contribute to low variability between these samples. An additional possible influence is the presence of anti-HRP2 antibodies in the serum of some patients. This may also affect RDT sensitivity and significance could be determined by quantitating anti-HRP2 in the serum of cases or by disrupting immune complexes prior to testing.

A reliable pLDH ELISA is available and an aldolase ELISA developed at CDC shows a good range of sensitivity. On initial results from screening parasites, pLDH concentration also shows a high variation with parasite density and, as with aldolase, with the parasite stage.

Antigen variation: assessment of HRP2, aldolase and pLDH variation

Further data since the Geneva consultation indicates little structural variation in pLDH and aldolase, and there appears to be no need for further investigation. High HRP2 variation was confirmed, but a lack of data exists on African and American strains and gathering this should be a priority before finalizing the specimen bank. The presence of multi-strain infection in African samples will complicate analysis, and it will be necessary to screen for single-strain infections to develop more data on HRP2 variation in this region.

As most African samples in the specimen bank will be multi-strain infections, antigen variation is likely to have less effect on RDT sensitivity. The Kisumu consultation determined that, while continued sequencing of African single-strain samples should be performed to gather further data on the extent of variation, for purposes of the specimen bank sequencing of HRP2 in all samples is not necessary. Asian samples should be sequenced, and outlying samples in terms of HRP2 structural variation should be kept in a sub-panel to test RDT sensitivity against parasite variants.

Sites for characterization of specimens.

Initial quantitation of antigen through ELISA will be focused at HTD (pLDH and HRP2) and CDC (aldolase), with the aim of transferring the focus of this work to the specimen banks and some laboratories collecting samples over the next 12 months. A similar process will be followed for sequencing of HRP2, currently performed at AMI. This will ensure consistency of results between samples collected at different sites, and allow the current work on testing and developing suitable ELISAs to continue.

Review of specimen bank composition

Cultured parasites

The importance of including cultured parasite samples in addition to recombinant was confirmed in Kisumu as this:

- allows identical samples to be produced indefinitely to allow standardization of testing across time;
- ensures HRP2 tertiary and quaternary structure is normal, which may not be the case with recombinant protein.

Cultured parasites in the specimen bank will initially be derived from samples from CDC and KEMRI, after confirmation that HRP2 primary structure is within a common range.

Recombinant antigen

HRP2: 3 clones will be expressed within the network for use in the specimen bank

Aldolase: current strain used for ELISA development will be used. CDC will determine whether a *P. vivax*-specific clone will also be required.

pLDH: Commercial sources of pLDH *P. falciparum* and *P. vivax*-specific protein will be investigated.

Wild-type parasites

It appears that four African collection sites and two to three Asian sites will be operating in time to contribute to the specimen bank for the initial round of RDT product testing. The consultation emphasized the importance of including American parasites, and recommended that priority be given to developing at least one collection site in South America to provide samples in time for this round.

The number of *P. vivax* samples in the bank, should be increased to 20, and include some South American samples to expand the power of the bank to discriminate between RDTs with border-line *P. vivax* sensitivity.

The number of *P. ovale* and *P. malariae* samples should be increased if possible.

Specimen collection: progress and issues from recent collections

Successful specimen collections had been undertaken at RITM and IPC in 2006 prior to the Kisumu consultation, and characterization is underway. IPC collections have been affected by clumping of RBCs after dilution. This was not seen at RITM. The causes are unclear, but investigation is urgently needed before further collections to reduce wastage of samples.

Discussion at the Geneva consultation had covered possible effects of preserved blood (EDTA, heparinized) versus fresh blood on RDTs, and on possible effects of mixing preservatives of donor and case blood during preparation. A review of several product instructions indicates that RDTs are commonly intended to test both fresh and preserved blood

by the manufacturers. Experience reported from IPC and RITM indicates no difference in RDT or ELISA results when fresh versus EDTA blood is used, and when blood bank versus EDTA preserved donor blood is used for dilution. This issue should be clarified further during sample collections in 2006.

Specimen bank database

A database of specimens at collection/lot testing sites and the specimen bank at CDC and KEMRI will be developed jointly by KEMRI and WHO. Initially, a database will be held at each site where samples are held and regularly updated to a central database, as either an MS Excel or MS Access software. A network database on a secure site is likely to present access problems due to firewall issues.

Review of draft standard operating procedures.

Standard operating procedures for specimen banks and product testing

Review of the specimen bank and product testing protocol from the Geneva consultation indicated some redundancy in sensitivity and stability testing, and a need to increase the number of RDTs at each stability testing interval. These are included in Section 6.3 in the main text, and include:

- reduction to two low-density and only one high-density sample should be used;
- increase in the number of RDTs in the stability test to 10 at each testing interval and temperature;
- a more structured approach to blinding of technicians from the contents of the tubes. A standard storage tube will be selected and distributed to all sample collection sites.

A trial run of product testing will be performed at CDC and KEMRI on RDTs purchased by WHO prior to formal product testing, to test procedures and determine the workload involved. This may delay the product testing programme by two to three months but ensure quality of results. It is likely that the initial product testing will take four to six months, depending on applications from manufacturers.

Ease of use testing was previously determined to be an essential part of product testing, and details were discussed at the Kisumu consultation. It was recommended that an overall scoring system not be used, as an appropriate score would depend on the intended use of the test (e.g. hospital versus remote clinic). Characteristics to be reported are detailed in Section 6.3 of the main text.

Standard operating procedures for specimen collection and lot testing

The 2006 Geneva consultation discussed the number of RDTs that should be tested for post-procurement lot testing, but did not make a final recommendation. As lot testing is intended to assure that RDTs are of acceptable sensitivity, but do not define all capabilities of a rapid test, variation within a lot should be small if quality manufacturing criteria are followed in procurement decisions. Costs of the procedure must be minimized to ensure acceptability and sustainability, therefore the total number of RDTs tested should be kept to the minimum necessary.

The Kisumu consultation recommended that:

- (1) the number of parasite-positive blood samples against a lot of which RDTs is tested at the initial test be expanded from two to four at low (200 parasites/ μ L) and high parasite density;
- (2) the number of samples against which RDTs are tested remain the same for subsequent testing at three month intervals during the shelf-life of the product (two cases, at low and high parasite density);

- (3) two RDTs be tested against lower parasite density samples, one RDT against the higher density sample.

This will be reflected in version 4 of the Methods Manual.

The Methods Manual will be updated to reflect modifications in specimen collection recommended for African collection sites. It was also considered that parasite dilutions between 200 parasites/ μL and high parasite density should be included (about 500 parasites/ μL). This could be achieved by including a third dilution from each case, or by including a panel of serial dilutions of recombinant antigen. Alternative protocols will be developed by WHO for review. Future modifications to lot testing should include more stable and easily standardized samples (e.g. lyophilized antigen).

Results of the comparative study on microscopy parasite counting indicate good equivalence between counting against white blood cells (true white cell count) and the Earl-Perez method, both giving lower counts than that obtained from a thin film. It was determined that both could be used for quantitation for the bank, with preference based on experience of the technician, and the Method Manual will reflect this.

Entry criteria for manufacturers for product testing

Significant modification was recommended to the proposed manufacturer stability testing recommended by the Geneva consultation (Annex 5). These modifications are intended to simplify testing, and to remove the requirement for manufacturers to test products under conditions well outside of their recommended storage specifications for that product. The Kisumu consultation recommended that, as a prerequisite for entry of products into the WHO product testing programme, manufacturers be required to undertake stability testing:

- At three-month intervals at the upper temperature limit recommended by the manufacturer
- At 35°C for 12 months
- Using a minimum of 10 RDTs at each interval, against recombinant or culture-derived standard sample provided by WHO.

Results should be reported to WHO and available for publication with WHO product testing results as they become available. Product testing may proceed in parallel.

It was recommended that a product-specific audit, recommended by the Geneva consultation should not be undertaken as part of product testing, but should be left to a WHO pre-qualification programme to be developed in the near future. A product-specific audit may be more appropriate as a post-testing activity, restricted to manufacturers of well-performing products.

It was confirmed that certification for ISO13485:2003 compliance (or the direct equivalent USFDA 21 CFR 820) should be required, and previous versions of ISO13485 should not be acceptable, as significant differences relevant to malaria RDTs exist between 1996 and 2003.

The definition of a product "Lot" was reviewed, and it was noted that the opinion from the Geneva consultation, that this is defined in ISO13485:2003, is not strictly true as scope for interpretation is wide in the case of malaria RDTs. However, a report on consultation with selected manufacturers and discussion within the consultation indicated that developing a precise definition would not be appropriate, due to the large number of components in an RDT, and possible adjustments commonly made during manufacture to optimize product quality. The definition of a "Lot" currently used by each manufacturer should be accepted.

External quality assurance of specimen collection sites and specimen banks

The laboratory assessment tool developed by the WHO/CSR office in Lyon was trialled at RITM and IPC in March 2006, and the results demonstrated at the consultation. Both institutions attained a high score. Minor recommendations for changes will be reported to CSR/Lyon for finalization of the tool. The consultation recommended an EQA assessment be performed every year.

Both specimen bank sites are involved in the Clinical Laboratory Improvement Amendments (CLIA) certification process, and it was recommended that the specimen banks maintain involvement in such EQA assessment. TDR is considering development of a specific assessment tool for other diagnostic laboratories and assessment of collecting sites may be incorporated into this in the future.

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REFERENCES

- Coleman, R. E., N. Maneechai, et al. (2002). "Field evaluation of the ICT Malaria Pf/Pv immunochromatographic test for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand." Am J Trop Med Hyg **66**(4): 379-83.
- Craig, M. H., B. L. Bredenkamp, et al. (2002). "Field and laboratory comparative evaluation of ten rapid malaria diagnostic tests." Trans R Soc Trop Med Hyg **96**(3): 258-65.
- Gaye, O., M. Diouf, et al. (1998). "Diagnosis of Plasmodium falciparum malaria using ParaSight F, ICT malaria PF and malaria IgG CELISA assays." Parasite **5**(2): 189-92.
- Huong, N. M., T. M. Davis, et al. (2002). "Comparison of three antigen detection methods for diagnosis and therapeutic monitoring of malaria: a field study from southern Vietnam." Trop Med Int Health **7**(4): 304-8.
- Iqbal, J., P. R. Hira, et al. (2001). "Diagnosis of imported malaria by Plasmodium lactate dehydrogenase (pLDH) and histidine-rich protein 2 (PfHRP-2)-based immunocapture assays." Am J Trop Med Hyg **64**(1-2): 20-3.
- Kolaczinski, J., N. Mohammed, et al. (2004). "Comparison of the OptiMAL rapid antigen test with field microscopy for the detection of Plasmodium vivax and P. falciparum: considerations for the application of the rapid test in Afghanistan." Ann Trop Med Parasitol **98**(1): 15-20.
- Mason, D. P., F. Kawamoto, et al. (2002). "A comparison of two rapid field immunochromatographic tests to expert microscopy in the diagnosis of malaria." Acta Trop **82**(1): 51-9.
- Ricci, L., I. Viani, et al. (2000). "Evaluation of OptiMAL Assay test to detect imported malaria in Italy." New Microbiol **23**(4): 391-8.
- Rubio, J. M., I. Buhigas, et al. (2001). "Limited level of accuracy provided by available rapid diagnosis tests for malaria enhances the need for PCR-based reference laboratories." J Clin Microbiol **39**(7): 2736-7.
- TDR (2003). SDI report. Geneva, UNDP / World Bank / WHO Special Programme for Research and Training in Tropical Diseases (TDR).
- WHO (2000). New Perspectives: Malaria Diagnosis. Report of a joint WHO/USAID informal consultation 25-27 October 1999. Geneva, World Health Organization.

- WHO (2001a). Hepatitis B surface antigen assays: operational characteristics (*Phase 1*) Report 2. Geneva, World Health Organization.
- WHO (2001b). Hepatitis C assays: operational characteristics (*Phase 1*) Report 2. Geneva, World Health Organization.
- WHO (2002). HIV simple/rapid assays (*Phase 1*) Report 12. Whole blood specimens. Geneva, World Health Organization.
- WHO (2003). Malaria Rapid Diagnosis: Making it Work. Meeting report 20-23 January 2003. Manila, World Health Organization.
- WHO (2004). HIV simple/rapid assays: operational characteristics (*Phase 1*) Report 14. Geneva, World Health Organization.
- WHO (2006a). Rapid Diagnostic Tests for Malaria: Methods Manual for Laboratory Quality Control Testing of Malaria Rapid Diagnostic Tests. Version 4. Manila, World Health Organization - Regional Office for the Western Pacific.
- WHO (2006b). Rapid Diagnostic Tests for Malaria: Methods Manual for Laboratory Quality Control Testing. Version 3. Manila, World Health Organization - Regional Office for the Western Pacific.
- WHO (2006c). Rapid Diagnostic Tests for Malaria: Methods Manual for Laboratory Quality Control Testing. Version 4. Manila, World Health Organization - Regional Office for the Western Pacific.